Expression of a Gene Cluster *kaiABC* as a Circadian Feedback Process in Cyanobacteria

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Cyanobacteria are the simplest organisms known to have a circadian clock. A circadian clock gene cluster *kaiABC* was cloned from the cyanobacterium *Synechococcus*. Nineteen clock mutations were mapped to the three *kai* genes. Promoter activities upstream of the *kaiA* and *kaiB* genes showed circadian rhythms of expression, and both *kaiA* and *kaiBC* messenger RNAs displayed circadian cycling. Inactivation of any single *kai* gene abolished these rhythms and reduced *kaiBC*-promoter activity. Continuous *kaiC* overexpression repressed the *kaiBC* promoter, whereas *kaiA* overexpression enhanced it. Temporal *kaiC* overexpression reset the phase of the rhythms. Thus, a negative feedback control of *kaiC* expression by KaiC generates a circadian oscillation in cyanobacteria, and KaiA sustains the oscillation by enhancing *kaiC* expression.

The circadian clock, a self-sustained oscillator with a period of about 24 hours, is found ubiquitously among eukaryotes and cyanobacteria. The clock controls a temporal program of cellular metabolism to facilitate adaptation to daily environmental changes (*1*). To elucidate the molecular mechanism of this oscillator, several clock genes of *Drosophila*, *Neurospora*, and mouse have been cloned and analyzed (*2*). Negative feedback-loop models have been proposed for the circadian clock of *Drosophila* and *Neurospora*, in which the clock genes *Drosophila per* and *Neurospora frq* are repressed by their products PER and FRQ, respectively (*3*). Recently, clock components that interact with each other and enhance expression of *per*, *mper*, and *frq* genes have been identified in *Drosophila*, mice (*4*), and *Neurospora* (*5*).

Cyanobacteria, the simplest organisms that display circadian rhythms, provide a model system for the circadian clock. We use bacterial luciferase as a reporter for the expression of a clock-controlled gene, *psbAI*, in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (*6*). The rhythm of bioluminescence from this reporter gene, like that of firefly luciferase in *Arabidopsis* and *Drosophila*, reliably reflects the activity of the native *psbAI* gene (*7*). Many mutants that display a wide range of circadian phenotypes have been isolated (*8*). We report here the cloning of a clock gene cluster *kaiABC* from *Synechococcus* and propose a model of the circadian oscillator of *Synechococcus* based on negative feedback control of *kaiABC* expression.

We constructed a library of wild-type (WT) genomic DNA in a plasmid vector (pNIBB7942) that targets the inserts to a specific site in the cyanobacterial genome termed NSII (*9*). A long-period clock mutant, C44a (period, 44 hours) (Fig. 1A, top), was transformed with the library (*10*), and transformants were screened by an automated apparatus (*8*). A few "rescued clones" (period, 25 hours) were found (Fig. 1A, middle) among 20,000 to 40,000 transformants. The library DNA from a rescued clone was recovered as a plasmid (p44N) in *Escherichia coli* (*11*). Targeting of p44N again, such that the transformant carried the mutated gene at its native locus and the WT gene or genes at NSII (*12*), rescued mutant C44a completely (Fig. 1A, bottom), suggesting that the plasmid contains an entire WT gene for the *kaiC11* mutation (Fig. 1D) of mutant C44a and that this mutation is recessive.

To localize the region essential for rescue, we subcloned genomic DNA fragments of p44N into the pSEQ1 vector (*13*) and introduced them into mutant C44a. Only the 4.7-kb Eco RI fragment of p44N rescued the mutant. We sequenced this fragment and analyzed putative protein-coding regions. Six open reading frames (ORFs) were found; three were known genes ("*cheY*," *rpL27*, and *rpL21*), and the other three form a cluster (Fig. 1B). To find the mutation site, we amplified the cluster from mutant C44a by polymerase chain reaction (PCR) and determined its nucleotide sequence (*14*). A single nucleotide alteration that caused a single amino acid substitution was found in the third gene. Insertion of the Ω fragment (*15*) into a Bam HI, Xho I, or Cla I site in either the first or second gene in the genome disrupted rhythmicity, suggesting that the entire gene cluster cloned here comprises clock genes. Southern (DNA) blot analysis of the genomic DNA with each *kai* gene probe suggested that WT *Synechococcus* sp. strain PCC 7942 has a single copy of this gene cluster.

We named the genes *kaiA*, *kaiB*, and *kaiC*, respectively (*kai* means cycle in Japanese). The predicted proteins have 284 (KaiA), 102 (KaiB), and 519 amino acids (KaiC) (Fig. 1C). KaiC has two putative adenosine 5′-triphosphate (ATP)– or guanosine 5′-triphosphate (GTP)–binding motifs (Walker's motif A), two imperfect Walker's motif Bs, and two putative catalytic carboxylate Glu residues that are found in ATP-binding proteins (*16*) (Fig. 1C). KaiC also has two putative DXXG sequences conserved in GTP-binding proteins (*17*). No other functional motifs were found in any of the Kai proteins.

