A Model for the Circadian Rhythm of Cyanobacteria that Maintains Oscillation without Gene Expression

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ABSTRACT An intriguing property of the cyanobacterial circadian clock is that endogenous rhythm persists when protein abundances are kept constant either in the presence of translation and transcription inhibitors or in the constant dark condition. Here we propose a regulatory mechanism of KaiC phosphorylation for the generation of circadian oscillations in cyanobacteria. In the model, clock proteins KaiA and KaiB are assumed to have multiple states, regulating the KaiC phosphorylation process. The model can explain 1), the sustained oscillation of gene expression and protein abundance when the expression of the *kaiBC* gene is regulated by KaiC protein, and 2), the sustained oscillation of phosphorylated KaiC when transcription and translation processes are inhibited and total protein abundance is fixed. Results of this work suggest that KaiA and KaiB strengthen the nonlinearity of KaiC phosphorylation, thereby promoting the circadian rhythm in cyanobacteria.

INTRODUCTION

Many species have evolved to have an endogenous clock with a period of nearly 24 h, presumably by adapting to the life in a periodically fluctuating environment. In *Neurospora*, *Drosophila*, and mammals, clock genes are known. In Drosophila, for example, the *period* gene (abbreviated as *per*) is transcribed to produce the mRNA, which is then translated into protein (PER protein). After entering the nucleus, PER protein suppresses the expression of *per* gene. This negative feedback regulation of the gene expression generates a sustainable oscillation, which has been regarded as a basis of the circadian rhythms in Drosophila. Negative feedback regulation of clock genes is also suggested to be responsible for the circadian rhythms in *Neurospora*, plants, and mammals (1–3).

Since a seminal work by Goodwin (4), who studied a mathematical model for the negative feedback regulation of a gene's expression by its own product, many models have been proposed and analyzed for Drosophila (5–9), Neurospora (10–12), and mammals (13,14). In the model proposed by Goldbeter (5), PER proteins are phosphorylated before entering the nucleus, and phosphorylated PER in the nucleus suppresses the *per* expression. Gene-protein kinetic networks observed in the real organisms are much more complex than ones assumed in those theoretically studied. A complex structure of the gene-protein network of the protein kinetics and the choice of kinetic parameters are likely to be the results of natural selection to make the system more readily produce stable oscillations (e.g., 4,5,15–20).

Cyanobacterium is a unicellular prokaryote but shows clear circadian rhythm. KaiA, KaiB, and KaiC proteins are identified as key components of the circadian clock in cyanobacteria (21). A single gene (*kaiBC* gene) is transcribed into *kaiBC* mRNA, which is then translated into two separate proteins, namely KaiB and KaiC (21). KaiA and KaiB proteins regulate the KaiC phosphorylation process (22–24). The KaiA protein activates accumulation of phosphorylated KaiC, and the KaiB protein promotes accumulation of nonphosphorylated KaiC (22,24). Phosphorylated KaiC proteins are suggested to suppress *kaiBC* gene expression (25). Thus the negative feedback regulation of the *kaiBC* gene expression, generating an autonomous oscillation, has been considered a key component for the cyanobacterial circadian clock (21).

Recently, Tomita et al. (26) discovered that the KaiC phosphorylation rhythm in cyanobacteria persists in constant dark conditions (abbreviated as DD). In DD conditions, transcription, translation, and degradation of KaiA, KaiB, and KaiC proteins are inhibited (26). Thus sustained oscillations of the cyanobacterial circadian clock can occur without regulation of clock genes. Moreover the KaiC phosphorylation rhythm occurs in an in vitro system if proper amounts of KaiA, KaiB, and KaiC proteins and ATP exist (27). The results of Tomita et al. (26) and Nakajima et al. (27) suggest that biochemical interactions among KaiA, KaiB, and KaiC proteins are likely to drive circadian oscillations of KaiC phosphorylation both in vitro and in vivo, although a regulatory mechanism of the KaiC phosphorylation process remains unclear.

In this work, we propose a new model for gene-protein dynamics that can generate a sustained oscillation both in DD and in constant light (abbreviated as LL) conditions. The basic idea underlying our modeling is that relaxation oscillations of KaiC phosphorylation can be the basis for

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circadian oscillations in cyanobacteria when neither transcriptional nor translational regulation operates. Our conjecture is that KaiB works to bring a nonlinearity in KaiC phosphorylation process. To express this, we assume that there are active and inactive forms of KaiB, just as for KaiC, and KaiC proteins affect the balance between these two forms of KaiB. For example active forms of KaiB (denoted by KaiB*) may suppress the phosphorylation of KaiC (i.e., KaiC-P), whereas phosphorylated KaiC may reduce the activation of KaiB. Such a mutually inhibitory interaction may result in bistability, namely coexistence of two stable states. In the DD condition in which the total amounts of the KaiA, KaiB, and KaiC proteins remain constant, a slow dynamics of active KaiA can generate a relaxation oscillation, whereas in the LL condition, another slow kinetics including regulation of the expression of the *kaiBC* gene produces an oscillation.

MODEL

Here we first focus on modeling of the regulatory mechanisms for circadian oscillations when transcriptional-translational regulations operate, which corresponds to LL conditions.

Mutual inhibition of KaiB and KaiC proteins

Autodephosphorylation of KaiC is known to be enhanced by KaiB (24,28). We here assume the regulatory mechanisms depicted in Fig. 1 *A*: 1), there are active and inactive forms of KaiB, 2), active KaiB enhances dephosphorylation of KaiC, and 3), phosphorylated KaiC inactivates KaiB.

