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# Reconstitution of Circadian Oscillation of Cyanobacterial KaiC Phosphorylation in Vitro

Masato Nakajima, Keiko Imai, Hiroshi Ito, Taeko Nishiwaki, Yoriko Murayama, Hideo Iwasaki, Tokitaka Oyama, Takao Kondo\*

Kai proteins globally regulate circadian gene expression of cyanobacteria. The KaiC phosphorylation cycle, which persists even without transcription or translation, is assumed to be a basic timing process of the circadian clock. We have reconstituted the self-sustainable oscillation of KaiC phosphorylation in vitro by incubating KaiC with KaiA, KaiB, and adenosine triphosphate. The period of the in vitro oscillation was stable despite temperature change (temperature compensation), and the circadian periods observed in vivo in KaiC mutant strains were consistent with those measured in vitro. The enigma of the circadian clock can now be studied in vitro by examining the interactions between three Kai proteins.

Circadian rhythms allow organisms to coordinate their lives according to the alteration of their environments by day and by night (*I*). In most model organisms, transcription-translation-derived oscillatory (TTO) processes based on negative feedback regulation of clock genes are proposed as the core generator of self-sustaining circadian oscillations (*I*, *2*). Cyanobacteria are the simplest organisms that exhibit circadian rhythms. In the cyanobacterium *Synechococcus elongatus* (PCC 7942), three genes (*kaiA*, *kaiB*, and *kaiC*) are essential components of the circadian clock (*3*). Negative-feedback regulation of the expression of the *kaiBC* operon by Kai proteins was proposed as a core loop of prokaryotic TTO (*3*). In the cyanobacterial TTO model, Kai proteins do not regulate a specific set of circadian-controlled genes, but they regulate genomewide gene expression, including that of the *kaiBC* operon (*4*). However, how this transcription-translation feedback loop achieves circadian periodicity and how it stabilizes circadian oscillation against alterations in temperature and metabolic activity (collectively referred to as circadian characteristics) have not been clear. These circadian characteristics are essential for the oscillator to adapt to the environment, so understanding their molecular basis is an important goal in circadian biology.

The phosphorylation state of KaiC robustly oscillates in the cell with a 24-hour period, even under conditions where neither transcription nor translation of *kaiBC* operon was permitted, raising doubts about the TTO model in cyanobacteria (*5*). The circadian

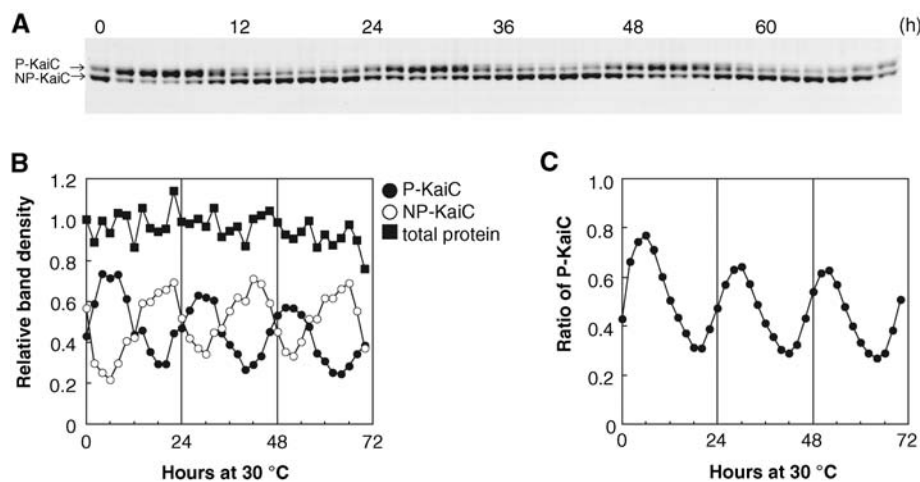
characteristics of this oscillator suggested that the pacemaker for the cyanobacterial circadian system was not a transcription-translation feedback loop but the KaiC phosphorylation cycle itself. KaiC has both autophosphorylation and autodephosphorylation activities, and KaiA enhances KaiC autophosphorylation (*6*), whereas KaiB attenuates the effect of KaiA (*7*, *8*). These results imply that, without additional kinases or phosphatases, an autonomous oscillation of KaiC phosphorylation could be generated by cooperation between KaiA and KaiB.

Recombinant KaiC protein was incubated with KaiA and KaiB at a ratio similar to that measured in vivo [KaiA:KaiB:KaiC=1:1:4 (by weight), (*7*)] in the presence of 1 mM ATP.

