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Semi-synthetic Analysis toward Validating Roles of a Transcription/Translation Feedback Process for the Cyanobacterial KaiC-protein-based Circadian System in *Escherichia coli*

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Abstract The use of synthetic biology to design artificial gene circuits is an important approach for understanding the principles underlying the complicated dynamic behaviors of biomolecular networks, such as genetic switching and biological rhythms. The synthetic approach is also useful in systems biology in that it can be used to create artificial bypasses for processes related to cellular phenomena of interest for their easier analysis. To validate the role of transcription feedback in the cyanobacterial circadian system, we propose an experimental design for such a "semi-synthetic" approach that involves transplantation of the kaiABC genes into Escherichia coli and the construction of chimeric transcriptional outputs. The design principle and some preliminary results have been reported.

1 Introduction

Synthetic biology involves the assembly of artificial or natural biomolecules that can be used to reconstitute dynamic molecular networks that mimic biological functions or to create artificial devices that do not exist in reality. One benefit of such synthetic approaches in systems biology is that they facilitate the elucidation of some characteristics of certain molecular networks via artificial synthetic pathways. Retrospectively, the yeast two-hybrid analysis is a pioneering example of this kind of

synthetic tools. It is based on the artificial reconstitution of a fully functional transcriptional activator through which the physical interactions of proteins of interest in biochemical pathways are elucidated. Such semi-synthetic approaches are important to reveal the more complicated nature of intra- and intercellular networks that cannot be easily analyzed.

In this paper, we propose a semi-synthetic experimental design for validating the role of transcription-translation feedback in the cyanobacterial circadian clock system. Circadian rhythms are endogenous self-sustaining biological oscillations with a period of $\sim$24 h. These rhythms are widely observed among various organisms ranging from bacteria to humans and higher plants. The phase of circadian rhythm is synchronized to environmental light/dark cycles, and the free-running period length is stable against temperature changes within the physiological range (temperature compensation). Cyanobacteria are the simplest organisms that exhibit circadian rhythms, and the genetically tractable strain *Synechococcus elongatus* PCC 7942 has been established as an excellent model organism to analyze the circadian clock at the molecular, biochemical, and physiological levels (Iwasaki and Kondo, 2004). Under continuous light (LL) conditions, this strain exhibits robust circadian oscillations in the activity of essentially all gene promoters. These genome-wide transcriptional rhythms require a gene cluster comprising the following three clock genes: *kaiA*, *kaiB*, and *kaiC* (Ishiura *et al.*, 1998). Most period mutations have been mapped to *kaiC*, which encodes an ATP-binding autophosphorylating protein. Interestingly, the autophosphorylation of KaiC at Ser431 and Thr432 is activated by KaiA, and this KaiA-mediated action is antagonized by KaiB (Iwasaki *et al.*, 2002; Williams *et al.*, 2002). Under LL conditions, the phosphorylation state of KaiC exhibits circadian cycling (Iwasaki *et al.*, 2002). Additionally, a bacterial two-component system comprising the KaiC-interacting histidine kinase SasA and its cognate response regulator RpaA probably mediates the timing signals from Kai proteins to transcriptional outputs (Iwasaki *et al.*, 2000; Takai *et al.*, 2006).

Until recently, transcription-translation feedback processes had been proposed to be essential for driving basic oscillations in the circadian systems of any organism. However, we demonstrated that a posttranslational circadian rhythm in KaiC phosphorylation persisted robustly even after the complete elimination of transcriptional and translational regulations under continuous dark (DD) conditions with an excess of inhibitors (Tomita *et al.*, 2005). It should be noted that *Synechococcus* is an absolute photoautotroph, essentially requiring light for viability, and *kaiBC* mRNA is rapidly destroyed after transfer from LL to DD conditions (Tomita *et al.*, 2005). More evidently, Nakajima *et al.* (2005) reconstituted the KaiC phosphorylation cycle by incubating KaiA, KaiB, and KaiC recombinant proteins alone at the appropriate concentrations with ATP *in vitro*, and they established that the Kai protein-based enzymatic reaction is the key to circadian timing in cyanobacteria.

