The EMBO Journal Vol.18 No.5 pp.1137–1145, 1999

Physical interactions among circadian clock proteins KaiA, KaiB and KaiC in cyanobacteria

Hideo Iwasaki, Yasuhito Taniguchi, Masahiro Ishiura[1] and Takao Kondo[1]

Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan

[1]Corresponding authors
e-mail: kondo@bio.nagoya-u.ac.jp or ishiura@bio.nagoya-u.ac.jp

The *kai* gene cluster, which is composed of three genes, *kaiA*, *kaiB* and *kaiC*, is essential for the generation of circadian rhythms in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942. Here we demonstrate the direct association of KaiA, KaiB and KaiC in yeast cells using the two-hybrid system, *in vitro* and in cyanobacterial cells. KaiC enhanced KaiA–KaiB interaction *in vitro* and in yeast cells, suggesting that the three Kai proteins were able to form a heteromultimeric complex. We also found that a long period mutation *kaiA1* dramatically enhanced KaiA–KaiB interaction *in vitro*. Thus, direct protein–protein association among the Kai proteins may be a critical process in the generation of circadian rhythms in cyanobacteria.

Keywords: circadian rhythm/*kai* genes/protein–protein interaction/*Synechococcus*

Introduction

Circadian rhythms have been observed ubiquitously in organisms from cyanobacteria to green plants and humans. The temporal regulation of various metabolic and behavioral activities by the rhythms is thought to be adaptive to daily changes in environmental conditions, such as light, temperature and humidity (Bünning, 1973). To elucidate the molecular mechanism of the circadian oscillator, several clock genes and clock-related genes have been cloned and analyzed in various organisms: the *Clock* (King *et al*., 1997; Allada *et al*., 1998; Darlington *et al*., 1998), *period* (Bargiello *et al*., 1984; Jackson *et al*., 1986; Zehring *et al*., 1984; Sun *et al*., 1997; Tei *et al*., 1997), *BMAL1* (or *cycle*) (Darlington *et al*., 1998; Gekakis *et al*., 1998; Rutila *et al*., 1998) and *timeless* (Gekakis *et al*., 1995; Myers *et al*., 1995; Sangoram *et al*., 1998; Zylka *et al*., 1998) genes in *Drosophila* and mammals; the *doubletime* gene (Kloss *et al*., 1998) in *Drosophila*; the *circadian clock associated-1* (Wang and Tobin, 1998) and *late elongated hypocotyl* genes (Schaffer *et al*., 1998) in *Arabidopsis*; and the *frequency* (*frq*; McClung *et al*., 1989) and *white collar-1* and *white collar-2* genes (Crosthwaite *et al*., 1997) in *Neurospora*.

Cyanobacteria are the simplest organisms to exhibit circadian rhythms, and molecular genetic techniques are well established for several cyanobacterial strains (reviewed by Golden *et al*., 1997). We introduced the bioluminescence reporter gene into the unicellular cyanobacterium *Synechococcus* sp. PCC 7942 to monitor circadian gene expression (Kondo *et al*., 1993) and isolated various rhythm mutants (Kondo *et al*., 1994). Using genetic complementation of these mutants, a gene cluster *kai* composed of three genes, *kaiA*, *kaiB* and *kaiC*, was recently cloned (Ishiura *et al*., 1998). Altered circadian phenotypes can arise from mutation in any of the *kai* genes. In particular, 19 distinct clock mutants that exhibit a wide range of period length and even arrhythmia map to the *kaiC* gene, and disruption of each of the *kai* genes abolishes circadian rhythmicity. Over-expression of *kaiC* completely suppresses rhythmic *kaiBC* expression, suggesting that KaiC acts as a negative factor in the clock gene feedback. In contrast, KaiA possibly acts as a positive regulator for *kaiBC* expression. Thus, it is thought that feedback of the *kai* gene products to their own transcription constitutes the circadian oscillator of cyanobacteria (Ishiura *et al*., 1998), as proposed for clock genes of *Drosophila* (Hardin *et al*., 1990; Zeng *et al*., 1994; Curtin *et al*., 1995; Sehgal *et al*., 1995; Allada *et al*., 1998; Rutila *et al*., 1998; Darlington *et al*., 1998), *Neurospora* (Aronson *et al*., 1994; Merrow *et al*., 1997) and mammals (Gekakis *et al*., 1998).

To understand roles of the *kai* genes in the formation of the circadian clock, it is essential to dissect the biochemical properties of the three Kai proteins. We did not find any similarity between the Kai proteins and any known clock proteins in other organisms. Moreover, the Kai proteins do not contain any known functional motifs except for several amino acid clusters in KaiC that are conserved in many ATP-/GTP-binding proteins (Ishiura *et al*., 1998). In prokaryotes, a cluster organization of genes is often correlated to their cooperative functions. In *Drosophila*, heterodimeric clock protein interactions between Period (PER) and Timeless (TIM), and between Cycle (CYC, dBMAL1) and Clock (CLK) proteins may be essential for the assembly of the circadian oscillator (Gekakis *et al*., 1995; Rutila *et al*., 1996; Saez and Young, 1996; Zeng *et al*., 1996; Darlington *et al*., 1998).

In the present paper, we show various hetero- and homotypic associations among three Kai proteins in yeast cells *in vitro*, and in *Synechococcus* cells, and enhancement of KaiA–KaiB interaction by a long period mutation *kaiA1* (Ishiura *et al*., 1998). These observations suggest that physical interactions among the Kai proteins are important for the generation of circadian rhythms in *Synechococcus*. Moreover, we found a duplicated structure in KaiC; the first half of the protein is strikingly similar to the second half. Both halves were functional in binding to Kai proteins and suppressing *kaiBC* expression.

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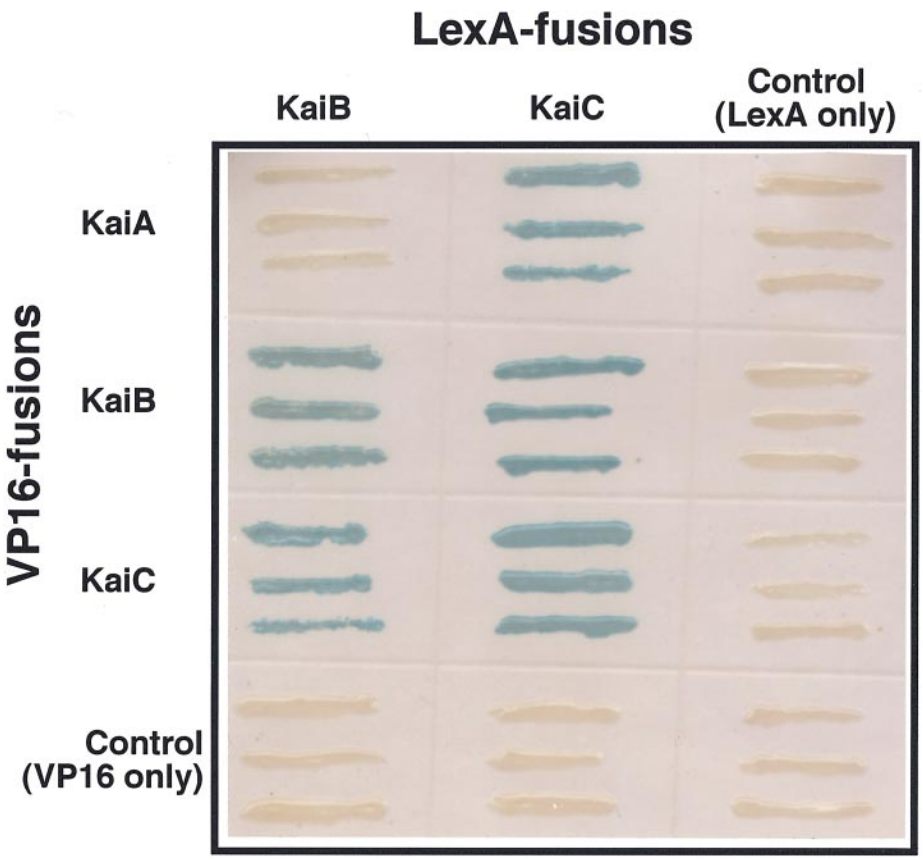