We examined the rescue by plasmid p44N of over 50 clock mutants that show short or long periods, or arrhythmia. All strains, including all mutants reported previously (*8*), were restored to the WT phenotype by p44N (*18*). Direct sequencing of the *kai* gene cluster from 19 mutants revealed that 14 have mutations in *kaiC*, three in *kaiA*, and two in *kaiB* (*19*) (Fig. 1D). All mutant phenotypes, even arrhythmia, were caused by single–amino acid substitutions (Fig. 1D). Thus, the *kai* gene cluster and Kai proteins have a crucial and central function or functions for the circadian clock of *Synechococcus*.

To further test the significance of the *kai* genes, we deleted the whole cluster from WT *psbAI*-reporter cells by replacement with a kanamycin-resistance gene (*20*). Transformants lacking the cluster (Δ*kaiABC*) grew as well as WT cells and emitted comparable bioluminescence, but they completely lost rhythmicity (Fig. 2B). Thus, the cluster is essential for the circadian oscillation, whereas it is not essential for growth, and may be specific for circadian rhythmicity. To determine whether the *kai* genes could function from another site in the genome, we reintroduced the cluster into NSII of the Δ*kaiABC* strain (*21*). Clones that carried the transposed cluster recovered rhythmicity completely (Fig. 2C). Thus, the segment contains the entire DNA sequence required for *kai* function. To assess the significance of each *kai* gene separately, we individually inactivated the three *kai* genes. The endogenous *kaiC* gene was disrupted by replacement with the

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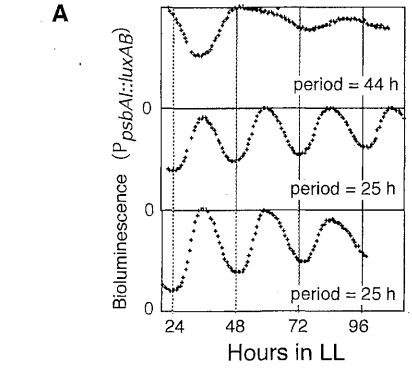
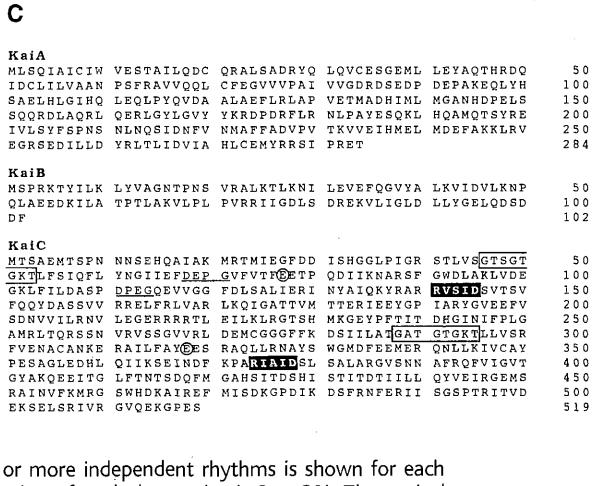
Ω fragment and the *kaiA* and *kaiB* genes were inactivated by introduction of a nonsense mutation just downstream of each start codon (*22*). All three strains carrying either an inactivated *kaiA*, *kaiB*, or *kaiC* gene were arrhythmic (Fig. 2, D to F). Thus, all three *kai* genes are essential to circadian clock function (*22*).

We examined expression of the *kai* genes by inserting a promoterless *luxAB* gene set just downstream of the cluster in the genome (*23*). The bioluminescence of the resulting *kaiABC*-reporter strain was rhythmic, with a period and phase like that of the previously characterized P_{psbAI}-reporter strain (Fig. 3, B and C). The level of bioluminescence of the reporter strain was ~2 to 3% of that of the P_{psbAI}-reporter strain. To localize promoters of the *kai* cluster, we fused each *kai* upstream region (*USR*) with the *luxAB* gene set and targeted the constructs into NSII of WT *Synechococcus* cells (*24*). Both USR_{kaiA}- and USR_{kaiB}-reporter strains showed bioluminescence rhythms whose period and phase were the same as those of the *kaiABC*-reporter strain, whereas a USR_{kaiC}-reporter strain showed no bioluminescence (Fig. 3A). Thus, a promoter exists upstream of *kaiA* and *kaiB*, but none could be detected upstream of *kaiC*, suggesting cotranscription of *kaiBC*. The average level of bioluminescence of the USR_{kaiA}- and USR_{kaiB}-reporter strains was about 1 and 3%, respectively, of that of the P_{psbAI}-reporter strain. The waveform of the *kaiB::luxAB* reporter matched that from the reporter in which *luxAB* was inserted downstream of *kaiC* (Fig. 3B), further supporting *kaiBC* cotranscription.

The transcriptional units of the *kai* cluster were confirmed by Northern (RNA) blot analysis, with *kaiA*-, *kaiB*-, and *kaiC*-specific probes (*25*). The *kaiA* probe gave a 1.0-kb hybridization band, whereas both the *kaiB* and *kaiC* probes gave a 2.3-kb band (Fig. 3D). These data support monocistronic transcription of *kaiA* and dicistronic transcription of *kaiBC*. Most of the *kaiBC* mRNA detected was degraded as shown by a smeared band (Fig. 3D). Both *kaiA* and *kaiBC* mRNAs displayed circadian cycling under continuous light conditions (LL) (Fig. 3E). The peaks of mRNA levels occurred at hours 9 to 12 and 33 to 36 in LL, which corresponded to those of the bioluminescence rhythms.