Two states of the KaiB protein may correspond to different cellular localizations of KaiB. The concentration of KaiB accumulated in the cytosol shows the maximum at LL 20 h, which is followed by the dephosphorylation of KaiC (24). In contrast KaiB concentration accumulated in the vicinity of the cellular membrane is maximized at LL 16 h, although the regulation of the localization of KaiB remains unclear. Alternatively, the multiple states of KaiB may be caused by chemical modification of KaiB, just as for KaiC.

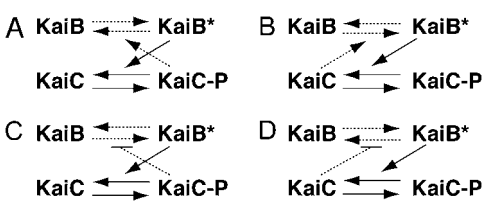

FIGURE 1 Regulatory mechanisms of KaiC phosphorylation by KaiB. Solid arrows indicate regulations or transitions, which are experimentally suggested. Broken arrows indicate ones, which we assume in this work. (*A*) KaiB is assumed to have multiple states in which only activated KaiB (KaiB*) enhances the dephosphorylation of KaiC. In panels *B–D*, three alternative regulations are also considered.

Let C_0s be the total abundance of the KaiC protein and B_0s be the total abundance of the KaiB protein. Since KaiB and KaiC are produced from the same transcription unit *kaiBC* gene, their numbers change in proportion to each other (see below), and for the convenience of modeling we use this notation for KaiB and KaiC abundance. There are phosphorylated and nonphosphorylated forms of KaiC. We denote their abundances by x and $C_0s - x$, respectively. In a similar manner, there are active and inactive forms of KaiB, the abundances of which are denoted by y and $B_0s - y$, respectively. They follow:

$$dx/dt = pa(C_0s - x) - bx(y + f), \tag{1a}$$

$$dy/dt = g(B_0s - y) - k_1yx^n/(q^n + x^n). \tag{1b}$$

The abundance of active KaiA is denoted by a. Equation 1a is for the balance of phosphorylation occurring at rate pa, which is proportional to active KaiA, and the inactivation of KaiC at rate $b(y+f)$, which increases with the active forms of KaiB protein. Equation 1b indicates the balance between active and inactive forms of KaiB whose sum is B_0s. The inactivation of KaiB protein is an increasing function of phosphorylated KaiC with cooperativity (or Hill constant) of n.

Kitayama et al. (24) and Xu et al. (29) demonstrated that KaiB dephosphorylates KaiC after KaiA forms a complex with phosphorylated KaiC. Thus KaiC dephosphorylation by KaiB depends on the presence of KaiA, which is not incorporated in Eq. 1a for simplicity. Later in this article, we will show that the conclusions derived from Eq. 1 also hold for the model in which the presence of KaiA is required for KaiC dephosphorylation by KaiB.

As a result of mutual inhibition between phosphorylated KaiC and active form of KaiB, Eq. 1 shows a bistability: a stable equilibrium with many active KaiB proteins but few phosphorylated KaiC, and another stable equilibrium with many phosphorylated KaiC but few active KaiB.

In Fig. 2 *A*, the equilibrium level of phosphorylated KaiC protein x is drawn as a function of C and total abundance of KaiC (i.e., $C = C_0s$). For small C, x is low, whereas for large C, x is high. There is an interval of C in which for each C there are two values of x that are simultaneously locally stable. These are represented as two branches of the graph on a C-x plane (see Fig. 2 *A*). The equilibrium with a high x corresponds to a high phosphorylated KaiC and a low active KaiB; whereas the other with a low x corresponds to a low phosphorylated KaiC and a high active KaiB. To be specific, let $s_1^* < s < s_2^*$ be the interval for the bistability (i.e., the existence of two stable solutions with different x for the same s): For $s < s_1^*$ and $s > s_2^*$, there is only a single x at equilibrium for a given s.

Concerning the manner in which active KaiB is affected by phosphorylated or unphosphorylated KaiC, there are several alternative ways that are also able to create bistability (see Fig. 1, *B*, *C*, and *D*). We will discuss them later.