To our surprise, KaiC phosphorylation robustly oscillated with a period of about 24 hours for at least three cycles without damping (Fig. 1A). The ratio of phosphorylated KaiC to total KaiC cycled between 0.25 and 0.65 (Fig. 1C). The amplitude of this in vitro KaiC phosphorylation rhythm was smaller than that observed in vivo under continuous light conditions (*5*). Also the total amount of KaiC remained constant during the incubation (Fig. 1B), which indicated that neither phosphorylated nor unphosphorylated KaiC was degraded during the reaction. Thus, oscillation of KaiC phosphorylation generates autonomously with a circadian period by cooperation of three Kai proteins.

One characteristic of circadian rhythms is that the free-running period remains stable for a relatively broad range of temperatures, referred to as “temperature compensation” of the period (*I*). Such temperature compensation of the in vitro oscillation of KaiC phosphorylation was observed with period lengths of about 22, 21, and 20 hours at 25°, 30°, and 35°C, respectively (Fig. 2A). The thermal sensitivity ( $Q_{10}$  coefficient) of the period (Fig. 2B) was about 1.1, consistent with that reported for in vivo gene expression rhythm (*9*). The rates of in vitro KaiC autophosphorylation and autodephosphorylation show a high degree of compensation for changes in temperature (*5*). Thus, temperature compensation of the in vitro rhythm is likely due to these reactions.

Many mutations of *kaiC* shorten or extend the period of circadian rhythm of *Synechococcus* (*3*). To assess whether the in vitro oscillation of KaiC phosphorylation was affected by such mutations, we incubated recombinant mutant KaiC with KaiA and KaiB. Three KaiC



**Fig. 1.** In vitro oscillation of KaiC phosphorylation. (A) Recombinant KaiC proteins (0.2  $\mu\text{g}/\mu\text{l}$ ) were incubated with KaiA (0.05  $\mu\text{g}/\mu\text{l}$ ) and KaiB (0.05  $\mu\text{g}/\mu\text{l}$ ) in the presence of ATP (1 mM) (*17*). Aliquots (3  $\mu\text{l}$  each) of the reaction mixtures were collected every 2 hours and subjected to SDS-polyacrylamide electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining. The upper and lower bands correspond to phosphorylated (P-KaiC) and unphosphorylated KaiC (NP-KaiC), respectively (*6*). (B and C) NIH image software was used to perform densitometric analysis of data (*5*) in (A). The relative densities of total, phosphorylated, and unphosphorylated KaiC are plotted in (B), and the ratios of P-KaiC to total KaiC are plotted in (C).

Division of Biological Science, Graduate School of Science, Nagoya University, and the Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (JST), Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan.

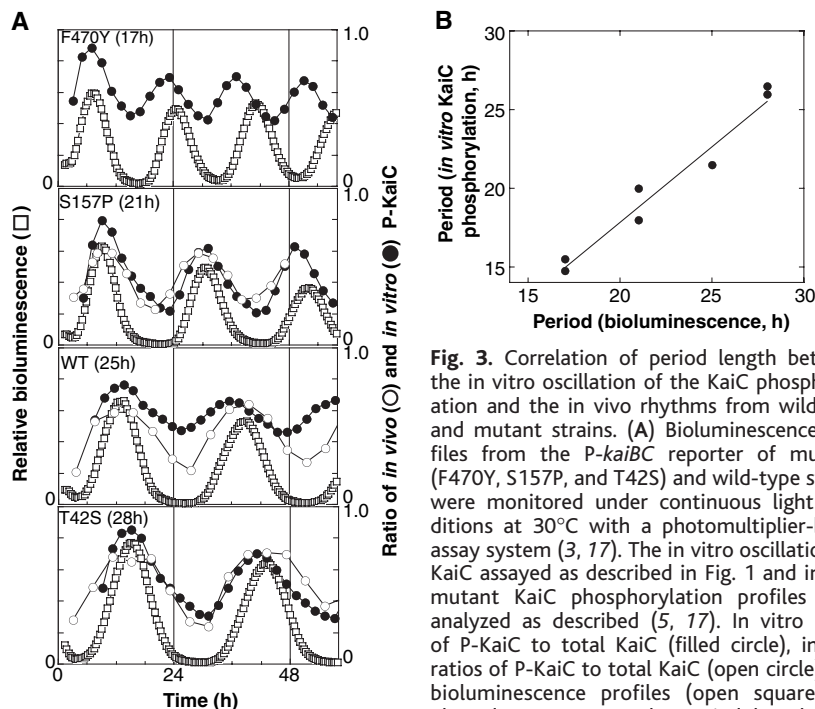
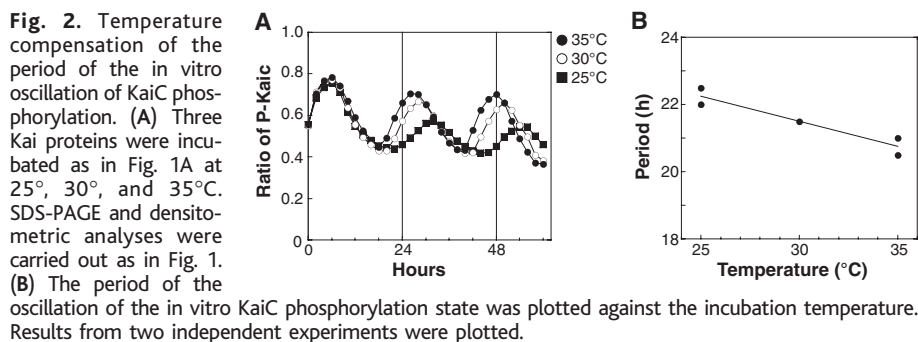
\*To whom correspondence should be addressed. E-mail: kondo@bio.nagoya-u.ac.jp