Autoregulation of *kai* expression could be a key point for generating circadian oscillation in *Synechococcus*. We confirmed that expression of the *kai* gene cluster was affected by *kai* mutations as observed for the P_{psbAI} reporter (*26*) (Fig. 4, A to D). We further examined the effects of inactivation of each *kai* gene on the bioluminescence of P_{kaiA}- and P_{kaiBC}-reporter strains (*22*). As was observed in a P_{psbAI}-reporter strain (Fig. 2), inactivation of either the *kaiA*, *kaiB*, or *kaiC* gene abolished rhythmicity of both the P_{kaiA}- and P_{kaiBC}-reporter strains (*27*). Inactivation of





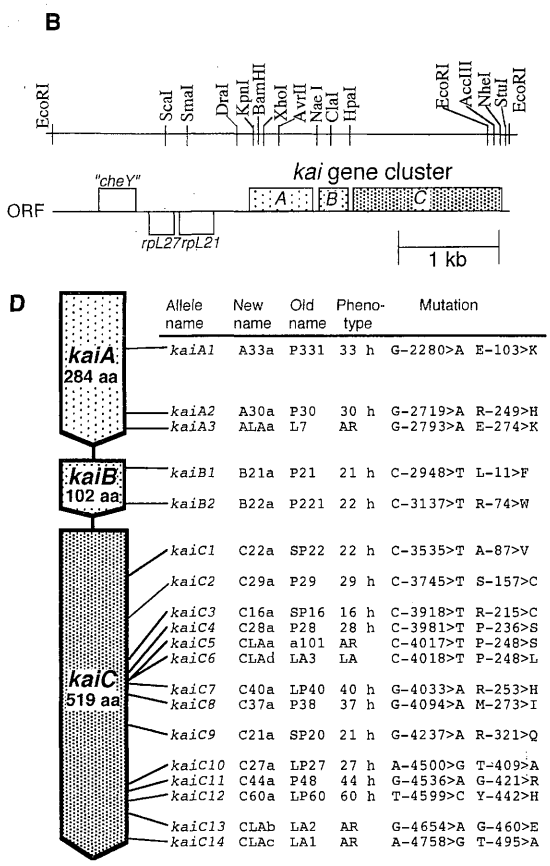


Fig. 1. Rescue of clock mutant C44a by transformation with a genomic DNA library, a map of the *kai* gene cluster, the deduced amino acid sequences of *kaiA*, *kaiB*, and *kaiC* gene products, and mapping of clock mutations. (**A**) Rescue. (Top) Mutant C44a; (middle) "rescued clones" obtained by transformation with the library; (bottom) rescue by targeting of plasmid p44N into NSII in the mutant genome. Colonies (1-mm diameter) of each strain were grown on solid medium in 90-mm plastic dishes under standard conditions (*8*). After a 12-hour darkness to synchronize the clock, the bioluminescence rhythm of each colony was monitored under standard conditions by a luminescent colony monitor system (*8*). A typical trace among 100 or more independent rhythms is shown for each strain. The standard deviation of each data point is 2 to 3%. The period was determined as described (*8*), and the standard deviation for 100 or more rhythms was less than 0.3 hours. Zero on the ordinate of each panel indicates darkness. The bioluminescence intensities among the panels were not normalized to each other, but the maximum level of bioluminescence by $P_{psbAI::lux}$ reporter varied little among these clones. (**B**) Restriction map and open reading frames. The six ORFs are shown as boxes. Those above the line are translated in the forward direction (left to right) whereas those below the line are translated in the opposite orientation. (**C**) Amino acid sequences. KaiA has 284 amino acids with a molecular mass of 32.6 kD and a calculated isoelectric point (pI) of 4.69. KaiB is composed of 102 residues(11.4 kD, pI = 7.11) and KaiC, 519 residues (58.0 kD, pI = 5.74). Walker's motif As (GXXXXGKT/S) (X, any residue) and imperfect motif Bs (ZZZZD; Z, hydrophobic residue) are boxed and reversed, respectively, and putative catalytic carboxylate Glu residues are circled. DXXG motifs are underlined. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**D**) Mutations mapped to the *kai* genes. Phenotypes of mutants: period in hours for period mutants; LA, low amplitude; AR, arrhythmic. Strain names are given along with the names previously published for these mutants (old names).

kaiA and *kaiC* lowered the bioluminescence level of P_{kaiBC}-reporter strains to 11 ± 1 and 28 ± 5% of the WT level (n = 3), respectively, although these inactivations did not significantly affect that of a P_{kaiA}-reporter strain.

To examine the effects of overexpression of the *kai* genes, we fused an *E. coli*–inducible promoter, P_{trc}, to the ORF of the *kaiA* or *kaiC* gene, targeted that construct into NSII of both the P_{kaiA}-and P_{kaiBC}-reporter strains, and induced overexpression of each *kai* gene by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (*28*). Overexpression of *kaiC* immediately nullified the bioluminescence of a P_{kaiBC}-reporter strain, whereas addition of IPTG did not affect the rhythm of a P_{kaiBC}-reporter strain that lacked the P_{trc}::*kaiC* transgene (Fig. 4, E and F). Thus, overexpressed KaiC strongly repressed the P_{kaiBC} promoter. In contrast, the presence of the P_{trc}::*kaiA* construct, even without IPTG addition, reduced the amplitude of the rhythm and markedly increased the average level of bioluminescence (Fig. 4G), suggesting that the promoter is not tightly repressed in the absence of inducer. Further *kaiA* overexpression induced by IPTG resulted in arrhythmicity and a further three- to fourfold increase in bioluminescence (*27*). In the P_{kaiA}-reporter strain, *kaiC* (Fig. 4, H and I) and *kaiA* overexpression (*27*) also abolished the rhythmicity but did not change the average level of bioluminescence significantly, as was the case for inactivation experiments. All these data suggest that regulated levels of expression of all three genes is needed for circadian rhythmicity.