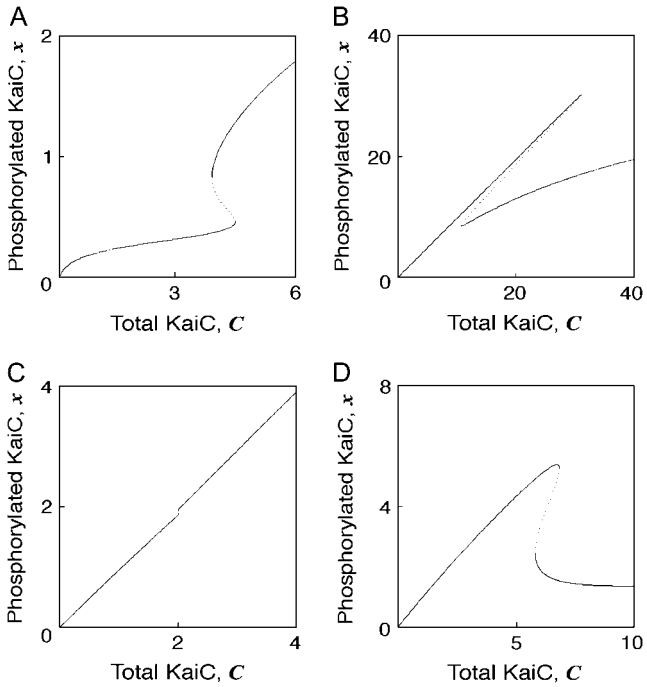

FIGURE 2 Bistability of KaiC phosphorylation. Abundance of phosphorylated KaiC protein x at equilibrium is plotted as a function of the total abundance of total KaiC, C. (*A*) Regulation of KaiC is described by Eq. 1 in the text. These diagrams have been obtained with XPPAUT (http://www.math.pitt.edu/~bard/xpp/xpp.html). Regulation of KaiC is described by Eqs. 1a and 5 in panel *B*, by Eqs. 1a and 6 in panel *C*, and by Eqs. 1a and 7 in panel *D*. Parameter values in panel *A* are as follows: $B_0 = 200$, $C_0 = 2$, $p = 6.3$, $a = 0.207$, $b = 0.063$, $f = 4$, $g = 25.2$, $k_1 = 630$, $q = 1.8$, $n = 2$. Alternative interactions between KaiC and KaiB are also shown (*B–D*). Parameter values are $a = 1.5$, $n = 8$ (*B*), $a = 1.5$, $n = 100$ (*C*), and $a = 1.5$, $n = 4$ (*D*), and other parameter values except a and n are the same as in panel *A*.

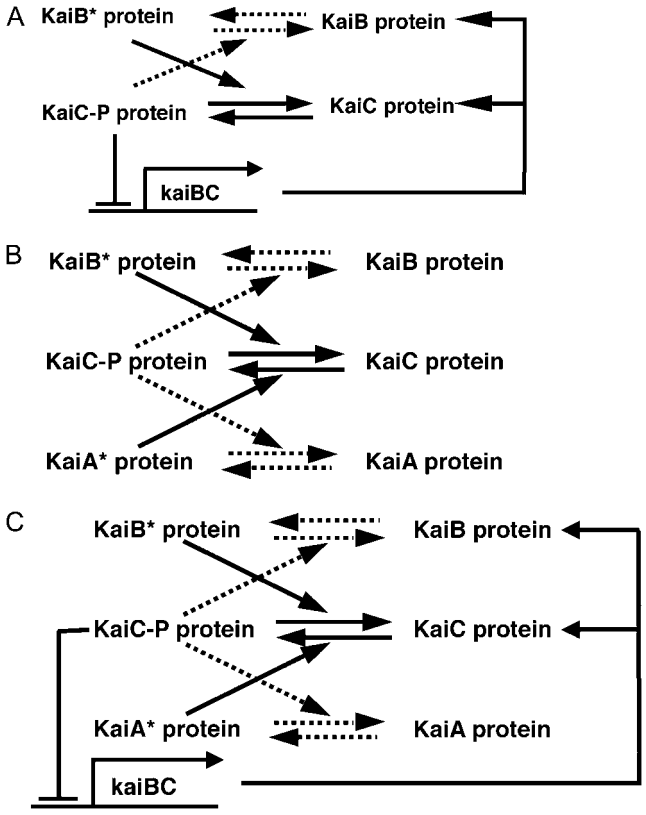

FIGURE 3 Gene-protein networks of circadian rhythms in cyanobacteria. (*A*) Negative feedback regulation of *kaiBC* expression by KaiC. (*B*) Regulations of KaiA, KaiB, and KaiC in the DD condition where transcription-translation feedback is inhibited. (*C*) Negative feedback regulation of *kaiBC* expression incorporating the dynamics of KaiA.

The regulation of the kaiBC gene

In continuous light (or LL), the total abundance of KaiB and KaiC fluctuate daily (24,29). KaiB and KaiC proteins are coded in the same transcriptional unit, named *kaiBC* gene. The expression of the *kaiBC* gene is suppressed by phosphorylated KaiC (Fig. 3 *A*). The mRNA of the *kaiBC* gene is translated into KaiB and KaiC proteins. KaiB and KaiC proteins are degraded in the light conditions.

In this section, we consider the regulation of the total abundance of the KaiB protein and the KaiC protein by phosphorylated KaiC, via the regulation of *kaiBC* gene expression. For the sake of simplicity, we consider the situation in which two proteins are assumed to change in proportion to each other, and the total abundance of the KaiB and KaiC proteins is suppressed by phosphorylated KaiC, denoted by x. To make the idea clear, we can start with the following equations:

$$dB/dt = \varepsilon_1\{B_0\lambda/(1 + h_1x^m) - \mu B\}, \quad (2a)$$

$$dC/dt = \varepsilon_1\{C_0\lambda/(1 + h_1x^m) - \mu C\}. \quad (2b)$$

Note that $\varepsilon_1\lambda$ is the transcription rate and is common for the two proteins because they are produced from the same gene. Here we assume that the decomposition rate $\varepsilon_1\mu$ is the same between these proteins. After a short transient, KaiB and KaiC come to change in proportion to each other, that is $B = B_0s(t)$ and $C = C_0s(t)$ where $s(t)$ follows,

$$ds/dt = \varepsilon_1\{\lambda/(1 + h_1x^m) - \mu\}. \quad (3)$$

The first term of the right-hand side in Eq. 3 indicates the production of KaiB and KaiC proteins by the rate decreasing with the phosphorylated KaiC abundance x, implying the inhibition of the *kaiBC* gene by phosphorylated KaiC proteins. The positive parameter m is the cooperativity in inhibition. The second term in Eq. 3 is the decay of KaiB and KaiC proteins. We assume that both production and decay of proteins occur slowly, indicated by a small factor ε_1.