mutants that display high-amplitude bioluminescence rhythms were examined. The period lengths of expression rhythm of *kaiBC* promoter, monitored with a bioluminescence reporter (3), were 17, 21, and 28 hours, respectively, in mutant strains with amino acid substitutions of Tyr for Phe<sup>470</sup> (F470Y), Pro for Ser<sup>157</sup> (S157P), and Ser for Thr<sup>42</sup> (T42S). The KaiC phosphorylation profiles, obtained when the mutant proteins were assayed *in vitro*, were consistent with those observed *in vivo* (Fig. 3A). We also confirmed that the bioluminescence profiles of these mutant strains were consistent with the *in vitro* oscillation of phosphorylation for each of the respective mutant KaiC proteins (Fig. 3, A and B). These results indicate that oscillation of KaiC phosphorylation is the molecular timer for the circadian rhythm of *Synechococcus*.

Phosphorylation of clock proteins has been reported in various prokaryotic and eukaryotic model organisms. PERIOD and TIMELESS in *Drosophila* and FREQUENCY in *Neurospora* degraded and/or translocated to the nucleus according to their circadian rhythms of phosphorylation state (10, 11). Because alterations of the phosphorylation of these clock proteins affect the period length, the phosphorylation processes were assumed to be important components of the TTO models, including those for cyanobacteria (2, 3). In these models, phosphorylation only contributes at a specific phase of the circadian cycle. However, our study demonstrates that the oscillation of KaiC phosphorylation is the pacemaker of the cyanobacterial circadian clock. In addition, the *in vitro* oscillation is generated in a homogeneous system, whereas heterogeneous com-

partments are assumed in eukaryotic models (2). KaiC forms hexamers (12, 13) and is phosphorylated at Ser<sup>431</sup> and Thr<sup>432</sup> (14, 15). These results imply that KaiC hexamer has multiple phosphorylation states that may have different biochemical characteristics, including autophosphorylation and autodephosphorylation activities and/or binding preferences with other Kai proteins. In addition, the reaction rates of KaiC phosphorylation and dephosphorylation are quite slow, with rate constants of 10<sup>-3</sup> to 10<sup>-4</sup> s<sup>-1</sup> [(16), SOM text]. Functionally, this feature would reduce the energy needed for timekeeping.

We propose a model of the cyanobacterial clock in which the autonomous oscillation of KaiC phosphorylation controls the expression of relevant genes including the *kai* genes to generate physiologically functional circadian oscillation (fig. S1). Simultaneously, the *in vivo* oscillation of KaiC phosphorylation could be amplified by coupling it with periodic changes in the concentrations of Kai protein and/or additional regulatory components of KaiC phosphorylation. The relationship between the phosphorylation of KaiC and the Kai transcription-translation cycle may be similar to that of a pendulum and an escapement mechanism that sustains the pendulum oscillation and transmits time signals to the hands of a wall clock.



phases of *in vitro* and *in vivo* oscillations of KaiC phosphorylation are shifted to put first peak of phosphorylation rhythms on that of the corresponding bioluminescence rhythm. Periods of bioluminescence rhythm are shown in parentheses. (B) For wild type and each mutant strain shown in (A), the period length of the *in vitro* oscillation of the KaiC phosphorylation state is plotted against that of the *in vivo* rhythms of the strain. Results from two independent experiments were plotted.

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16. The order of rate constant of KaiC phosphorylation was estimated by fitting the model equation  $P(t) = P_{equi} + LP(0) - P_{equi} \exp[-(k_1 + k_2)t]$  (SOM text) to experimental data shown in figure 3, C and D, of our previous report (5).
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