We suggest a feedback-loop model for the circadian oscillator of *Synechococcus* (Fig. 5). The following four sets of data—(i) mapping of various clock mutations to the *kai* cluster, (ii) rhythmicity in the expression of the *kai* genes, (iii) alteration of the rhythmicity of *kai* expression by the mutations mapped to the *kai* cluster, and (iv) elimination of rhythms caused by inactivation or overexpression of each *kai* gene—all support a model in which the *kai* genes are essential to the circadian clock, and the feedback regulation of the expression of the *kai* genes by their gene products generates the circadian oscillation in cyanobacteria (Fig. 5). In particular, negative feedback on the P_{kaiBC} promoter by KaiC (Fig. 4F) suggests that KaiC could play a role as a "state variable" of the circadian oscillator (*29*), as appears to be the case for *Drosophila* PER and *Neurospora* FRQ proteins (*3*). To test this hypothesis, we overexpressed the *kaiC* gene temporarily by pulse administrations of IPTG (Fig. 4, J to N) and found that the phase of the oscillation was reset in a way that the model predicts. Temporal elevation of *kaiC* expression advanced the oscillation during a phase in which native *kaiC* expression was increasing (the subjective-day phase, hours 0 to 12) and delayed the oscillation during a phase in which the expression was decreasing (the night phase, hours 12 to 24). The magnitude of the phase shifts was proportional to the phase difference between the time of *kaiC* overexpression and the time when the *kaiC* gene was maximally expressed. Thus, the

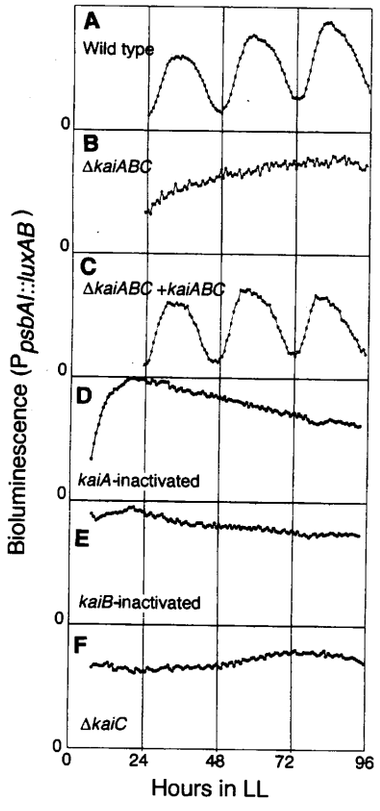


Fig. 2. Arrhythmia of bioluminescence caused by inactivation of the *kai* genes. (**A**) WT cells. (**B**) *kaiABC*-deleted cells (Δ*kaiABC*) in which the entire *kai* gene cluster was deleted by replacement with the Ω fragment. (**C**) *kaiABC*-transposed cells (Δ*kaiABC* + *kaiABC*) in which the entire WT cluster was transposed to NSII in the genome of Δ*kaiABC* cells. (**D**) *kaiA*-inactivated cells in which the *kaiA* gene was inactivated by a nonsense mutation. (**E**) *kaiB*-inactivated cells in which the *kaiB* gene was inactivated by a nonsense mutation. (**F**) *kaiC*-disrupted cells in which the *kaiC* ORF was replaced with the Ω fragment. Monitoring of rhythms and representation of data were as described in the legend to Fig. 1.