Oscillation with gene expression in the LL condition

Sustained oscillations are produced if the dynamics of phosphorylated KaiC given by Eq. 1 and the regulation of

kaiBC gene given by Eq. 3 are combined. The model has three variables: x, y, and s. Fig. 4 *B* illustrates a stable oscillation (or limit cycle) of phosphorylated KaiC (x) and activated KaiB (y) with a circadian period. The mechanism of oscillation can be explained as follows. If the total protein abundance is constant (s is fixed), the dynamics of phosphorylated KaiC and active KaiB represented by Eq. 1 shows bistability. As illustrated in Fig. 2 *A*, the branch with the higher x is connected to the solution for $s > s_2^*$ at $s = s_2^*$, but it is not connected to the solution for $s < s_1^*$ at $s = s_1^*$. In contrast the lower branch is connected to the solution for $s < s_1^*$, but it ends at a saddle-node bifurcation with $s = s_2^*$.

Equation 3 describes the dynamics of total protein abundance s, which changes slowly (as ε_1 is small). According to Eq. 3, s decreases with time if x is larger than a threshold, whereas s increases with time if x is smaller than it. The threshold is the value of x that changes the sign of Eq. 3. In Fig. 4 *A*, the threshold x is chosen to be between the upper and lower branches. Then if we start from a point on the branch with the higher x, the slow change of s makes the point move leftward while it stays near the upper branch and comes to $s = s_1$ at which x jumps down to the lower branch through a saddle-node bifurcation. On the lower branch, the total protein abundance s increases with time slowly and the state point moves to the right until it reaches $s = s_2$. At this point the state point jumps to the upper branch, and then s starts decreasing again. Thus perpetual oscillation is maintained.

This is a typical way of creating limit cycle oscillation, named ''relaxation oscillation''. One cycle includes a long stay near a quasiequilibrium branch and occasional fast changes describing the transition from one branch of quasiequilibria to the other. The argument depends on the fact that the time change in s is much slower than that of x, which is guaranteed if ε_1 in Eq. 3 is very small. The period and the amplitude of KaiC oscillations depend on ε_1, the speed of transcription-translation regulations. When ε_1 is small, the amplitude of the KaiC phosphorylation rhythm is large and the period of oscillation is long. As ε_1 increases, the amplitude and the period of the oscillations decrease. When transcription-translation regulations are fast, i.e., $\varepsilon_1 = 1$ in Eq. 3 is large, the abundances of phosphorylated KaiC and activated KaiB (x, y) converge to a stable steady state without oscillation. Thus a slow regulation of *kaiBC* promotes the rhythm of KaiC phosphorylation (Fig. 4, *A* and *B*).

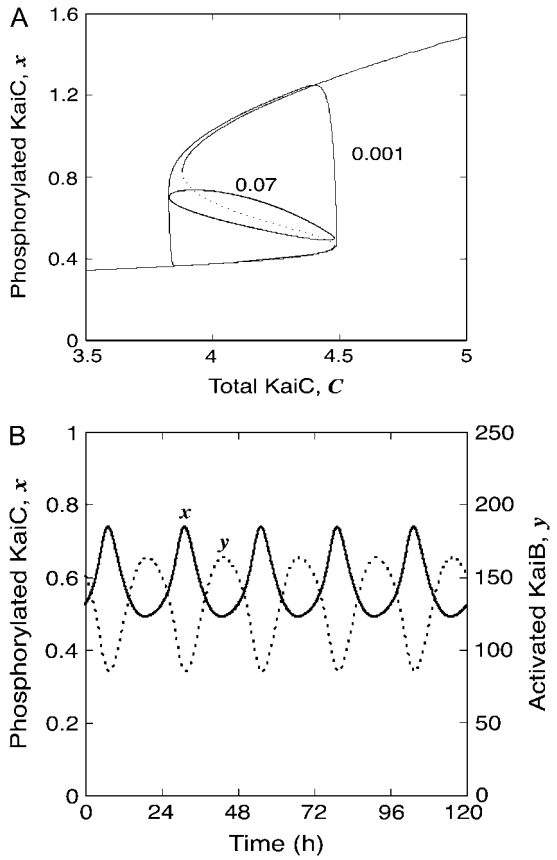

FIGURE 4 Relaxation oscillations in the cyanobacterial circadian clock. (*A*) Abundance of phosphorylated KaiC oscillates under the regulation of total KaiB and KaiC (see Eq. 3). The timescale of transcription and translation is varied by changing ε_1. Numbers by the graphs are for ε_1. When ε_1 is small (i.e., $\varepsilon_1 = 0.001$), the amplitude of the cycle is large with a large period. When ε_1 is very large (i.e., $\varepsilon_1 = 1$), abundance of phosphorylated KaiC converges to steady state. This diagram has been obtained with XPPAUT. Parameters are as follows: $\lambda = 15$, $h_1 = 1$, $\mu = 6.3$, and $m = 4$. Other parameter are the same as in Fig. 2 *A*. (*B*) Oscillations of the abundance of phosphorylated KaiC (indicated by the *smooth curve*) and activated KaiB (indicated by the *dotted curve*) for $\varepsilon_1 = 0.07$.