Fig. 1. Interactions among the three Kai proteins in yeast cells. Plasmids that carried chimeric gene fusions to express the DNA-binding protein LexA fused to KaiB or KaiC, and the activation domain of VP16 fused to KaiA, KaiB or KaiC, were used as bait and prey, respectively. Unfused LexA and VP16 genes were used as negative controls. Three colonies of each doubly transformed clone (carrying one bait and one prey construct) were streaked onto an agar plate deficient in tryptophan and leucine, and β-galactosidase activity was then monitored by the filter assay in the presence of X-gal at 30°C for 20 min.

Results

Kai proteins associate in yeast

We first tested direct association among Kai proteins using the yeast two-hybrid system (Fields and Song, 1989; Vojtek *et al*., 1993). Each of the three Kai proteins was expressed as a fusion with either a DNA-binding protein, LexA, or the transcriptional activation domain of VP16. The filter assay for β-galactosidase (Ausubel *et al*., 1996) demonstrated both heterotypic interactions (KaiA–KaiB, KaiA–KaiC and KaiB–KaiC) and homotypic interactions (KaiB–KaiB and KaiC–KaiC) in doubly transformed yeast cells that expressed two types of fusion protein (Figure 1). These interactions were detected within 20 min incubation at 30°C (Figure 1), except for the KaiA–KaiB interaction whose faint signal was detected after 8 h incubation (Figure 4A). This observation suggests that the KaiA–KaiB interaction is much weaker than the other combinations. Results obtained by both the liquid assay for β-galactosidase and the *His3* reporter assay on agar medium lacking histidine confirmed interactions among the Kai proteins (data not shown). LexA–KaiA fusion was not used for the assay because it gave a background positive signal (data not shown), which may have been caused by the highly acidic property of KaiA (pI =4.6).

Kai proteins associate in vitro

Next, we examined interactions among the three Kai proteins using an *in vitro* biochemical assay. Glutathione *S*-transferase (GST) fused to KaiA, KaiB or KaiC and unfused control GST were expressed separately in *Escherichia coli* and immobilized to an affinity resin (Figure 2A). KaiA and KaiC were labeled with ^{35}S *in vitro*, using a rabbit reticulocyte system (Figure 2B). Each of the ^{35}S-labeled proteins was incubated with the resin that carried immobilized GST or each GST–Kai fusion protein. The ^{35}S-labeled proteins bound to the resin were then eluted and analyzed using SDS–PAGE followed by autoradiography. The results demonstrated that both KaiA and KaiC bound KaiA, KaiB and KaiC *in vitro* (Figure 2C). The KaiA–KaiB interaction was weaker *in vitro*, as observed in yeast cells (Figure 1). The doublet bands of *in vitro* translated KaiA (Figure 2B and C) may be due to alternative translation or degradation.

KaiB contains no internal methionine residues to be labeled by ^{35}S. Therefore, a His_6–KaiB fusion was used to examine interactions between KaiB and each of three Kai proteins. Purified and immobilized His_6–KaiB fusion (Figure 2A) was incubated with purified GST–Kai fusion proteins. The GST–Kai fusions, which were bound to His_6–KaiB, were eluted and fractionated by SDS–PAGE,

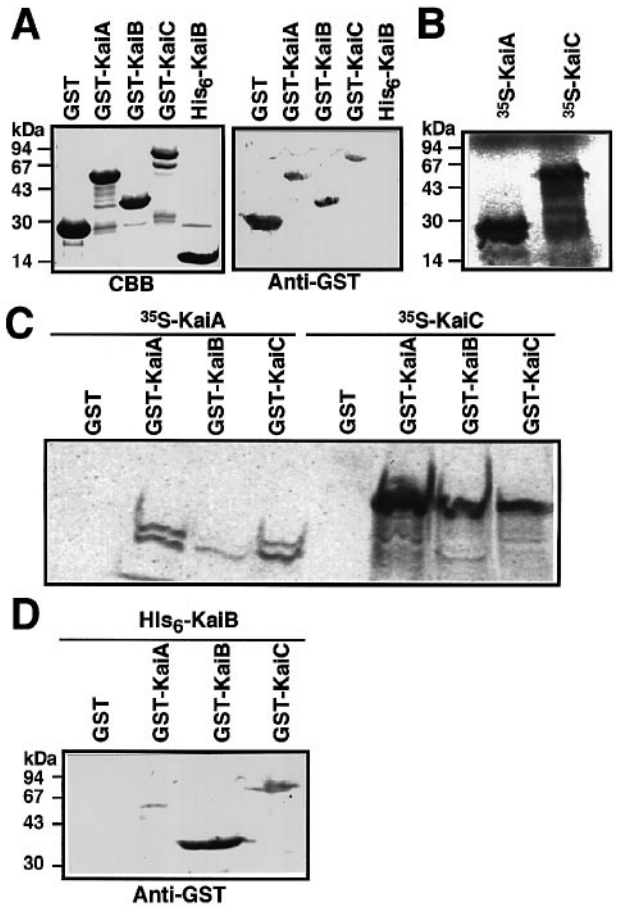

Fig. 2. *In vitro* assay for interactions among the three Kai proteins. (**A**) Production of fusion proteins in *E.coli*. GST, GST fused to KaiA, KaiB or KaiC, and His_6-tagged KaiB proteins were produced in *E.coli* and purified by affinity chromatography with a matrix appropriate for the GST or His_6 tag. The fusion proteins (~5 μg each) were eluted from the matrices and fractionated by SDS–PAGE on 10–15% gradient gels, then stained with CBB and analyzed by immunoblotting experiments using an antibody against GST. (**B**) *In vitro* translated, ^{35}S-labeled KaiA and KaiC. Proteins were generated and labeled with ^{35}S *in vitro* using the rabbit reticulocyte system. Aliquots (1.5 μl) of the reaction mixtures were size-fractionated using SDS–PAGE on 10% gels and analyzed by autoradiography. (**C**) Interactions between GST–Kai fusions and ^{35}S-labeled KaiA or KaiC. GST, GST–KaiA, GST–KaiB and KaiC proteins (10 μg) were immobilized on glutathione–Sepharose 4B and then incubated with 3 μl of the reticulocyte reaction mixture containing the ^{35}S-labeled KaiA or KaiC protein. Proteins associated with the resin were detected by SDS–PAGE on 10% gels followed by autoradiography. (**D**) Interactions between GST–Kai fusions and His_6-tagged–KaiB. His_6–KaiB protein (10 μg) was immobilized on TALON metal-affinity resin and incubated with 5 μg of GST, GST–KaiA, GST–KaiB or GST–KaiC. Proteins bound to the resin were eluted and fractionated by SDS–PAGE on 10–15% gradient gels and subjected to immunoblot analysis using an antibody against GST.

then detected by Western blotting using an anti-GST antibody. The results shown in Figure 2D demonstrate the homotypic interaction of KaiB as well as KaiB–KaiC and weak KaiA–KaiB interactions.