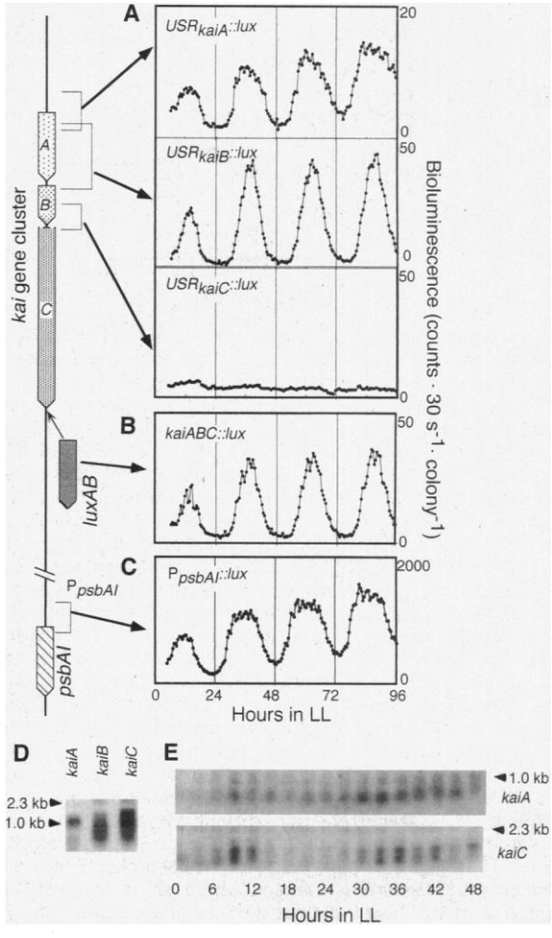


Fig. 3. Expression of the *kai* genes. (**A**) USR_{kaiA}-, USR_{kaiB}-, and USR_{kaiC}-reporter strains carrying *USRs* of the *kaiA*, *kaiB*, *and kaiC* genes, respectively, fused to a promoterless *luxAB* gene set at the Bst EII site of NSII in the genome. (**B**) A *kaiABC*-reporter strain carrying the *luxAB* gene set inserted at the Nhe I site just downstream of the *kaiC* gene. (**C**) A *psbAI*-reporter strain carrying a P_{psbAI}::*lux* construct in another specific genomic site (NSI) (*6*). Cells were grown to give 30 to 100 colonies (0.1-mm diameter) on solid medium in 40-mm plastic dishes in LL (standard conditions) (*8*) for 3 days. After a 12-hour dark treatment to synchronize the clock, the dishes were set in a sample changer (luminescent dish monitor, 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 by a photomultiplier tube (Hamamatsu R466S). Signal pulses were processed as described (*31*). Bioluminescence intensity was normalized to the number of colonies. A representative rhythm among three to eight replicates was shown for each reporter strain. The standard deviation at each time point was <10% of measurements. (**D**) Northern (RNA) blots prepared at hour 8 in LL (*25*). Probes were *kaiA*-, *kaiB*-, or *kaiC*-specific. The arrowheads indicate a 1.0-kb *kaiA* transcript and a 2.3-kb *kaiBC* transcript. (**E**) Northern blots of RNA prepared at hours 0 to 48 in LL probes were *kaiA*- or *kaiC*-specific.

level of *kaiC* expression is directly linked to the phase of the oscillation.

Activation of clock genes is also important for the negative feedback loop to oscillate. Some threshold level of KaiC appears to be necessary for full P_{kaiBC}-promoter activity because *kaiC* expression was reduced by *kaiC* inactivation. Enhancement of P_{kaiBC}-promoter activity by KaiA (Fig. 4G) might be important to sustain the circadian oscillation of *kaiC* expression. Severe lowering of P_{kaiBC}-promoter activity by *kaiA* inactivation is also compatible with this hypothesis. Recently, positive activators for the expression of circadian clock genes have been identified in *Neurospora* (*5*), *Drosophila*, and mouse (*4*). The Kai proteins have no DNA binding motifs as are found in some other clock-gene products (*4*, *5*). Thus, unidentified DNA binding proteins might be involved in the KaiA- and KaiC-mediated regulation of the P_{kaiBC}-promoter activity. At present, we cannot suggest any specific role for KaiB.

Although KaiA would be crucial to sustain the oscillation, its expression is not likely to be controlled directly by the proposed feedback loop because P_{kaiA}-promoter activity was affected differently than P_{kaiBC}-promoter activity by inactivation and overexpression of the *kai* genes (Fig. 4, E to I). Differences in waveforms of the bioluminescence rhythms of the P_{kaiA}- and P_{kaiBC}-reporter strains (Fig. 3, A and B) also support this distinction.

Although the basic feedback structure of a clock model for cyanobacteria is similar to those for eukaryotes, Kai proteins do not have any similarity to known clock proteins in *Drosophila*, *Neurospora*, or mouse (*2*–*5*). In *Drosophila* and *Neurospora*, time delays in the transcription of a clock gene or genes, the processing, transport into cytoplasm, and translation of its mRNA or mRNAs, and the phosphorylation and nuclear translocation of a clock protein or proteins are postulated to