For very large *n* and slow/fast splitting the dynamics

The oscillation is caused by the existence of bistability for an interval of s, and the way the upper and lower branches of x are connected with the solution outside of the bistability interval. In general cases, the exact location of the bistability branches can be known only numerically. However when the cooperativity n is very large, we can simplify Eq. 1 and derive an explicit solution. In the Appendix, we explain the analytic solution of the equilibrium of Eq. 1 when n is large, from which we can tell how the two branches for bistability region are connected with the solution outside of the bistability interval.

OSCILLATION WITHOUT GENE EXPRESSION IN THE DD CONDITION

If the gene expression and decomposition of proteins are suppressed experimentally, the total abundance of KaiC is kept constant, but the fraction of phosphorylated KaiC can oscillate with a period close to 24 h (26). This observation cannot be explained by traditional clock gene models, which assume oscillation caused by a negative feedback regulation

of gene expression. In these clock mechanisms, the fluctuation of the total abundance of proteins plays an essential role.

In the model we here propose, the bistability of the KaiC phosphorylation can be generated by the regulation of the active KaiB by phosphorylated KaiC. The bistability provides an opportunity for the oscillation even when total protein abundance is kept constant. We consider the case in which transcription-translation regulation is suppressed. Since the total abundances of proteins KaiB and KaiC are kept constant, s in Eq. 1 is unchanged. Instead we consider the slow change in a. We assume that 1), there are active forms of KaiA and inactive forms of KaiA, and 2), the parameter a in Eq. 1 increases with the abundance of active forms of KaiA; we also assume that 3), a high phosphorylated KaiC slowly inactivates KaiA. The kinetic equation for the parameter a due to change in activity level of KaiA is given by

$$da/dt = \varepsilon_2\{l(w-a) - k_2(1+h_2x)a\}. \quad (4)$$

Equation 4 is for the balance of KaiA activation occurring at constant rate $\varepsilon_2 l$ and inactivation of KaiA at rate $\varepsilon_2 k_2(1+h_2x)$, which increases with phosphorylated KaiC. A large x decreases a, which implies that a high level of phosphorylated KaiC (large x) inactivates ''KaiA'', denoted by a. Then a reduced a makes KaiC phosphorylation slower, as indicated in the first term of Eq. 1a.

In Eq. 4, KaiA is assumed to be inactivated by phosphorylated KaiC. One possible mechanism for this inactivation is the depletion of free KaiA when some KaiC are phosphorylated. It is known that the number of molecules of KaiC in a cell is from 5000 to 15,000, which is an order of magnitude greater than that of KaiA molecules, which is from 250 to 500 (see Kitayama et al. (24)). Hence if phosphorylated KaiC is able to form a complex with KaiA, it can deplete free KaiA without reducing the relative abundance of phosphoryrated KaiC much.

The regulation of the activity of ''KaiA'' by phosphorylated KaiC can cause the transition between the state with high phosphorylated KaiC and the one with low phosphorylated KaiC. By incorporating the regulatory mechanism of ''KaiA'', self-sustained oscillations can occur.

In the DD condition, transcription-translation regulation and degradation of protein are suppressed in cyanobacteria (26,30,31). Dynamics in DD is given by Eqs. 1 and 4 with s fixed (see Fig. 3 *B*). In contrast, KaiC dynamics in LL is given by Eqs. 1, 3, and 4, which incorporate both the regulation of the *kaiBC* gene and the dynamics of KaiA activation (Fig. 3 *C*). Fig. 5 illustrates KaiC phosphorylation rhythms in LL and DD conditions. In DD without transcriptional regulation, only phosphorylated KaiC oscillates but the total KaiC remains unchanged. In contrast, in LL with transcriptional regulation both the total KaiC proteins and phosphorylated KaiC oscillate. We can see that the amplitude of oscillation in phosphorylated KaiC is similar in DD and in LL, and also that the period is similar between these two. These results correspond to the experimental finding in which the period in LL is close to the period in DD (27).

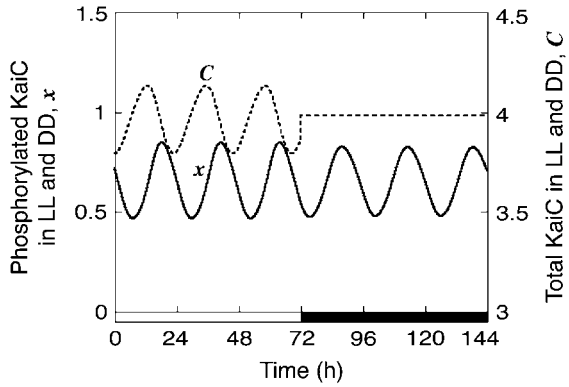

FIGURE 5 Persistence of KaiC phosphorylation rhythm in LL and DD. The dotted curve indicates abundance of KaiC. Abundance of KaiC oscillates in LL conditions in which the period of the oscillation is ~24 h. Transcription, translation, and degradation of proteins are suppressed in DD conditions. Therefore abundance of KaiC is almost constant in DD. However the abundance of phosphorylated KaiC shown by the solid curve still oscillates in DD. Moreover the period of the oscillation of phosphorylated KaiC is similar in DD and in LL. Parameters are as follows: $p = 2.5$, $b = 0.025$, $g = 10$, $k_1 = 250$, $\varepsilon_1 = 0.02$, $\lambda = 5.95$, $\mu = 2.5$, $\varepsilon_2 = 0.02$, $l = 2.5$, $w = 0.551$, $k_2 = 2.5$, and $h_2 = 1$. The other parameters are the same as in Fig. 4 *B*.