Kai proteins interact in Synechococcus cells

Physical associations among the Kai proteins in cyanobacterial cells were examined using an affinity co-purification method. We constructed a DNA fragment that contained the entire *kai* gene cluster in which the *kaiA*,

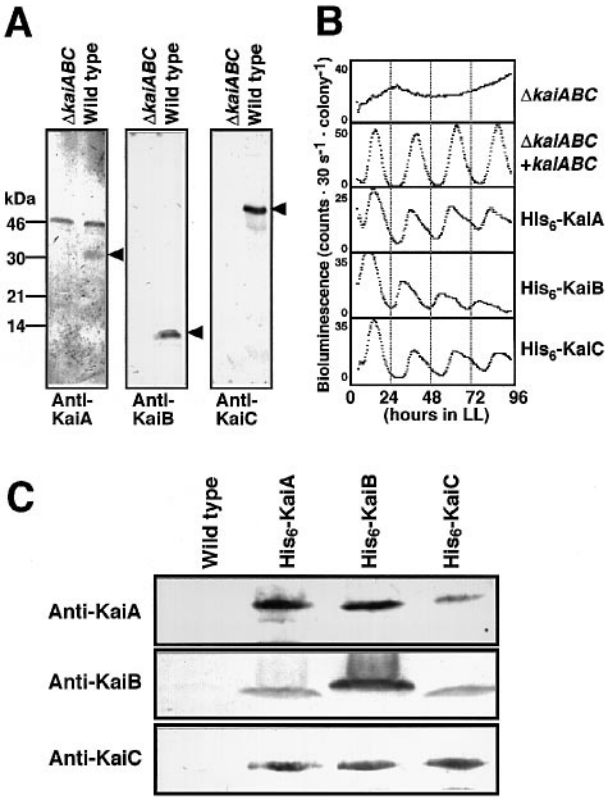

Fig. 3. Associations among Kai proteins in cyanobacterial cells. (**A**) Immunological detection of Kai proteins in *Synechococcus*. Soluble proteins (5 μg) were extracted from cells of a wild-type strain or *kai*-cluster-deficient (*ΔkaiABC*) strain and then analyzed by SDS–PAGE on 10–15% gradient gels followed by immunoblotting experiments using anti-KaiA, anti-KaiB and anti-KaiC antisera. The arrows indicate specific signals detected only in the wild-type strain. (**B**) Recovery of bioluminescence rhythms by introducing modified *kai* gene clusters. The *ΔkaiABC* strain was transformed with plasmids to carry a *kai* gene cluster segment at the original *kai* locus, which produced wild-type Kai proteins (*ΔkaiABC* + *kaiABC*), or two wild-type Kai proteins and one Kai protein fused to a His_6-tag (His_6–KaiA, His_6–KaiB and His_6–KaiC). Cells were grown for 3–4 days on solid medium in LL conditions to give 30–60 colonies (0.1 mm diameter). After a 12 h dark treatment, the bioluminescence from the dish was measured using a photomultiplier tube. (**C**) Co-purification of Kai proteins. Soluble proteins extracted from wild-type *Synechococcus* cells or cells that were producing either His_6-tagged KaiA, His_6-tagged KaiB or His_6-tagged KaiC protein were incubated with TALON metal-affinity resin. Proteins associated with the resin were analyzed using SDS–PAGE on 10–15% gradient gels followed by immunoblotting using anti-Kai antisera.

kaiB or *kaiC* ORF was modified to encode KaiA, KaiB or KaiC fused to a His_6-tag at the N-terminus. Each DNA construct was introduced into a *kai*-deficient strain in which the *kai* cluster had been replaced with a kanamycin resistance gene. Thus, each transformant regained a different modified *kai* cluster at the original *kai* locus. Our previous analyses demonstrated that inactivation of each *kai* gene completely nullified the circadian oscillation (Ishiura *et al.*, 1998). However, *Synechococcus* producing the His_6–Kai fusion protein showed circadian expression of the *kaiBC* operon, as monitored by bioluminescence with a P_{kaiBC}*::luxAB* reporter (Figure 3B), although the amplitude of the rhythms was weaker in the transformants than in a control strain that had regained an unmodified *kai* gene cluster (Figure 3B, second panel). Thus, each of the His_6–Kai fusion proteins was functional

in *Synechococcus*. Next, we performed affinity-purification of the His_6–Kai protein from cell extracts of each transformants using TALON metal-affinity resin. Eluted proteins were separated by SDS–PAGE, blotted onto nitrocellulose membranes and incubated with each anti-Kai antiserum. Each His_6-tagged Kai fusion protein was co-purified with the other two Kai proteins, as detected by each antiserum (Figure 3C). This result indicates heterotypic interactions among the Kai proteins in cyanobacterial cells.

KaiC enhances KaiA–KaiB interaction

KaiA–KaiB interaction was weak both in yeast cells and *in vitro*. Therefore, we examined whether the presence of KaiC in the reaction would alter the KaiA–KaiB interaction. To test this in yeast cells, we modified the LexA–KaiB expression vector to carry an extra expression unit for unfused KaiC. Transformants that simultaneously expressed VP16–KaiA, LexA–KaiB and unfused KaiC exhibited significantly higher β-galactosidase activity than those producing VP–KaiA and Lex–KaiB (Figure 4A). The β-galactosidase activity from the KaiABC-expressing cells was detected by 2 h incubation at 30°C (data not shown), whereas KaiA–KaiB interaction was detected only after 8 h incubation (Figure 4A). Negative control transformants that produced LexA–KaiB, unfused VP16 and KaiC did not show such activity. We confirmed that the accumulation levels of the KaiA- and KaiB-hybrid proteins were almost equivalent both in KaiABC-expressing yeast cells and in KaiAB-expressing cells, as estimated by Western blotting using anti-KaiA and anti-KaiB antisera (data not shown). Thus, the presence of KaiC is not likely to affect the stability of KaiA and KaiB proteins. These results suggest that KaiC dramatically enhanced the KaiA–KaiB interaction. We also examined the effect of KaiC on the KaiA–KaiB interaction *in vitro*. The purified and immobilized His_6-KaiB protein was incubated with the ^{35}S-labeled KaiA protein in the presence of GST or GST–KaiC. The GST–KaiC fusion protein markedly enhanced KaiA–KaiB interaction (Figure 4B).

