Mutations in KaiA, a clock protein, extend the period of circadian rhythm in the cyanobacterium *Synechococcus elongatus* PCC 7942

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KaiA KaiB and KaiC are essential circadian clock proteins in the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942. KaiA protein activates transcription of the *kaiBC* operon, which is believed to be a crucial step in the oscillating feedback loop of cyanobacteria. In this study, ~400 mutations were introduced into *kaiA* by PCR-based mutagenesis, and rhythmic phenotypes of these mutants were studied by a bioluminescence reporter. In contrast to mutations in KaiB or KaiC, the vast majority of KaiA mutations extended the period and only rarely shortened it. The period could be extended to 35 h without lowering the mean or peak levels of *kaiBC* expression. However, several mutations resulted in low-amplitude oscillations or arrhythmia, which were accompanied by lowered *kaiBC* transcription. These results imply that the KaiA protein can change the period length of the circadian rhythm directly (through an unknown biochemical mechanism) or indirectly (by lowering *kaiBC* expression). Specific mutations of KaiA were identified in 34 mutants. While mutations mapped to various locations of the KaiA sequence, two clusters of period-altering mutations were found. This suggested that these regions are important domains of the KaiA protein for defining the period length. On the other hand, different sequences within KaiA to which arrhythmic mutations were mapped are important to enhance *kaiBC* expression.

Keywords: circadian clock, *kai* genes, *kaiBC* expression, amplitude of rhythm, bioluminescence rhythm

INTRODUCTION

Circadian rhythms, biological oscillations with a period of approximately 24 h, have been found in many diverse organisms, ranging in complexity from bacteria to green plants and mammals. These rhythms regulate various metabolic and behavioural activities and are believed to be adaptable to daily changes in environmental conditions, such as light, temperature and humidity (Bünning, 1973; Pittendrigh, 1993). The circadian clock, an intracellular mechanism that generates oscillations with ~24 h periods, has been postulated as a basis for these

rhythms, while feedback models of molecular cycling have been proposed for *Neurospora*, *Drosophila* and mammals (Dunlap, 1999; Young & Kay, 2001). In these models, clock protein interactions function as crucial steps in feedback loops with a circadian time-course.

Cyanobacteria are the simplest organisms that display circadian rhythms (Golden *et al.*, 1998; Kondo & Ishiura, 2000; Iwasaki & Kondo, 2000). We have previously introduced a bioluminescence reporter gene into the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 to monitor circadian gene expression (Kondo *et al.*, 1993), isolated various clock mutants (Kondo *et al.*, 1994), and both identified and cloned the clock gene cluster *kaiABC*, composed of *kaiA*, *kaiB* and *kaiC* (Ishiura *et al.*, 1998). While no

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Abbreviations: LL, constant illumination; PMT, photomultiplier tube.

similarity was found between Kai proteins and the clock proteins of other organisms, disruption of any single *kai* gene completely abolished circadian rhythm. Experiments in which *kai* genes are overexpressed suggest that KaiC represses its own (*kaiBC*) expression while KaiA enhances it. Thus, KaiC and KaiA have been proposed to be negative and positive elements, respectively, in the molecular feedback loop of *kaiBC* expression (Ishiura *et al.*, 1998).

Nineteen distinct mutants exhibiting a wide range of period lengths and arrhythmia mapped their mutations onto the *kaiC* gene (Ishiura *et al.*, 1998). In light of this, it seems highly likely that understanding the biochemistry of the KaiC protein will be key in understanding circadian characteristics. Previous studies have shown that KaiA and KaiC interact with each other in the yeast two-hybrid system, *in vitro*, and in *Synechococcus*; closer examination revealed that the two half-units of KaiC (CI and CII, the first and second half domains, respectively) both interact with KaiA (Iwasaki *et al.*, 1999). The ATP-binding and autophosphorylation capacities of KaiC have also been shown to be important parts of the circadian timing mechanism (Nishiwaki *et al.*, 2000). Furthermore, two KaiA-binding domains (CKABD1 and CKABD2) in KaiC were found on the C-terminal regions of CI and CII, respectively, by using the yeast two-hybrid system and an *in vitro* interaction assay (Taniguchi *et al.*, 2001). More recently, SasA, a regulatory element consisting of a two-component sensory kinase, has been found to amplify the *kai*-based oscillations through its interaction with KaiC (Iwasaki *et al.*, 2000).