In our model, if the abundance of KaiA is enhanced, the fraction of phosphorylated KaiC increases and saturates to 1. This is consistent with the experimental results reported by Kitayama et al. (24).

ALTERNATIVE INTERACTIONS

In previous sections we studied the case in which phosphorylated KaiC inactivates KaiB, as given by Eq. 1b (see Fig. 1 *A*). This created bistability, which is a potential basis for generation of circadian oscillations in DD and in LL. In this section, we consider alternative regulatory mechanisms of KaiB activity by phosphorylated KaiC protein that can generate bistability. We study 1), the enhancement of the activation of KaiB by nonphosphorylated KaiC (as illustrated in Fig. 1 *B*); 2), the suppression of the activation of KaiB by phosphorylated KaiC (Fig. 1 *C*); and 3), the suppression of the inactivation of KaiB by the nonphosphorylated KaiC (Fig. 1 *D*). We examine whether any of them can be promising as the basis for sustained oscillations.

KaiB is activated by nonphosphorylated KaiC

We consider the case in which nonphosphorylated KaiC enhances the activation of KaiB, as illustrated in Fig. 1 *B*. The dynamics of activated KaiB is given by

$$dy/dt = g(B_0s - y)(C_0s - x)^n/(q^n + (C_0s - x)^n) - k_1y. \quad (5)$$

The first term of the right-hand side of Eq. 5, KaiB activation is enhanced by nonphosphorylated KaiC denoted by $C_0s - x$.

We combine Eqs. 1a and 5, and the equilibrium is illustrated in Fig. 2 *B*. The vertical axis is phosphorylated KaiC x at equilibrium, and the horizontal axis is $C = C_0s$, the total abundance of KaiC. For an intermediate level of total KaiC abundance, there are two simultaneously stable steady states differing in the phosphorylated KaiC abundance, the one with a large x and the second with a small x (Fig. 2 *B*). Therefore Eq. 5 can also generate bistability of KaiC phosphorylation.

Again let $s_1^* < s < s_2^*$ be the interval for the bistability. Then the branch of a higher x is connected with the solution of $s < s_1^*$ but is not connected with the solution of $s > s_2^*$ (Fig. 2 *B*). There is a discontinuity of the solution of a higher x at $s = s_2^*$. In contrast the lower branch solution is connected with the solution for $s > s_2^*$, but is not connected with the solution for $s < s_1^*$ (Fig. 2 *B*). Hence the way two branches in the bistability interval are connected with the solution outside of the interval is different from the case studied before (see Fig. 2 *A*).

When the total protein abundance s changes with time following Eq. 3, the point on the upper branch moves to the left and smoothly connects to the solution for $s < s_1^*$. The point moves until it reaches the cross point of the solution and the threshold level of x at which Eq. 3 is equal to zero, and the state point stays there forever. If the state starts from the lower branch of the bistability interval, s increases and the state point moves toward the right, until finally it reaches the equilibrium at which the solution of $s > s_2^*$ crosses with the threshold level of x at which Eq. 3 is equal to zero. Hence the perpetual oscillation is not possible. When n is very large, we can derive these results mathematically, as explained in the Appendix.

We can conclude that a relaxation oscillation in LL cannot occur in the model in which nonphosphorylated KaiC activates KaiB.

Activation of KaiB is suppressed by phosphorylated KaiC

We consider the case in which phosphorylated KaiC suppresses the activation of KaiB (see Fig. 1 *C*). The dynamics of active KaiB is given by

$$dy/dt = gq^n(B_0s - y)/(q^n + x^n) - k_1y. \quad (6)$$

Equations 1a and 6 are combined, and at equilibrium, the abundance of phosphorylated KaiC (x) increases with the total abundance of KaiC (s). This model can generate bistability but the magnitude of difference in x between upper and lower branches is very small (see Fig. 2 *C*), and it is very difficult to create the oscillation based on this mechanism. We numerically calculated the model for a wide range of parameters and observed that the region of bistability for the model given by Eq. 6 is always much narrower than that in the model given by Eq. 1b.

Inactivation of KaiB is suppressed by nonphosphorylated KaiC

When nonphosphorylated KaiC suppresses the inactivation of KaiB (as illustrated in Fig. 1 *D*), the dynamics of active KaiB is studied by

$$dy/dt = g(B_0s - y) - k_1yq^n/(q^n + (C_0s - x)^n). \quad (7)$$

This combined with Eq. 1a gives the dynamics for KaiB and KaiC. The abundance of phosphorylated KaiC (x) at equilibrium as a function of total KaiC protein ($C = C_0s$) is illustrated in Fig. 2 *D*. For an intermediate value of s, there is an interval in which bistability is shown (see Fig. 2 *D*). The way two branches within this bistability interval are connected to the solution outside of the interval is similar to Fig. 2 *B*. Hence the model does not show oscillation when Eqs. 7 and 1a are combined with the dynamics of s, given by Eq. 3.

In short, these three alternative modes of regulations are either unable to produce cycles or the amplitude of the cycle is much smaller than the case studied earlier in which phosphorylated KaiC inactivates KaiB.

DISCUSSION

In cyanobacteria, KaiC protein suppresses the expression of its own gene, causing a feedback loop with a long time delay, resulting in a sustained oscillation. KaiB is coded in the same transcriptional unit as KaiC (i.e., the *kaiBC* gene) and oscillates together with KaiC in the LL condition. This study suggests that KaiB and KaiA might work to create nonlinearity within the feedback loop of KaiC for the organisms without nuclear membrane.