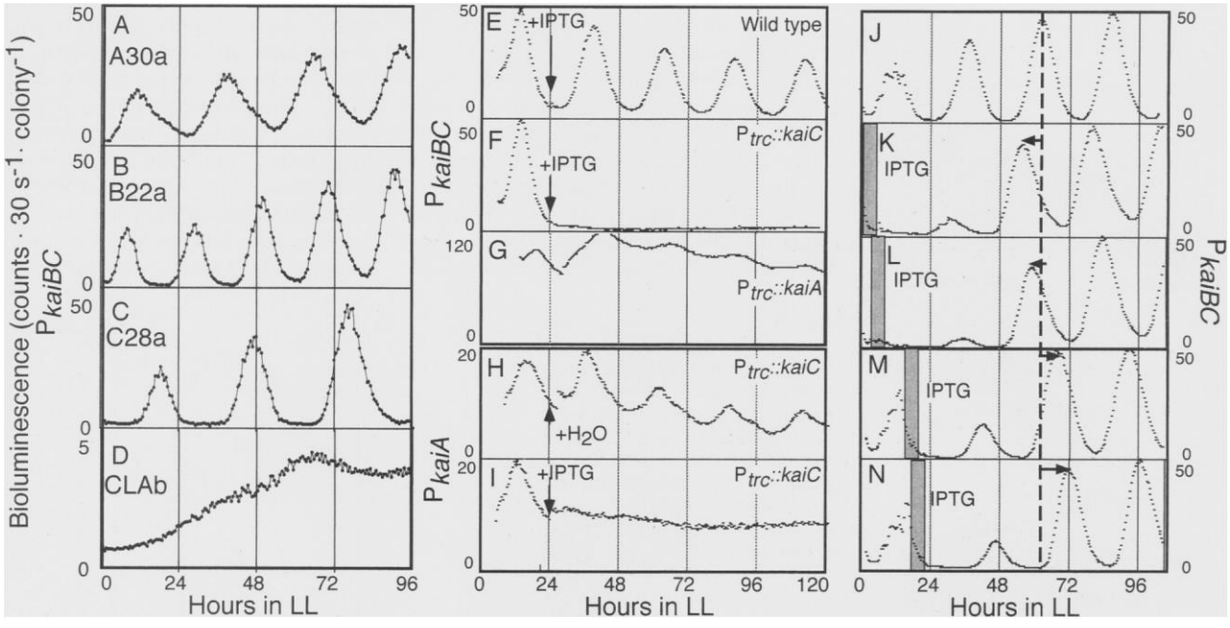


Fig. 4 (above). Expression of the *kai* genes in clock mutants, the effects of overexpression of the *kaiA* and *kaiC* genes on the activity of the P_{kaiA} and P_{kaiBC} promoters, and phase shifts by temporal overexpression of the *kaiC* gene. (**A** to **D**) Expression of the *kai* genes in clock mutants. Bioluminescence of P_{kaiA} (USR_{kaiA})-, P_{kaiBC} (USR_{kaiB})-, and P_{psbAI}-reporter derivatives of mutants A30a (*kaiA2*) (A), B22a (*kaiB2*) (B), C28a (*kaiC4*) (C), and CLAb (*kaiC13*) (D) was monitored. Only the data for P_{kaiBC} reporter derivatives are shown. In each period mutant (A to D), the period and phase of the rhythms in both P_{kaiA}- and P_{kaiBC}-reporter strains were similar to those of the P*psbAI*-reporter strain. In the arrhythmic mutant (D), P_{kaiA}- and P_{kaiBC}-reporter strains as well as the P_{psbAI}-reporter strain showed arrhythmic bioluminescence. (**E** and **F**) Effects of overexpression of the *kaiC* gene on P_{kaiBC}-promoter activity. WT cells (E) and cells carrying a P_{trc}::*kaiC* construct for overexpression (F) were treated with 1 mM IPTG. The arrows indicate the time of addition of IPTG. (**G**) Effects of overexpression of the *kaiA* gene on P_{kaiBC}-promoter activity. Cells carried a P_{trc}::*kaiA* construct for overexpression. (**H** and **I**) Effects of overexpression of the *kaiC* gene on the P_{kaiA} promoter. Cells of a P_{kaiA}-reporter strain carrying a P_{trc}::*kaiC* construct were treated with water (arrows) (H) or 1 mM IPTG (I). (**J** to **N**) Phase shifting by temporal *kaiC* overexpression by IPTG pulses. Cells of a P_{kaiBC}-reporter strain carrying a P_{trc}::*kaiC* construct were not treated (J) or treated with 1 mM IPTG for 6 hours from hours 0 (K), 3 (L), 15 (M), and 18 (N) in LL. The vertical broken line and the arrows indicate a standard phase and phase shifts, respectively. Similar treatments with water did not shift the phase at any time. Monitoring of bioluminescence and representation of data were as described in the legend to Fig. 3.

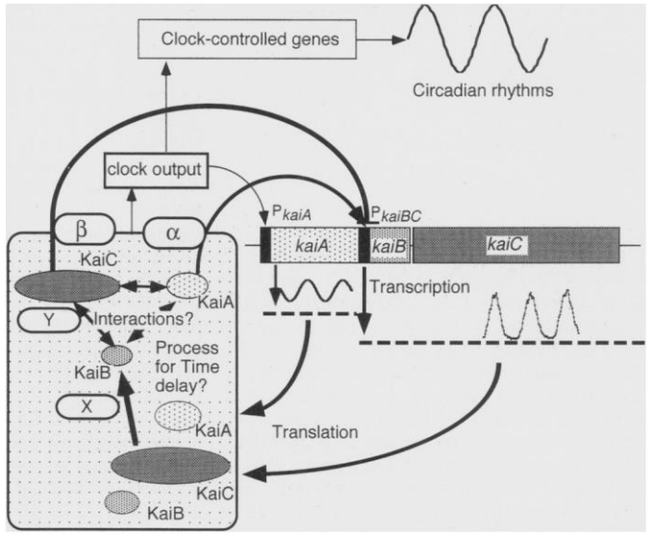


Fig. 5 (right). Feedback model for the circadian oscillator of cyanobacteria. A possible feedback loop (bold-arrows) that generates a circadian oscillation is illustrated. Hatched box at left represents an unknown part of the feedback loop. X and Y are unidentified clock components. α and β are unidentified DNA binding proteins. The positive effects of KaiC on the P_{kaiBC} promoter are not shown. See text for explanation.

account for a period as long as 24 hours (*3*). However, some of these processes are absent in cyanobacteria, which are prokaryotic (*30*). Thus, the phylogenetic relationship of clock systems among various organisms still remains to be elucidated. Putative KaiB and KaiC homologs have been found in archaeal genome databases (*31*). Although a physiological function for those homologs has not yet been found, this finding might uncover the evolution of circadian systems.