Under LL conditions, *Synechococcus* expresses an operon comprising the *kaiB* and *kaiC* genes in an extremely high-amplitude circadian fashion (Ishiura *et al.*, 1998; Iwasaki *et al.*, 2000), indicating the presence of transcriptional feedback processes for

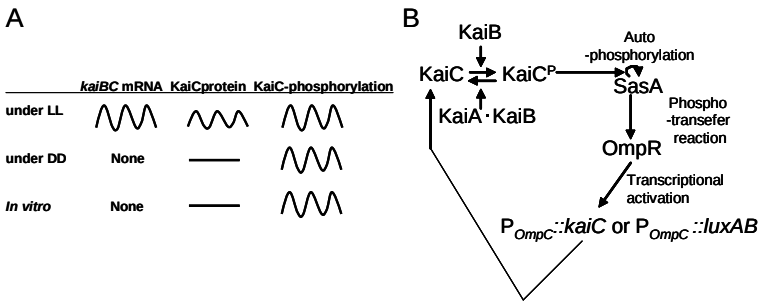


Figure 1: Schematic representation of clock gene expression profiles and the design of semisynthetic experiments. (A) Summary of *kaiBC* mRNA and KaiC level/phosphorylation profiles in *Synechococcus* under continuous light (LL) or continuous dark (DD) conditions, and the *in vitro* reconstitution system. (B) Schematic view of semisynthetic clock gene circuit in *E. coli*.

kaiBC expression in addition to a core posttranslational oscillator. Thus, under LL conditions, *Synechococcus* exhibits rhythms with regard to *kaiBC* mRNA expression, KaiB and KaiC abundance, and KaiC phosphorylation. On the other hand, under DD and *in vitro* conditions, Kai levels are maintained at a constant, although *kai* gene mRNA is not expressed, and KaiC phosphorylation oscillates (Fig. 1A).

These observations immediately raise a question regarding whether *kaiBC* transcription-translation feedback, which generates clock protein-abundance rhythms, would affect the core KaiC phosphorylation cycle. In addition to the phosphorylation rhythm, what is the possible role of clock protein-abundance rhythms in the circadian system? One of the plausible hypotheses could be that such secondary (transcriptional) feedback is important to render the core timing loop robust against environmental or intracellular noise. It should be noted that under LL conditions, cells are metabolically active and propagate rapidly, and intracellular fluctuations in cellular dynamics are thereby generated. Therefore, the clock should be sufficiently robust against such internal noise. *Synechococcus* does exhibit circadian transcription rhythms even when its doubling time is considerably less than 24h (Kondo *et al.*, 1997; Mori *et al.*, 1996). On the other hand, under DD conditions (and *in vitro* conditions), the chemical conditions must be more stable because metabolic activity drastically reduces and cell division is completely arrested.

Some multiple feedback processes have been proposed to enhance the robustness of oscillation against ambient fluctuations under some parameter sets (Rand *et al.,* 2006;Ueda *et al.*, 2001). Although several theoretical modeling and biochemical analyses have been reported for the Kai-based oscillator, the actual biochemical mechanism underlying the manner in which the protein network drives the KaiC phosphorylation rhythm remains to be elucidated. Thus, determining the role of the transcription-translation rhythms of clock genes in the cyanobacterial circadian system requires some experimental validation, such as comparison of the core phospho-