Theoretical studies of clock systems have shown that many of the structures possessed by the reaction kinetics of circadian clock systems play a role in helping to form sustained oscillation. For example, in Drosophila, a cell is compartmentalized as a nucleus and cytosol, and there is a strong nonlinearity of the nuclear transport of protein. As PER proteins accumulate in the cytosol, they do not enter the nucleus until a threshold amount of PER protein is accumulated in the cytoplasm, which creates all-or-none-like transport (32). This nonlinearity in the nuclear transport makes the oscillation more likely according to a theoretical study (19). However in cyanobacterial, cells have no nucleus separated from cytosol.

Moreover circadian oscillations can occur in an in vitro system without any spatial structure (27). Hence an additional source of nonlinearity in the feedback loop is required

to realize oscillation, and this additional nonlinearity might be realized by KaiB.

The most peculiar aspect of the circadian rhythm system of cyanobacteria is that, in the DD condition, a large fraction of the metabolic activity (including RNA and protein syntheses) stops (31), presumably to save energetic expenditure under photosynthetically unfavorable environment. On the other hand, before the onset of daytime, to prepare the sufficient metabolic activity would be useful and having a rhythm based on an internal clock would be energetically economical (31). An accurate clock that works under the DD condition without gene expression would be important for their lives. Hence from an energetic viewpoint, having two separate modes of clock kinetics, with and without gene expression, is quite advantageous for the cyanobacteria's life.

In this work we explored a new model that can explain the stable oscillation in the condition both when there is no gene expression with the protein abundance constant and when there is gene expression that fluctuates periodically. The basic idea underlying the model in this work is that a single bistability created by nonlinear interaction between KaiB and KaiC would create an opportunity for relaxation oscillation. And the slow change in the active KaiA level and the slow change in the *kaiBC* gene expression level create oscillations in DD and in LL, respectively. If this scenario is correct, we predict that the oscillation in the DD condition (promoted by KaiA dynamics) and the oscillation in the LL condition (promoted by *kaiBC* gene regulation) should have a similar amplitude, although they are unlikely to be exactly the same. According to the experimental study in vivo (26), the fraction of phosphorylated KaiC fluctuates between 0.2 and 0.8 in the LL condition, but it fluctuates between 0.1 and 0.8 in the DD condition, suggesting a slightly larger amplitude in the DD condition. However experiments in vitro with KaiA, KaiB, KaiC, and ATP conclude that the fraction of phosphorylated KaiC fluctuates between 0.25 and 0.65, a smaller amplitude than that in LL (27). These results seem to be consistent with the prediction that the amplitude of oscillation must not be very different in LL and in DD, and at least must have the same order of magnitude.

The period of a relaxation oscillation is controlled by the dynamics of a slow variable, and it is inversely proportional to the speed of slow dynamics. Hence the period of oscillation in the LL condition is inversely proportional to ε_1 in the *kaiBC* gene regulation given by Eq. 2. In contrast, the period of oscillation in the DD condition is inversely proportional to ε_2 in the dynamics of KaiA given by Eq. 4. Both of these periods are close to ~24 h. We conjecture that this coincidence is the result of natural selection working on the speed of KaiA dynamics and that of gene regulation. The natural selection makes the period of oscillation in DD and in LL conditions close to 24 h, so that the system functions well as a circadian clock in both conditions.

We concluded that phosphorylated KaiC inactivates KaiB, which is the most likely way of interaction that generates oscillation. This is based on the analysis assuming that phosphorylated KaiC inactivates the *kaiBC* promoter, which is consistent with the experimental observation by Nishiwaki et al. (25). On the other hand, Iwasaki et al. (22) reported that mutant with perfectly inactivated KaiC has a reduced *kaiBC* expression. If *kaiBC* expression is assumed to be enhanced by nonphosphorylated KaiC, we observed the relaxation oscillation depicted in Fig. 4 *A*. Thus we can derive the same conclusion. However, if *kaiBC* expression is enhanced by phosphorylated KaiC, we did not observe oscillations when phosphorylated KaiC inactivates KaiB. We can incorporate activation of *kaiBC* expression by phosphorylated KaiC by introducing, $ds/dt = \varepsilon_1\{\lambda x/(1+h_1x) - \mu s\}$. Notably, sustained oscillations occur when inactivation of KaiB is suppressed by nonphosphorylated KaiC (data not shown). In this case we concluded that nonphosphorylated KaiC suppresses KaiB inactivation, which is the most likely way of interaction.

Concerning the role of KaiA, we assumed that KaiA activates the phosphorylation of KaiC protein and also that phosphorylated KaiC inactivates KaiA. Kageyama et al. (23) reported that a clear peak of a KaiA-KaiC complex can be observed only after a sufficient amount of phosphorylated KaiC is accumulated in a cell. This may suggest that KaiA first helps phosphorylation of KaiC, which in turn depletes free KaiA by forming a KaiA-KaiC complex, which is consistent with the assumption of our model.

Kitayama et al. (24) and Xu et al. (29) demonstrated that KaiA is required for KaiC dephosphorylation by KaiB. We can incorporate KaiA-dependent KaiC dephosphorylation by KaiB if the second term in the right-hand side of Eq. 1a is multiplied by $\gamma a/(1+\gamma a)$ in which γ is the kinetic constant. This factor is close to 1 except for small a, if γ is sufficiently large. Then we can show that bistability of the ratio of phosphorylated KaiC in the KaiB-KaiC subsystem appears and also the sustained oscillations in LL and in DD occur by assuming that phosphorylated KaiC inactivates KaiB (data not shown).