A long period kaiA allele enhances the KaiA–KaiB interaction

To test whether such protein–protein interactions among the three Kai proteins are involved in the timing mechanism in *Synechococcus*, we examined the effects of *kai* mutations on associations among the Kai proteins. We found that the *kaiA1* mutation enhanced the KaiA–KaiB interaction *in vitro* (Figure 5A, lanes 1 and 3), whereas it did not alter the strength of the KaiA–KaiC association (Figure 5B). As shown in Figure 5C, the A33a mutant that carries this mutation (an amino acid substitution of Glu103 to Lys in KaiA; Ishiura *et al*., 1998) lengthens the period of the rhythm by 8–9 h. Moreover, KaiC enhanced KaiA–KaiB interaction as described above, but did not enhance the KaiA1 (KaiA[*kaiA1*])–KaiB interaction (Figures 5A, lanes 3 and 4, and 4A). The abnormality in Kai-binding profiles caused by the long period mutation suggests that association among the Kai proteins may be an important step in the generation of the circadian period in *Synechococcus*.

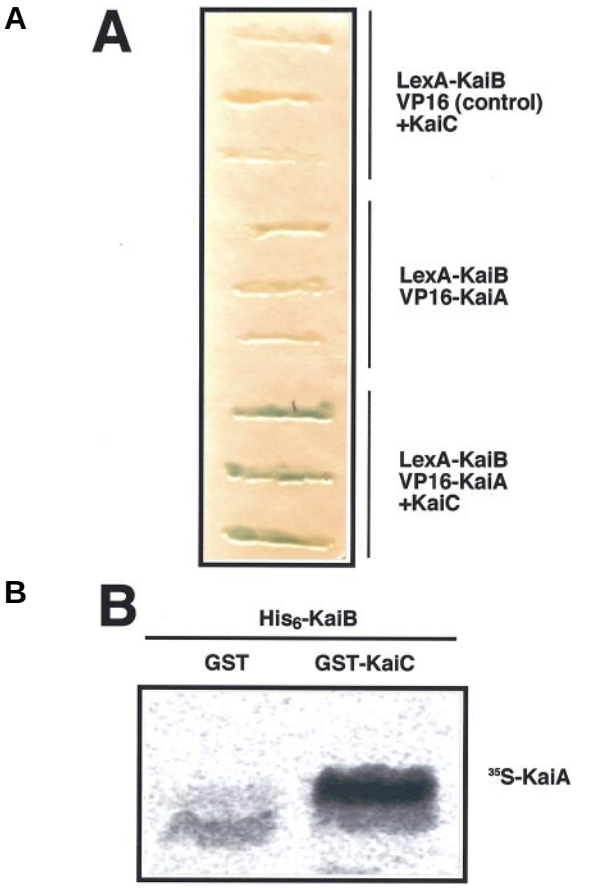

Fig. 4. KaiC protein enhances KaiA–KaiB interaction. (**A**) The yeast two-hybrid assay. β-galactosidase activities of three colonies expressing LexA–KaiB, VP16 and unfused KaiC (upper), LexA–KaiB and VP16–KaiA (middle) or LexA–KaiB, VP16–KaiA and unfused KaiC (lower) were estimated using the filter assay at 30°C for 8 h. (**B**) *In vitro* binding assay. The His_6–KaiB fusion protein was immobilized on the affinity resin and then incubated with ^{35}S-labeled KaiA in the absence (left lane) or presence (right lane) of GST–KaiC. The resin-associated proteins were subjected to SDS–PAGE followed by autoradiography.

Duplication of Kai-binding activities in KaiC

Analysis of the deduced amino acid sequence of KaiC revealed significant similarity between the first and second halves of the sequence. As shown in Figure 6A, identity and similarity between the first half (amino acid residues 1–260) and the second half (residues 261–519) are 20.8 and 41.6%, respectively. Each pair of ATP-/GTP-binding motifs (P-loop or Walker's motif A, imperfect Walker's motif Bs and putative catalytic carboxylate Glu residues in ATP-binding proteins) in KaiC (Ishiura *et al*., 1998) maps to corresponding locations in both halves. Therefore, KaiC is composed of tandemly duplicated domains.

We designated the first and second half domains of KaiC as CI and CII, respectively, and examined the *in vitro* binding of each to Kai proteins. GST–CI (KaiC; residues 2–250), GST–CII (KaiC; residues 253–519), ^{35}S-labeled CI (KaiC; residues 1–250) and CII (KaiC; residues 252–519) were prepared (Figure 6B and C). Both ^{35}S-labeled CI and CII bound GST–KaiA, GST–KaiB and GST–KaiC (Figure 6D). These results indicate that both CI and CII associate with all three Kai proteins. In addition, each of the half domains associated with GST–CI and GST–

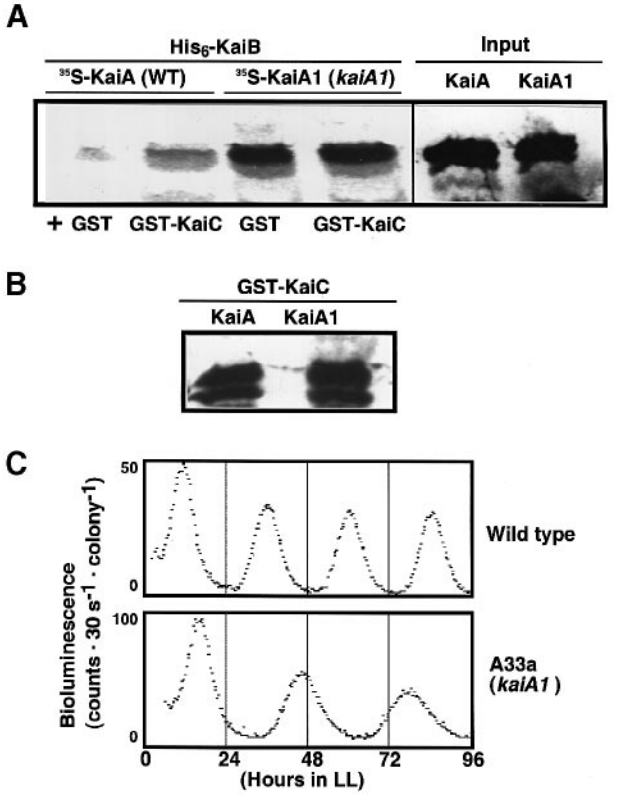

Fig. 5. *kaiA1* mutation enhances the KaiA–KaiB interaction. (**A**) *In vitro* binding assay. His_6-tagged KaiB was immobilized on affinity resins and then incubated with ^{35}S-labeled wild-type KaiA (lanes 1 and 2) or KaiA1 (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of GST–KaiC. Proteins associated with the resins were eluted and subjected to SDS–PAGE followed by autoradiography. Experimental conditions were the same as described in Figure 4B. To ensure that the amounts of KaiA and KaiA1 used for the assay were equivalent, input controls are also shown (lanes 5 and 6). (**B**) *kaiA1* does not alter the KaiA–KaiC interaction. GST-tagged KaiB was immobilized on affinity resins and then incubated with ^{35}S-labeled wild-type KaiA (lane 1) or KaiA1 (lanes 2). Experimental conditions were the same as those described in Figure 2C. (**C**) Circadian rhythms of *kaiBC* expression in wild-type and A33a (*kaiA1*) mutant strains. Bioluminescence profiles of P_{kaiBC}-reporter strains are shown. Monitoring of the bioluminescence and the representation of data were the same as in the legend for Figure 3.