rylation states in the presence or absence of rhythmicity in the *kai* gene expression with equivalent average expression levels. However, it is known that even a minimal core promoter from *E. coli* can drive the rhythm of reporter (luciferase) gene expression in *Synechococcus* (Katayama *et al.*, 1999) and thus this organism experiences difficulty in constitutively expressing *kai* genes at constant levels. Thus, we attempted to transplant cyanobacterial clock genes into *E. coli* in order to reproduce posttranslational Kai-protein dynamics and to synthesize an artificial secondary transcriptional feedback process in order to examine the effect of secondary feedback on the core enzymatic network. Although complete KaiC cycling has not been reconstituted, we observed some dynamic profiles of KaiC phosphorylation that varied depending on the growth conditions. These observations are consistent with the notion that some regulatory mechanisms are necessary for circadian KaiC phosphorylation rhythms to be sufficiently robust under different metabolic conditions. Moreover, we partially reconstituted the KaiC-dependent transcriptional output in *E. coli* by using an artificial chimeric two-component regulatory system comprising SasA and OmpR.

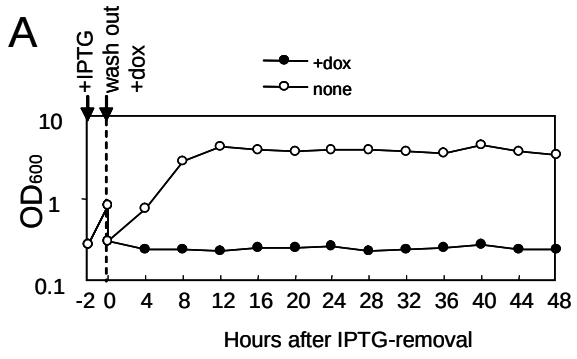
Cells were initially grown to an OD_{600} of $\sim$0.3, treated with IPTG for 2 h, rinsed with fresh LB medium, and diluted to an OD_{600} of $\sim$0.3. The cells were then cultured in LB medium with or without doxycycline and collected at the indicated times. (A) Cell density during the course of the experiments. OD_{600} values are shown. (B) Total protein (4 μg) prepared from the cells was analyzed by immunoblotting with antisera to KaiA and KaiC by using 10% SDS-PAGE, as described previously (Iwasaki *et al.*, 2002). Note that the upper and lower signals in the graph for KaiC represent its phosphorylated (P-KaiC) and non-phosphorylated (NP-KaiC) forms, respectively. (C) Quantification of KaiC by densitometric analysis of the blots shown in (B).

2 Results and Discussion

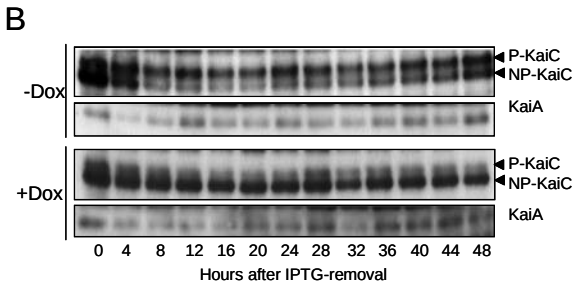
Transplantation of cyanobacterial clock genes into *E. coli*

Under LL conditions, the molecular ratio of KaiA:KaiB:KaiC in *Synechococcus* is $\sim$1:40:20 (Kitayama *et al.*, 2003), while in *in vitro* conditions, it is an optimum of $\sim$1:4:4 for the generation of the KaiC phosphorylation cycle (Nakajima *et al.*, 2005). This discrepancy in the ratio remains to be understood. We required a lower level of KaiA than those of KaiB and KaiC. Therefore, we used a p15A-based lower copy shuttle vector for *kaiA* expression in *E. coli* and a pBR322-based higher copy plasmid for expression of the *kaiBC* operon. We initially attempted the induction of *kaiA* and *kaiBC* by using different inducers under distinct promoters (the *tac*, *trc*, or *bad* promoter); however, clock gene expression could not be induced from the *bad*-promoter constructs, but the *E. coli*-derived isopropyl thio-galactopyranoside (IPTG) -inducible *trc* promoter (P_{trc}) could be used to induce *kaiBC* expression and the *lac* promoter (P_{lac}), to induce *kaiA* expression. The activity of P_{trc} is higher than that of P_{lac}.