References and Notes

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9. Construction of pNIBB7942 and a *Synechococcus* DNA library will be described elsewhere (M. Ishiura *et al.*, in preparation). The vector was designed to target the insert DNA together with the *km* gene and ori_{p15A} from pACYC177 [A. C. Y. Chang and S. N. Cohen, *J. Bacteriol.* **134**, 1141 (1978); R. E. Rose, *Nucleic Acids Res.* **16**, 356 (1988)] into the Bst EII site of the *Synechococcus* NSII segment (GenBank accession number U44761) of the genome.
10. Clock mutant cells were transformed with the library DNA, plated on solid medium containing BG-11 medium and 1.5% Bacto-Agar, grown in continuous light of 46 $\mu E\ s^{-1}\ m^{-2}$ (1 E = energy of 1 mol of photons) from white fluorescent lamps at 30°C (standard conditions), and selected with kanamycin sulfate (33 μg/ml).
11. Genomic DNAs prepared from rescued clones were digested with Not I, circularized with T4 DNA ligase, and electroporated into *E. coli* DH10B.
12. p44N was digested with Not I, ligated with the larger Not I fragment of pNIBB7942, and introduced into mutant C44a cells to target p44N into the Bst EII site of NSII in the genome.
13. M. Ishiura and H. Nakamura, unpublished data.
14. We amplified by PCR a 3.3-kb DNA segment carrying the *kai* gene cluster from the genomic DNAs prepared from clock mutants, using primers 5′-ACCGGCCACGTAGGGCTGTCCA-3′ and 5′-GGTGCTCGGGTTGACGACTG-3′, and determined its nucleotide sequence.
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19. Previously, we isolated plasmid pS1K1 as a gene that appeared to complement the *kaiC1* mutation (*8*). However, pS1K1 carries a suppressor gene for the mutation [T. Kutsuna, T. Kondo, S. Aoki, M. Ishiura., *J. Bacteriol.* **180**, 2167 (1998)].
20. A plasmid for disruption of the *kai* gene cluster, p*DkaiABC*, carries three segments in tandem: a 0.55-kb DNA segment carrying an upstream region of the *kaiA* gene [nucleotides (nt) 1424 to 1975 relative to the left Eco RI site of the 4.9-kb Eco RI segment shown in Fig. 1B]; a 1.2-kb Nhe I—Bsm FI segment from pACYC177 carrying a *km* gene [A. C. Y. Chang and S. N. Cohen, *J. Bacteriol.* **134**, 1141 (1978); R. E. Rose, *Nucleic Acids Res.* **16**, 356 (1988)]; a 2.2-kb Nhe I–Pvu II segment carrying a downstream region of the *kaiC* gene (nt 4831 to 7071) on the pSEQΩ (M. Ishiura and S. Kutsuna, unpublished data) backbone. CR1 [a *psbAI*-reporter strain carrying a chloramphenicol-resistance gene as a selective marker gene (*19*)] was transformed with p*DkaiABC* and selected with kanamycin sulfate (33 μg/ml) (CR1/Δ*kaiABC* cells).
21. pTS2*CkaiABC* is a targeting plasmid carrying a *kaiABC* cassette in the Bst EII site in NSII. The cassette is composed of a 3.0-kb Dra I–Nhe I segment carrying the *kai* gene cluster and the 2.0-kb Hind III Ω fragment (*15*). CR1/Δ*kaiABC* cells (*20*) were transformed with pTS2*CkaiABC* and selected with spectinomycin dihydrochloride (40 μg/ml).
22. A 1.5-kb Hpa I–Bcl I segment of p60N (another plasmid carrying the *kaiABC* gene cluster) (M. Ishiura, S. Kutsuna, T. Kondo, unpublished data) was replaced with the 2.1-kb Bam HI Ω segment (p*DkaiC*) and introduced into CR1 cells to disrupt *kaiC* in the genome. A nonsense mutation (TAA) was introduced by PCR into the fourth codon CAA of *kaiA* or the fifth codon AAA of *kaiB* in pSEQ*kaiABC*, which carries a 3.7-kb Sma I—Bss HII fragment from p44N on the pSEQ1 backbone (*13*). p*CkaiABC* is a derivative of pSEQ*kaiABC*, which carries the 2.0-kb Hind III Ω segment in the Nhe I site just downstream of the *kaiC* gene. CR1/Δ*kaiABC* cells (*20*) were transformed with derivatives of p*CkaiABC* that carried an inactivated *kaiA* or *kaiB* gene to reintroduce the modified *kai* gene cluster into the original *kai* locus in the genome. Transformants were selected with spectinomycin. Insertion of a nonsense mutation into an upstream gene may result in reduction of the transcription of a downstream gene if these two genes are organized as an operon, although the extent of the polar effect is unpredictable. Nevertheless, it is evident that the *kaiB* gene has an important role in the clock function because point mutations in the *kaiB* gene affect rhythmicity (Fig. 2B).
23. p*kaiABC::lux* carries a 2.8-kb Pvu II segment carrying the *kaiC* gene and its downstream region on the pSEQ1K (M. Ishiura and S. Kutsuna, unpublished data) backbone, in which segment a 2.7-kb Hind III–Eco47 III promoterless segment of a *luxAB* gene set from *Vibrio harveyi* [T. O. Baldwin *et al.*, *Biochemistry* **23**, 3663 (1984)] and the 2.0-kb Hind III Ω fragment (*15*) were inserted at the Nhe I site of the segment. WT *Synechococcus* cells were transformed with p*kaiABC::lux* and selected with spectinomycin.