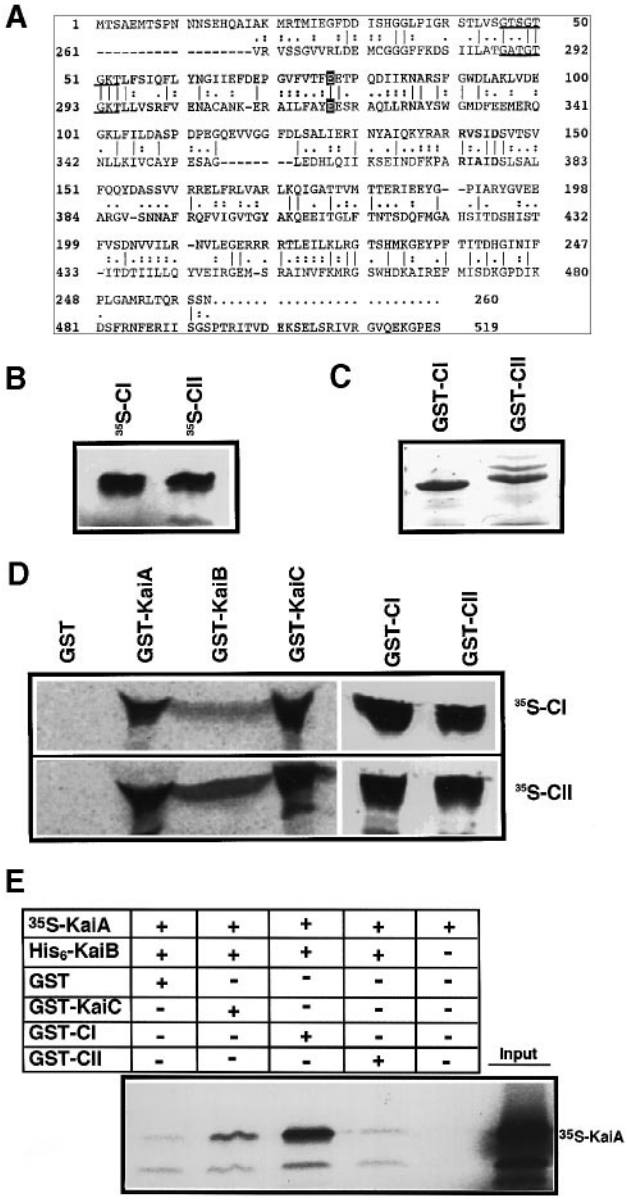

Fig. 6. Duplication of the primary structure and Kai-binding activities of KaiC. (**A**) The predicted amino acid sequence of the first half of KaiC (residues 1–260) is aligned with that of the second half (residues 261–519). Identical and similar amino acids are indicated by vertical lines and colons, respectively. Hyphens were introduced to optimize alignment. Walker's motifs A (GXXXXGKT/S) and imperfect motifs B (ZZZZD; Z, hydrophobic residue) are underlined and bold, respectively. Possible carboxylate Glu residues are reversed. (**B**) *In vitro* translated, ^{35}S-labeled CI (KaiC; 1–250) and CII (KaiC; 252–519) proteins. CI and CII were produced and labeled in a rabbit reticulocyte system. A 1.5 μl aliquot of each reaction mixture was subjected to SDS–PAGE on a 10% gel followed by autoradiography. (**C**) Affinity-purified GST–CI (KaiC; 3–250) and GST–CII (KaiC; 253–519) fusion proteins. Five micrograms of each of the proteins was analyzed by SDS–PAGE and stained with CBB as described in Figure 2A. (**D**) *In vitro* binding assay. GST, or GST fused to KaiA, KaiB, KaiC, CI or CII (10 μg) was immobilized on glutathione–Sepharose 4B and then incubated with 3 μl of the reticulocyte reaction mixture containing ^{35}S-labeled CI or CII. Proteins associated with the resin were analyzed by SDS–PAGE on 10% gels followed by auto-radiography. (**E**) Effect of CI or CII domain on KaiA–KaiB interaction *in vitro*. The His_6-KaiB fusion protein was immobilized on the affinity resin and then incubated with ^{35}S-labeled KaiA in the presence of GST control, GST–KaiC, GST–CI or GST–CII. The resin-associated proteins were subjected to SDS–PAGE followed by autoradiography.

CII fusion proteins *in vitro* (Figure 6D). Moreover, we examined whether CI or CII is sufficient to enhance the KaiA–KaiB interaction *in vitro*. Interestingly, GST–CI dramatically enhanced interaction between [^{35}S]KaiA and His_6-KaiB association, even more than the full-length KaiC fusion did, whereas GST–CII did not alter the strength of the interaction (Figure 6E). This result indicates that one of the half domains (CI) is sufficient to enhance the KaiA–KaiB interaction, and suggests distinct functional properties between CI and CII domains.

Over-expression of CI/CII domain suppresses KaiBC transcription

Previously, we demonstrated that over-expression of KaiC immediately suppressed *kaiBC* expression and blocked circadian oscillation (Ishiura *et al*., 1998). This negative feedback may constitute molecular cycling underlying the circadian clock of cyanobacteria. Thus, we examined the *kaiBC*-regulating activity of the CI and CII

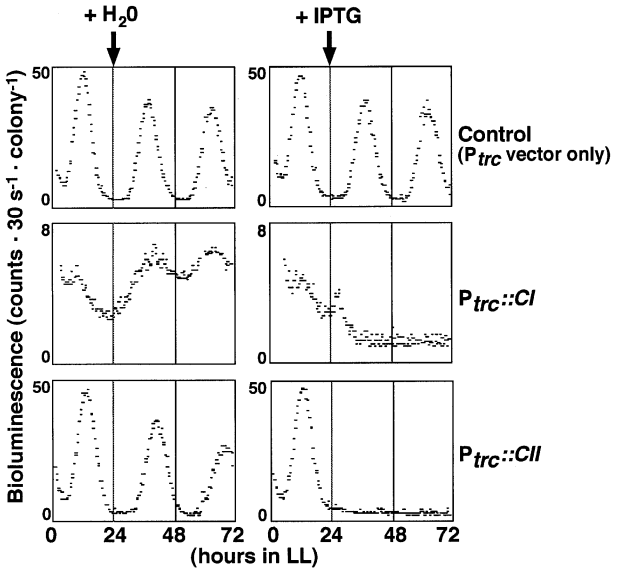

Fig. 7. The effects of over-expression of CI and CII domains of KaiC on the activity of the P_{kaiBC} promoter. Bioluminescence from P_{kaiBC} reporter strains carrying a P_{trc} vector only (control), or a P_{trc}*::CI* or P_{trc}*::CII* construct, was monitored. Cells were grown on agar plates in continuous light incubation (LL), exposed to darkness for 12 h to synchronize the clocks, returned to LL and treated with 2 mM IPTG or water at hour 24 in LL after the dark exposure (the arrows indicate the timing of addition of IPTG or water). Monitoring of the bioluminescence and the representation of data were the same as those described in the legend for Figure 3.

domains using the same strategy. As in intact KaiC, we found that over-expression of either CI or CII under the control of the inducible *E.coli trc* promoter (P_{trc}) with isopropyl-β-D-thiogalactopyranoside (IPTG) immediately abolished circadian rhythmicity and dramatically reduced the level of bioluminescence from a P_{kaiBC}*::luxAB* reporter (Figure 7). Circadian expression of the photosynthesis-related *psbAI* gene (Kondo *et al*., 1993) was also abolished by either CI or CII over-expression (data not shown). These results suggest that *kaiBC* regulating as well as Kai binding activities may be conserved between both halves of KaiC. Even in the absence of IPTG, the P_{trc}*::CI* construct lowered *kaiBC* transcriptional activity to 20% of the normal level and reduced the amplitude of the rhythm (Figure 7). Thus, CI generated by the background activity of the P_{trc} promoter probably affected the clock machinery. Note that the presence of the P_{trc}*::kaiC* construct without IPTG did not affect the rhythmicity (Ishiura *et al*., 1998), and the presence of the P_{trc}*::CII* construct did not affect the rhythm as much.