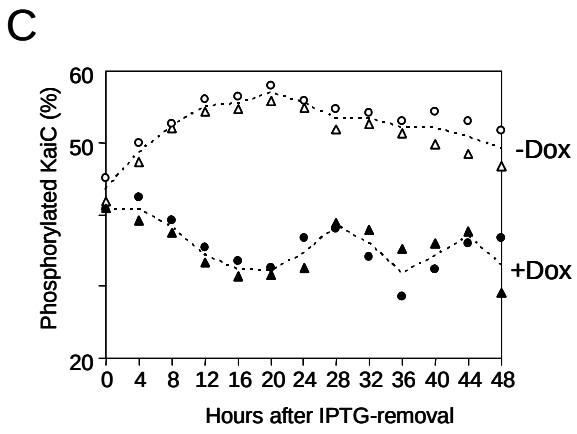
In *Synechococcus*, a transient increase in the levels of KaiB and KaiC under P_{trc} sets the clock (Ishiura *et al.*, 1998). Thus, *E. coli* transformants were transiently treated with IPTG for 2 h; the cells were then rinsed to remove the inducer (defined as



(a)



(b)



(c)

Figure 2: Temporal KaiA and KaiC protein expression profiles in *E. coli* under different growth conditions.

time 0) and cultured for 48 h in the presence or absence of antibiotics. We used doxycycline, a translation inhibitor, to arrest the cell propagation of *E. coli*. As observed in *Synechococcus* under DD conditions, the level of KaiC accumulation was not altered after doxycycline treatment (Fig. 2B). Even in the absence doxycycline, the increase of the KaiA and KaiC proteins can be observed during 48 h after IPTG removal (Fig. 2B). This observation could probably be attributed to the leaky expression of *kaiA* and *kaiBC* from the *lac* and *trc* promoters, respectively.

Under our experimental conditions, we observed some fluctuations in the KaiC phosphorylation ratio under both growth conditions, although we have not established the optimum conditions for the generation of reliable circadian oscillations. Interestingly, the KaiC phosphorylation ratio is higher in the absence of doxycycline than in its presence (Figs. 2B and C). In the absence of doxycycline, the phosphorylated form of KaiC gradually became dominant from 4 to 30 h after IPTG removal. The transient reduction in the level of KaiC that was observed from 4 to 8 h may be due to rapid log phase-dependent propagation (Fig. 2A). Consequently, the KaiC/KaiA molecular ratio would decrease, and this may affect the KaiC phosphorylation state. However, a difference in growth conditions and altered doubling times do not essentially affect the phase of circadian rhythms in either continuous or batch cultures of *Synechococcus*. Thus, such growth/metabolism-dependent variations in the KaiC phosphorylation state observed in *E. coli* (Figs. 2B and C) must be prevented in the cyanobacterial circadian system. This does not contradict our hypothesis that transcription/translation feedback might be a key to the noise-escaping mechanism.

Reconstitution of KaiC-SasA signaling in *E. coli*

In *Synechococcus*, the posttranslational Kai protein-based oscillation feeds back in order to regulate *kaiBC* expression and generate the rhythms in *kaiBC* mRNA and KaiB and KaiC protein abundances The two-component SasA-RpaA His-to-Asp regulatory system is a major mediator of timing signals from the core oscillator to transcriptional activities (Iwasaki *et al.*, 2000; Takai *et al.*, 2006). KaiC binds to the N-terminal sensory domain of SasA. Depletion of the *sasA* gene dramatically attenuates genome-wide transcription rhythms, while very unstable and short-period rhythms are maintained only under low light conditions (Iwasaki *et al.*, 2000). *In vitro* KaiC was found to activate SasA autophosphorylation (Smith and Williams, 2006), thereby enhancing phosphotransfer from SasA to RpaA (Takai *et al.*, 2006). The phosphotransfer reaction is modified by a mixture of the KaiA, KaiB, and KaiC recombinant proteins, and it depends on the phase of the *in vitro* reconstituted clock (Takai *et al.*, 2006). RpaA contains a possible DNA-binding domain, although the transcriptional targets remain to be identified.