24. pUSR_{kaiA}*::lux*, pUSR_{kaiB}*::lux*, and pUSR_{kaiC}*::lux* carry a DNA segment including each upstream region of the *kaiA* (an 859–base pair Sma I–Xho I segment from p44N), *kaiB* (nt 1984 to 2920; the 5′-end nucleotide of the 4.9-kb Eco RI segment carrying the *kaiABC* gene cluster is numbered +1), and *kaiC* (nt 2961 to 3278) genes, respectively, on the pTS2Slux plasmid (*19*). To target these reporter constructs into the Bst EII site of NSII in the genome, we transformed WT *Synechococcus* cells with the plasmids and selected the cells with spectinomycin.
25. Cells were grown with aeration in LL of 100 $\mu E\ s^{-1}\ m^{-2}$ at 30°C until the optical density at 730 nm (OD_{730}) of culture reached 0.3. The OD_{730} was maintained between 0.27 and 0.45 by dilution with fresh BG-11 medium. The culture was exposed to 12 hours of darkness to synchronize the circadian clock, and then returned to LL. At 3-hour intervals in LL, cells were harvested, immediately frozen, and stored at −80°C. RNA was extracted from each frozen sample as described [A. Mohamed and C. Jansson, *Plant Mol. Biol.* **13**, 693 (1989)]. RNA was subjected to electrophoresis, blotted onto nylon membranes (Boehringer GmbH, Mannheim, Germany), and hybridized with digoxigenin-labeled *kaiA*, *kaiB*, *and kaiC* ORF probes. The bioluminescence rhythm of the culture was confirmed by measuring the bioluminescence at each time point. Experiments were carried out three times and typical data are shown in Fig. 3, D and E.
26. To replace the *psbAI*-reporter construct in clock mutants with a kanamycin-resistance gene (*km*), we transformed cells with pTS1KT (M. Ishiura and S. Kutsuna, unpublished data), which carries a *km* gene in the Xho I site of NSI [S. A. Bustos and S. S. Golden, *J. Bacteriol.* **173**, 7525 (1991)] and selected the cells with kanamycin. Then, pUSR_{kaiA}*::lux* and pUSR_{kaiB}*::lux* were targeted into NSII in the non-bioluminescent derivatives of the mutants as described (*24*).
27. M. Ishiura, S. Kutsuna, T. Kondo, unpublished data.
28. Plasmids p$TS2KP_{trc::kaiA}$ and p$TS2KP_{trc::kaiC}$ were used for overexpression of the *kaiA* and *kaiC* genes, respectively. Each plasmid carries the ORF of the respective *kai* gene on p$TS2KP_{trc}$ (*19*). The start codon GTG of the *kaiA* gene was modified to an ATG codon. Cells were transformed with these plasmids and selected with kanamycin. Twenty to 100 colonies carrying each construct were allowed to develop on solid medium. After 24 hours in LL, IPTG was added under the agar medium at a final concentration of 1 mM. For pulse administration of IPTG, colonies were grown on nitrocellulose membranes placed on solid medium, and the filter was transferred onto solid medium containing 1 mM IPTG for 6 hours and then returned to unsupplemented solid medium. Other conditions were the same as described in the legend to Fig. 4.
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31. Homologies to and phylogeny of the *kaiB* and *kaiC* genes in cyanobacteria and Archaea will be reported elsewhere (M. Ishiura *et al.*, in preparation).
32. Cloning of the *kai* genes was carried out at the National Institute for Basic Biology (Okazaki, Japan) and further studies at Nagoya University with M.I., S.K., H.I., A.T., and T.K. We are grateful to H. Shinagawa (Osaka University), H. Aiba (Nagoya University), and S. Itoh (National Institute for Basic Biology) for helpful discussions; to S. Ishikawa (Instrument Development Center of School of Science, Nagoya University) for help in developing LDM; and to T. Suto and H. Kondo for technical assistance. This work was supported by grants from the Japanese Ministry of Education, Science and Culture (08454244, 08877053, 07558103, 08404053, 07554045), the Ishida Foundation, the Nissan Foundation, the Yamada Foundation, the Chiba-Geigy Foundation for the Promotion of Science, the Kurata Research Grant, grants from the Shimadzu Foundation, Research for the Future program of Japan Society for the Promotion of Science (JSPS) (JSPS-RFTF96L00601), the competitive research grant from the Agency of Industrial Science and Technology, Ministry of International Trades and Industry and the Mitsubishi Foundation (to T.K.), and from the U.S. NSF (MCB-9311352 and MCB-9513367 to S.S.G. and MCB-9633267 to C.H.J.), the U.S. National Institute of Mental Health (MH01179 to C.H.J.), and the Human Frontier Science Program. S.K. and H.I. were supported by the Research Fellowships of the JSPS for Young Scientists.

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