Discussion

We demonstrated that KaiA, KaiB and KaiC associated in various combinations both in yeast cells and *in vitro*. The binding profiles using the two methods were consistent: (i) any two Kai proteins can associate with each other, including homotypic interactions; (ii) the KaiA–KaiB interaction is possibly weaker than the other interactions; and (iii) KaiC enhances the KaiA–KaiB interaction. Moreover, we presented evidence that the heterotypic interactions actually occur between the Kai proteins in *Synechococcus* cells.

We do not know whether these results reflect heterodimeric interactions between two clock proteins or the formation of a heteromultimeric protein complex(es). Enhancement of the KaiA–KaiB interaction by KaiC both in yeast cells and *in vitro* may imply the formation of a large heteromultimeric complex composed of the three Kai proteins. KaiC, or a homomultimer of KaiC, may act as a bridge between KaiA and KaiB. Alternatively, KaiC could initially form a heterodimer with either KaiA or KaiB, and this binding could induce a conformational change in the partner to allow direct interaction with the other Kai protein. However, we can not rule out the possibility that transient KaiC binding alters the Kai-binding property of KaiA and/or KaiB, and the 'KaiC-activated' KaiA and KaiB may be able to form a heterodimer after releasing KaiC. Studies on the stoichiometry of the Kai protein complex will be necessary to answer these questions.

We found that the *kaiA1* mutation of *kaiA* enhanced the *in vitro* interaction of KaiA–KaiB markedly, whereas it did not affect the KaiA–KaiC interaction. Interestingly, the GST–KaiC fusion protein failed to enhance the KaiA1–KaiB interaction. Enhancement of the KaiA–KaiB interaction by the *kaiA1* mutation may be too strong to allow further enhancement by GST–KaiC. Alternatively, it is possible that an abnormality in the KaiA1–KaiB interaction in the mutant cells disallowed the interaction of KaiC with KaiA1 and/or KaiB.

How do the interactions among the Kai proteins contribute to the cyanobacterial circadian oscillator? In the *Drosophila* clock system, it is proposed that the CLK–CYC complex activates the transcription of *per* and *tim* (Allada *et al*., 1998; Darlington *et al*., 1998; Rutila *et al*., 1998). Cytoplasmic accumulation of PER and TIM leads to the formation of a heterodimer that can move to the nucleus (Saez and Young, 1996; Zeng *et al*., 1996). The nuclear PER–TIM complex is thought to negatively regulate *per* and *tim* transcription by inhibiting CLK–CYC activity (Darlington *et al*., 1998). In mammalian cells, heterodimers of Clock and BMAL1 (mammalian homologs of *Drosphila* CLK and CYC, respectively) transactivate a mammalian homolog of the *per* gene (*mper1*; Gekakis *et al*., 1998). Therefore, interactions among clock proteins may be crucial in generating the circadian oscillation. In this report, we clearly demonstrated that the three Kai proteins of cyanobacteria associate in various combinations. Although the *Drosophila* case can not simply be applied to the *Synechococcus* clock, physical association among the Kai proteins may be a critical process in the cyanobacterial clock also. As in the PER–TIM complex, formation of the Kai protein complex(es) could be involved in regulation of, as yet unknown activities of the Kai proteins in a phase-dependent manner. Alternatively, binding among KaiA, KaiB and KaiC could be important for constitutive functions of the Kai proteins. For further biochemical and functional description of the interactions among Kai proteins, it is necessary to follow temporal and spatial profiles of the Kai proteins in *Synechococcus* at various circadian phases and to examine the effects of environmental cues, such as light and temperature, on them.

Our previous analyses (Ishiura *et al*., 1998) suggest that KaiC possibly inhibits its own transcription. In contrast, KaiA may act as a positive factor for *kaiBC* expression.

Thus, it is possible that feedback of the *kaiC* gene product to its own transcription constitutes the basic circadian oscillation itself, as proposed for the *per* and *tim* genes of *Drosophila* and for the *frq* gene of *Neurospora*. In this study, we found duplication of the primary structure in KaiC and demonstrated that, like the full-length KaiC, both half-domains have Kai-binding activities. Moreover, we demonstrated that over-expression of either CI or CII was sufficient to suppress *kaiBC* expression in *Synechococcus*. Thus, the half-domains of KaiC might be functional units of the molecule.

However, both domains are apparently important for normal circadian rhythmicity because single amino acid mutations mapped on each domain (*kaiC5* in CI, *kaiC13* and *kaiC14* in CII) could abort the rhythmicity (Ishiura *et al*., 1998). Alteration of the rhythmicity by background expression of P_{trc}*::CI* (Figure 7) also implies that a balance of the two domains may be important for normal clock function. It is also possible that CI and CII have distinct functional properties. Different effects of CI and CII on KaiA–KaiB interaction *in vitro* (Figure 6E) support this possibility. In either case, Kai-binding properties of both domains may be responsible for the physiological function of KaiC in the assembly of the circadian clock. The homotypic and heterotypic associations between the half domain(s) (CI–CI, CI–CII and CII–CII) could reflect intramolecular association within a KaiC molecule and/or intermolecular association profiles among two or more KaiC molecules. Therefore, analysis for the duplicated structure of the KaiC protein could provide important biochemical information on KaiC function in the circadian oscillator of cyanobacteria. Furthermore, it should be noted that the duplication of KaiC will provide significant insights into the evolution of the circadian clock component, because ORFs similar to the CI or CII domain are found in several species of *Archaea* (M.Ishiura *et al*., manuscript in preparation).

Materials and methods

Bacterial and yeast strains

Escherichia coli strain DH5α was used as a host for both plasmid constructions and bacterial expression of the GST-fusion proteins. *E.coli* strain M15 carrying the pREP4 repressor plasmid (Qiagen) was used for bacterial induction of the His_6-tagged proteins. *Saccharomyces cerevisiae* strain L40 was used for the two-hybrid analysis (Vojtek *et al*., 1993). *Synechococcus* P_{psbAI}-reporter strain AMC149 (Kondo *et al*., 1993) and P_{kaiBC}-reporter strain NUC39 were used as positive and negative controls, respectively, to check the antigen specificity of the anti-Kai antisera. NUC39, in which the whole *kai* gene cluster was replaced with a kanamycin-resistance gene, was constructed by transformation of NUC38 (a P_{kaiBC}-reporter strain carrying the chloramphenicol-resistance gene as a selective marker gene at a specific targeting site called NSI; S.Kutsuna and M.Ishiura, unpublished results) with pD*kaiABC* (Ishiura *et al*., 1998). This strain was also used for construction of the transformants that endogenously produced each of the His_6-tagged Kai proteins. NUC38 was also used as a host for CI and CII-over-expression. The clock mutant A33a (renamed from P331; Ishiura *et al*., 1998), which carries the *kaiA1* mutation on *kaiA*, was originally isolated from chemically mutagenized AMC149 (Kondo *et al*., 1994), and the P_{psbAI}-reporter construct at NSI was replaced with the P_{kaiBC}-reporter construct (M.Ishiura and S.Kutsuna, unpublished results).