The aim of our semisynthetic experiments is to validate the role of *kaiBC* mRNA oscillation in the KaiC phosphorylation cycle under different metabolic conditions via the synthesis of a transcription-translation feedback loop for the genetically transplanted Kai-clock system in *E. coli*. Therefore, we constructed a transcriptional

feedback loop containing SasA in *E. coli* (Fig. 1B). The *sasA* gene was originally identified in *Synechococcus* as one encoding histidine kinase that complemented an *E. coli envZ* mutant (Nagaya *et al.*, 1993). The *E. coli* EnvZ-OmpR two-component system is one of the best characterized bacterial His-to-Asp signaling factors, and it senses osmolarity for the regulation of some genes including *ompC*, which is a direct target of the DNA-binding regulator OmpR. In the *envZ* mutant, endogenous *ompC* expression is severely reduced, while inducible *sasA* expression recovers the *ompC* expression monitored by a *lacZ* reporter (Nagaya *et al.*, 1993). As an initial attempt to reconstitute transcriptional output from the clock, we expanded Nagaya's system to examine the effect of the KaiC protein by monitoring *ompC* promoter (P_{ompC}) activity with a luciferase reporter. The *sasA* and *kaiBC* genes were introduced into *E. coli* JW3367, which lacks the *envZ* gene (Baba *et al.*, 2006). The expression of *sasA* and *kaiBC* was controlled by the TetR and LacI repressors, respectively. In this system, KaiC is expected to activate SasA phosphorylation to enhance the phosphotransfer reaction to OmpR, thereby activating P_{ompC}*::luxAB* (Fig. 1).

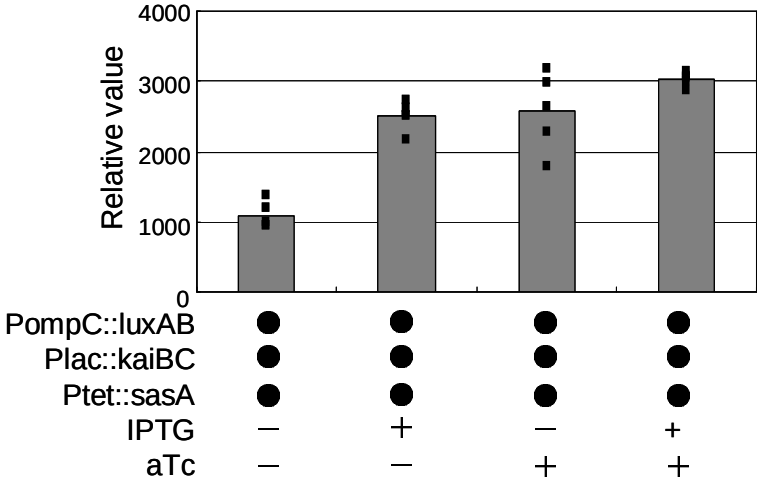


Figure 3: Examination of KaiC-SasA signaling in *E. coli*.

The *E. coli* strain ILE18 was grown in LB medium in the presence or absence of inducers (aTc for *sasA* and IPTG for *kaiBC*) for 11 h. Then, equivalent amounts of the cells were subjected to P_{ompC} bioluminescence analysis. Bars indicate the relative average values of luminescence in 5 experiments.

As shown in Fig. 3, when SasA was induced, P_{ompC}*::luxAB* was significantly activated (Fig. 3), confirming the results of a previous study by Nagaya *et al.* (1993). Interestingly, P_{ompC}activation was also observed when KaiB and KaiC were co-induced, regardless of the presence of anhydrotetracycline (aTc) used for SasA induction (Fig. 3). This aTc independency can be interpreted in two alternative ways. One possibility is that the leaky expression of *sasA* in the absence of an inducer is sufficient for the KaiC-dependent activation of the SasA-OmpR chimeric signaling pathway.