The models in this work are just a first-step attempt of modeling the circadian rhythm of cyanobacteria, which has features very different from the circadian clock of Drosophila, mouse, or Neurospora, well studied by theoretical models in the past.

APPENDIX

To examine the generation of oscillations for nonlinear dynamics, we derive an explicit solution of equilibrium for very large n (33). In the limit of very large n, we can analyze the model explicitly. From Eq. 1a, we can obtain a null cline $dx/dt = 0$ as:

$$y = paC_0s/bx - (f + pa/b). \tag{A.1}$$

We examine different assumptions of how the dynamics of active KaiB might be affected by the phosphorylated or unphosphorylated KaiC proteins.

KaiB is inactivated by phosphorylated KaiC

In the limit of very large n, from Eq. 1b we can obtain a null cline $dy/dt = 0$ as:

$$y = \begin{cases} B_0 s & \text{if} \quad x < q \\ B_0 s/(1 + k_1/g) & \text{if} \quad x > q \end{cases}. \qquad \text{(A.2)}$$

Combining these two, we have the following three cases: 1), one equilibrium with a small y and a large $x > q$; 2), one equilibrium with a large y and a small x ($<q$); and 3), there are three equilibria, namely one with a large y and small x ($<q$), and an intermediate equilibrium with $x = q$, and another with a small y and a large x ($>q$). These can be distinguished by noting the cross point of $x = q$ and Eq. A.1 and see if the value of y at the cross point is above $B_0 s$ or below $B_0 s/(1+k_1/g)$ or between these two.

Suppose $paC_0 > qbB_0$. For convenience, introduce $s_1^* = (pa+bf)/[paC_0/q - bB_0/(1 + k_1/g)]$, and $s_2^* = (pa+bf)/[paC_0/q - bB_0]$. Then, we have the following three regions:

Substituting Eq. A.2 into Eq. A.1, we get $x^* = C_0 s/[1+(b/pa)(f+B_0 s)]$ for $s < s_1^*$. This equilibrium is locally stable.

In a similar manner, for $s > s_2^*$, we get one stable equilibrium: $x^* = C_0 s/[1 + (b/pa)(f + B_0 s/(1 + k_1/g))]$.

For $s_1^* < s < s_2^*$, there are three equilibria: $x^* = C_0 s/[1 + (b/pa)(f + B_0 s/(1 + k_1/g))]$ and $x^* = C_0 s/[1 + (b/pa)(f + B_0 s)]$ are stable, but $x^* = q$ is unstable.

We can conclude that the branch with a higher x in the bistable region is connected to the solution for $s > s_2^*$, but not to the one for $s < s_1^*$. In contrast the branch with a lower x is connected to the solution for $s < s_1^*$, but not to the one for $s > s_2^*$. If this dynamics is combined with the slow dynamics of Eq. 3 or Eq. 4, we have relaxation oscillation.

Kai B activation enhanced by nonphosphorylated KaiC

The dynamics of active KaiB is given by Eq. 5. In the limit of very large n, the null cline of Eq. 5 provides the following y:

$$y = \begin{cases} B_0 s/(1+k_1/g) & \text{if} \quad x < C_0 s - q \\ 0 & \text{if} \quad x > C_0 s - q \end{cases}. \qquad \text{(A.3)}$$

Combining these two, we have the following three cases: 1), one equilibrium with a small y and a large $x > C_0 s - q$; 2), one equilibrium with a large y and a small x ($< C_0 s - q$); and 3), there are three equilibria, namely one with a large y and small x ($< C_0 s - q$), an intermediate equilibrium with $x = q$, and another with a small y and a large x ($> C_0 s - q$). These can be distinguished by noting the cross point of $x = C_0 s - q$ and Eq. A.3 and seeing if the value of y at the cross point is above $y = B_0 s/(1+k/g)$ or $y = 0$ or between these two.

Suppose $C_0 s > q$. For convenience, introduce $s_1^* = q(1 + pa/bf)/C_0$, and $s_2^* = [-b(C_0 f(g + k_1) - B_0 gq) + \{b(4B_0 C_0 g(g+k_1)(bf+ap)q + b(C_0 f(g+k_1) - B_0 gq)^2)\}^{1/2}]/(2bgB_0 C_0)$ Then we have the following three regions:

Substituting Eq. A.3 into Eq. A.1, we obtain $x^* = C_0 s/[1+(b/pa)(f+B_0 s/1+k_1/g))]$ for $s < s_1^*$. This equilibrium is locally stable.

In a similar manner, for $s > s_2^*$, there is one equilibrium: $x^* = C_0 s/[1+(bf/pa)]$ is stable.

For $s_1^* < s < s_2^*$, there are three equilibria: $x^* = C_0 s/[1+(b/pa)(f+B_0 s/(1+k_1/g))]$ and $x^* = C_0 s/[1+(bf/pa)]$ that are stable, and $x^* = C_0 s - q$ that is unstable.

Note that for small s there is one solution that corresponds to the larger x than $C_0 s - q$, whereas for large s, there is one solution that corresponds to a smaller x. This is different from case 1 studied in the text.

The dynamics of Eqs. 1a and 5 has bistability, but the way two stable branches are connected to the solutions in $s < s_1^*$ and in $s > s_2^*$ is different from the one required for the relaxation oscillation, as explained in the text. Hence this dynamics combined with the slow dynamics of s, in Eq. 3, or with the slow dynamics of a, as given by Eq. 4 cannot produce oscillations.

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