Antisera against Kai proteins

Each of the purified GST–Kai fusion proteins was used to immunize female, 10-week-old New Zealand white rabbits (total 1–1.2 mg protein/rabbit). To test the antigen specificity of the antisera, AMC149 and NUC39 were grown in BG-11 liquid medium in continuous light of 46 μE/m^2/s from white fluorescent lamps at 30°C (standard conditions) until the cell density reached 1.0 OD_{730}. Cell pellets from the 30 ml culture were resuspended in 500 μl of phosphate-buffered saline (PBS) and disrupted using the Mini Bead-beater (Biospec Products) with 1 ml of zirconium beads (0.1 mm in diameter, Biospec Products) as described previously (Kutsuna *et al*., 1998). The suspension was centrifuged at 15 000 r.p.m. for 20 min at 4°C and then the supernatant fraction was collected as a soluble cell extract. Equivalent protein amounts of cell extracts (5 μg) were size-fractionated using SDS–PAGE on 10–15% gradient gels and transferred onto nitrocellulose membranes. The membranes were immersed for 2 h in blocking buffer [10% (w/v) skim milk, 0.3% (v/v) Tween-20] at 37°C, reacted for 1 h with anti-KaiA, anti-KaiB or anti-KaiC antiserum at 200-, 1000- and 5000-fold dilution, respectively in blocking buffer at 37°C, and then reacted for 1 h with anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, St. Louis, MO, USA) diluted at 1:1000 in blocking buffer at 37°C. Signals were visualized using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) (Gibco-BRL) as substrates. As shown in Figure 3A, each of the anti-Kai antisera detected a specific signal for endogenous KaiA, KaiB or KaiC at the expected molecular size in a size-fractionated *Synechococcus* extract, although anti-KaiA antibody also gave a nonspecific ~46 kDa signal.

Yeast two-hybrid analysis

Expression vectors for Kai proteins fused to LexA and VP16 (pLexA–KaiA, pLexA–KaiB, pLexA–KaiC and pVP16–KaiA, pVP16–KaiB, pVP16–KaiC) were generated by inserting the full-length ORFs of the *kai* genes into the *Bam*HI site of pBTM116 and pVP16 vectors (Vojtek *et al*., 1993). For simultaneous expression of the Lex–KaiB and unfused KaiC proteins, a modified two-hybrid vector pLB-UC was constructed as follows. A promoter region of the alcohol dehydrogenase gene (P_{adh}) carried on pACT2 (Clontech) was amplified by PCR using primers 5′-AACGCGCGCAGCTGCAACTTCTTT-3′ (*Pvu*II site underlined) and 5′-TTATCCATCTTTGCGGCCGCTGGAGTTG-3′ (*Not*I site underlined), and then digested with *Pvu*II and *Not*I. A DNA fragment carrying a terminator region (T_{adh}) was obtained by digestion of pACT2 with *Not*I and *Sma*I. The P_{adh} and T_{adh} fragments were fused at the *Not*I termini and the ligated fragment was inserted into the *Pvu*II site of pLex–KaiB to obtain pLexB–Padh. A modified *kaiC* ORF, which carried a Kozak sequence, ACCATGG (ATG translational initiation codon underlined; Kozak, 1986), was inserted into the *Not*I site of pLexB–Padh to obtain pLB-UC.

These fusion plasmids were introduced into yeast L40. Doubly transformed cells were selected on Complete Mineral (CM) dropout medium lacking leucine and tryptophan (Ausubel *et al*., 1996) at 30°C for 2–3 days. For the β-galactosidase filter assay (Ausubel *et al*., 1996), three colonies were inoculated and grown on nylon membranes (Hybond N+; Amersham) on agar medium at 30°C for an additional 1.5 days. The membrane was immersed in liquid nitrogen for 30 s, thawed at room temperature, placed on a filter paper laid on 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) containing 250 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated at 30°C.

Fusion protein production in E.coli

Plasmids expressing GST fused to each full-length Kai at the N-terminus (pGST–KaiA, pGST–KaiB or pGST–KaiC) were constructed by insertion of a PCR product for each *kai* ORF into the *Bam*HI site of the pGEX-3X vector (Pharmacia). The *kaiB* ORF fragment was also inserted into the *Bam*HI site of the pQE32 vector (Qiagen) for His_6-tagged KaiB production. For production of GST–CI and GST–CII fusion proteins, DNA fragments encoding the CI domain (KaiC; residues 3–250) and the CII domain (KaiC; residues 253–519) were inserted into the *Bam*HI site of pGEX-3X to obtain pGST–CI and pGST–CII, respectively.

To induce GST, GST–KaiA, GST–KaiB, GST–CI and GST–CII proteins, DH5α transformed with each pGEX-3X derivative was cultured overnight in 100 ml of TB medium (Sambrook *et al*., 1989) containing 50 μg/ml of sodium ampicillin. The cultures were diluted in 1 l of TB containing ampicillin, incubated at 37°C for 3 h and IPTG was added to a final concentration of 1 mM. The culture containing IPTG was incubated for an additional 15 h before harvest. For production of GST–KaiC, DH5α transformed with pGST–KaiC was cultured at 37°C for 50 h without IPTG, because IPTG induction caused the formation of inclusion bodies. Cells collected as a pellet were resuspended in 100 ml of cold PBS and lysed by sonication at level 5 for 2 min on ice (Astrason, XL2020). Proteins in the sonicates were solubilized by the addition of

Triton X-100 (final 1%) followed by gentle mixing at 4°C for 2 h. The lysates were centrifuged at 20 000 r.p.m. for 20 min (at 4°C) and the supernatant was incubated with 100 µl of 50% (v/v) suspension of glutathione–Sepharose 4B (Pharmacia) at 4°C overnight. The resins were washed six times with 20 ml of cold PBS containing 0.7% Triton X-100 and suspended in 50 µl of storage buffer [50 mM HEPES, pH 8.0, 150 mM NaCl, 15% (v/v) glycerol]. His_6-tagged KaiB protein was produced by the same procedure as described for GST–KaiA and GST–KaiB proteins, except that TALON metal-affinity resin was used (Clontech). The quantity and purity of the immobilized proteins were estimated by densitometric measurement of coomassie brilliant blue stained proteins fractionated on 10–15% SDS–polyacrylamide gels.