In this case, IPTG-independent P_{ompC} activation in the presence of aTc may be due to saturated SasA accumulation. If this is the case, then SasA induction would be sufficiently low for the difference in KaiC-dependent timing signals to be sensed. Consistently, *sasA* expression in *Synechococcus* is very weak compared with that of *kaiBC* (Iwasaki *et al.*, 2000). An alternative possibility is that KaiC can affect P_{ompC} activity independent of the SasA protein. Previously, KaiC was proposed to be a DNA-binding protein that regulates genomic DNA conformation, thereby controlling genome-wide transcription (Mori and Johnson, 2001). Moreover, even in the *sasA*-null mutant, residual Kai-dependent rhythmicity can be observed, suggesting the presence of an as yet unknown pathway from the Kai proteins to the transcription system that functions even in the absence of SasA (Iwasaki *et al.*, 2000; Takai *et al.*, 2006; Taniguchi *et al.*, 2007). In order to address this issue, we are currently testing the effect of KaiC on the *ompC* reporter in the absence of transgenic *sasA* constructs, which will be reported in the meeting.

3 Materials and Methods

Plasmids and Strains

The plasmids used in this study are listed in Table 1. *E. coli* JM109 and JW3367 (Δ*envZ*) were used as the host cells. The strains transformed with each vector are listed in Table 2.

Western blot analysis

The JM109 strain containing pIL82 and pIL52-2 was cultured overnight at 30.˚C in LB medium containing the 2 above-mentioned antibiotics. This modified *E. coli* was designated ILE18. Then, 10 mM IPTG was added to the medium, and the cells were cultured for another 2 h. The cells were washed and diluted to an OD_{600} of 0.3 in 150 ml of fresh LB medium containing only 20 μl/ml of chloramphenicol and kanamycin, or also containing 40 μg/ml of doxycycline (Becton Dickinson) in addition to the antibiotics. The cells were cultured at 30.˚C for 48 h, and they were sampled every 4 h. Whole-cell extracts were prepared as described (Iwasaki *et al.*, 2002) with minor modifications. As described (Iwasaki *et al.*, 1999), the immunoblots were incubated with anti-KaiC antibodies and analyzed by enhanced chemiluminescence (ECL; GE Bioscience).

KaiC-SasA signaling assay

The ILE21, 24 or 25 were cultured overnight at 37.˚C in 2 ml of the LB medium containing 13.3μl/ml of carbenicillin, chloramphenicol and streptomycin. Next, 2 ml of fresh medium was inoculated with 4 μl of the overnight culture. Moreover, if necessary, in order to induce SasA and KaiC, 500 ng/ml of aTc and 10 mM of IPTG were added to the media, respectively. The cells were cultured for 10 - 11 hours. During the culture, the cells were diluted several times in order to maintain the logarithmic phase of growth. After the density of the cultures was equalized, 1 μl

of *n*-decanal (Sigma) was added to the media, and the luminescence was measured using the microplate reader, Infinite M200-W (TECAN).

Acknowledgments

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Table 1: Plasmid list

Name	Gene cassette	Origin	Resistance marker	Reference
pIL82	P_{lac}*::kaiA*	p15A	chloramphenicol	This study
pIL83	P_{lac}*::kaiBC*	p15A	chloramphenicol	This study
pIL84	P_{LtetO1}*::sasA*, P_{ompC}*::luxAB*	pSC*101	spectinomycin	This study
pIL85	P^{q}_{lacI}*::lacI*, P_{N25}*::tetR*	pBR322	ampicillin	This study
pIL86	P_{trc}*::kaiBC*	pBR322	kanamycin	This study

Table 2: Strain list

Name	Plasmids	Source	Reference
ILE18	pIL82, pIL86	JM109 (Takara)	This study
ILE21	pIL83, pIL84, pIL85	JW3367 (Keio collection)	This study

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