In vitro transcription-coupled translation of Kai proteins

Full-length ORFs of *kaiA*, *kaiA1* and *kaiC* were inserted separately into the *Bam*HI site of pSP64-poly(A) vector (Promega). For production of CI (KaiC; 1–250) and CII (KaiC; 252–519) proteins *in vitro*, PCR products were inserted separately into the *Sal*I–*Bam*HI site of pSP64-poly(A). The translational initiation codon (ATG) for each protein was modified to harbor the typical Kozak sequence as described above. A TNT rabbit reticulocyte system (Promega) was used for *in vitro* production of Kai proteins. Twenty-five microliters of reaction mixture containing 1 µg each of the appropriate pSP64 poly(A)-derivatives and 12.5 µl of reticulocyte lysate was incubated for 2 h at 30°C in the presence of 15 mCi/ml of ^{35}S-methionine (1 Ci/mmol). The sizes and amounts of labeled proteins were analyzed by SDS–PAGE (10% gels) followed by autoradiography using the BAS2000 image analyzer (Fuji).

In vitro assay for protein–protein interaction

GST–Kai fusion proteins and GST (~5 µg each), which were immobilized on glutathione–Sepharose 4B, were incubated with 3 µl of *in vitro* translated, ^{35}S-labeled KaiA, KaiC, CI or CII at 30°C for 40 min in 200 µl of binding buffer [20 mM HEPES, pH 8.0, 50 mM KCl, 5 mM EDTA, 2.5 mM $MgCl_2$, 2 mM DTT, 10% (v/v) glycerol, 0.2% NP-40 and 2.5% BSA]. The resin beads were washed with binding buffer and then with binding buffer minus BSA, and boiled for 10 min in SDS–PAGE sample buffer. The reduced proteins were fractionated on 10% SDS–PAGE gels and analyzed by autoradiography. We also used the His_6-tagged KaiB protein for the interaction assay *in vitro*. His_6-KaiB protein (~10 µg) immobilized on TALON metal-affinity resin was incubated with ~5 µg of purified GST or GST fusion protein in 200 µl of binding buffer without EDTA and DTT at 30°C for 1 h. The resin was washed and boiled as described above, and the reduced proteins were analyzed by SDS–PAGE using 10–15% gradient gels (Pharmacia). GST fusion proteins bound to His_6-KaiB were detected by Western blotting on a nitrocellulose membrane (Hybond-C; Amersham), using a goat anti-GST primary antibody (Pharmacia) and a secondary anti-goat IgG antibody conjugated to alkaline phosphatase (Sigma), according to the Pharmacia protocol. Signals were visualized using NBT and BCIP as substrates. To test the effects of KaiC on the KaiA–KaiB interaction, resin-immobilized His_6-KaiB (~10 µg protein) and the ^{35}S-labeled KaiA protein (5 µl of the reaction mixture of reticulocyte system) were incubated with 10 µg of GST control, GST–KaiC, GST–CI or GST–CII protein in 200 µl of the binding buffer for 1 h at 30°C.

Protein interactions in cyanobacterial cells

To produce each of the His_6-tagged Kai proteins in *Synechococcus*, p*CkaiABC* that carried the entire *kai* gene cluster with a spectinomycin-resistance gene (Ishiura *et al*., 1998) was modified by PCR-based *in vitro* site-directed mutagenesis (Horton, 1993) to harbor a His_6-encoding sequence (5′-CACCACCACCACCACCAC-3′) just downstream of the translational initiation codon of *kaiA*, *kaiB* or *kaiC* ORF. The *kai*-deficient strain NUC39 was transformed with each of modified p*CkaiABC* to regain the modified *kai* gene cluster at the original *kai* locus of the chromosome. Transformant clones were selected with spectinomycin dihydrochloride (40 µg/ml). Bioluminescence rhythms were assayed as described below. Soluble protein extracts were prepared separately from the *kai*-modified clones and AMC149 as described above. Each extract (500 µl aliquot) was mixed with 500 µl of 2× binding buffer (without EDTA and DTT) and then incubated with 25-µl bed volume of TALON metal-affinity resin at 4°C for 18 h. The affinity beads were washed seven times with 1 ml of 1× binding buffer and boiled in 20 µl of SDS–sample buffer for 10 min. Eluted proteins were size-fractionated by SDS–PAGE on 10–15% gradient gels and subjected to immunoblotting analysis using anti-Kai antisera.

Over-expression of the CI and CII domains of KaiC in Synechococcus

*Nco*I–*Bam*HI segments of pSPK–CI and pSPK–CII that contained the whole CI- and CII-encoding sequences were ligated with *Nco*I- and *Bam*HI-digested $p322P_{trc}$ (Kutsuna *et al*., 1998) to obtain $p322P_{trc}$*::CI* and $p322P_{trc}$*::CII*, respectively. The smaller *Bgl*II-fragments from $p322P_{trc}$*::CI* and $p322P_{trc}$*::CII* were inserted into the *Bam*HI site of the pTS2KC (Kutsuna *et al*., 1998) to obtain pTS2C-P_{trc}*::CI* and pTS2C-P_{trc}*::CII*, respectively. The P_{kaiBC}-reporter strain NUC38 was transformed with each plasmid as described previously (Kutsuna *et al*., 1998) to harbor the expression construct at a specific genomic targeting site of the chromosome (NSII; GenBank/EMBL/DDBJ accession No. U44761). Conditions for over-expression were the same as described previously (Ishiura *et al*., 1998), except that 2 mM IPTG was used for induction. Briefly, transformant colonies were allowed to develop on solid medium in 40 mm plastic dishes under standard conditions for 3–4 days. At hour 24 of LL for the bioluminescence rhythm assay, 2 mM (final concentration) IPTG was added under the agar medium.

Assay for bioluminescence rhythms

Bioluminescence rhythms were monitored as described previously (Ishiura *et al*., 1998). *Synechococcus* cells were grown on solid BG-11 agar medium in 40 mm plastic dishes under standard conditions for 3–4 days to give 30–60 colonies (0.1 mm diameter). After a 12 h dark treatment to synchronize the clock, the dishes were set in a sample changer (LDM) that alternated the dishes between the standard light conditions (27 min) and darkness (2 min). The bioluminescence from the dish was measured for 30 s using a photomultiplier tube (Hamamatsu R466S). The signal pulses were processed as described previously (Kondo *et al*., 1993). Bioluminescence intensity was normalized to the number of colonies. A representative rhythm among two to four replicates was shown for each reporter strain. Standard deviation (SD) at each time point was $<10\%$ of measurements.

Acknowledgements

We thank Dr Stanley Hollenberg (Fred Hutchinson Cancer Research Center) for providing the pBTM116, pVP16 and yeast L40. We wish to thank the members of our laboratories, in particular Dr Shinsuke Kutsuna for the construction of NUC39 and technical suggestions, Dr Mitsunori Katayama for the construction of NUC38, and Ms Mioko Kohno for assistance with the preparation of anti-Kai antisera. We also thank Dr Kazuhiro Furukawa (Nagoya University) and Dr Kazuo Ogawa (National Institute for Basic Biology, Okazaki, Japan) for technical suggestions. We acknowledge Dr Susan S.Golden (Texas A&M University) and Dr Carl H.Johnson (Vanderbilt University) for valuable comments and help in preparing the manuscript. This research was supported by grants from the Japanese Ministry of Education, Science and Culture (08454244, 08404053), Research for the Future Program of the Japan Society for the Promotion of Science (JSPS-RFTF96L00601), the Human Frontier Science Program and the Mitsubishi Foundation to T.K. H.I. was supported by the Research Fellowships of JSPS for Young Scientists.

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Received November 24, 1998; revised January 5, 1999; accepted January 7, 1999