In previous work (Ishiura *et al.*, 1998), only four KaiA mutants were identified after chemical mutagenesis. While the phenotypes of these mutants include arrhythmia, which indicates that KaiA is crucial in the establishment or maintenance of circadian rhythm, further mutational analysis is necessary to understand the function of KaiA. In this study, we performed PCR-based mutagenesis on the *kaiA* gene and screened for abnormal rhythmic phenotypes. Using targeting of gene integration, followed by high throughput screening for rhythmic phenotypes with bioluminescence, we obtained ~400 mutants and analysed their phenotypes. Point mutations of representative *kaiA* mutants were also identified and mapped onto the KaiA protein. These mutations were concentrated in two distinct regions. We discuss the implications of our findings on the potential role of KaiA in generating circadian oscillations in cyanobacteria.

METHODS

Cyanobacteria strains and culture conditions. *Synechococcus elongatus* PCC 7942 was used as the background strain in this study. All *Synechococcus* strains were cultured in either modified BG-11 liquid medium or solid BG-11 medium containing 1.5% Bacto agar (Bustos & Golden, 1991) under constant illumination (LL) of $46 \mu\text{mol m}^{-2} \text{s}^{-1}$ from white fluorescent lamps at 30 °C. NUC42 and NUC43 are the wild-

Fig. 1. Introduction of mutations into *kaiA* by PCR error. *Synechococcus* DNA was digested with *Sma*I and *Afl*II to obtain a DNA segment that contains the entire *kaiA* sequence. A PCR reaction was performed to amplify this DNA. PCR errors (labelled X) were introduced into DNA copies. At the same time, the pCkaiABC plasmid containing the whole *kaiABC* operon was digested by *Sma*I and *Afl*II, to obtain *kaiA*-deleted DNA. The PCR product and the *kaiA*-deleted plasmid were fused to obtain *kaiABC* operons that carry an error-prone PCR product of *kaiA*. Fused pCkaiABC was introduced into NUC43 *Synechococcus* to obtain *kaiA* mutants that could be screened by a CCD camera system.

type and *kaiABC*-deleted strains, respectively. Both strains carry the *PkaiBC::luxAB* reporter construct at neutral site I (S. Kutsuna, M. Ishiura & T. Kondo, unpublished results). The construct is a fusion of a region upstream of *kaiB* (−91 to +6) and a promoterless *luxAB* gene set that reports expression of the *kaiBC* operon. Four *kaiA* mutations previously obtained by chemical mutagenesis (EMS, ethylmethane sulfonate; Ishiura *et al.*, 1998) are also listed in Fig. 4. Kanamycin-resistant and spectinomycin-resistant transformed clones were selected with 40 μg kanamycin or spectinomycin ml^{-1} . *Escherichia coli* strains DH5 α and DH10B were used as hosts for plasmid construction.

PCR-based mutagenesis of *kaiA* and construction of mutant strains. PCR-based mutagenesis is a well-documented method in bacteria (Diaz *et al.*, 1991) including a cyanobacterium, *Synechocystis* sp. PCC 6803 (Narusaka *et al.*, 1999). Fig. 1 illustrates the method of constructing *kaiA* mutants by PCR-based mutagenesis. pCkaiABC is a targeting plasmid carrying the *kaiABC* gene and an omega fragment (Prentki & Krisch,

1984). Using *rTaq* DNA polymerase (Takara Shuzo), the *kaiA* gene was amplified by PCR, using pCkaiABC as a template and two oligonucleotides [B48-up (5'-AACCAATAGGCCG-AAATCGGC-3') and 715(1) low (5'-TTGAAGTTCGCCGT-AGAGTA-3') (i.e. nt +1068 to +1087 of *kaiA*)] as primers. The PCR cycle was set at 94 °C (30 s), 54 °C (30 s), 72 °C (2 min extension phase) and repeated 30 times. Because *Taq* DNA polymerase aberrantly recognizes DNA sequences at a constant rate, thereby making incorrect PCR products, some PCR-amplified products of *kaiA* will have a point mutation at a random location (represented by an X in Fig. 1). The resulting products were digested by *Sma*I and *Afl*II and ligated to *Sma*I/*Afl*II-digested pCkaiABC with T4 DNA ligase. NUC43 was obtained by transforming the wild-type reporter strain NUC42 with pDkaiABC (Ishiura *et al.*, 1998), which carries three segments in tandem: a region upstream of the *kaiA* gene, a kanamycin-resistance gene and a region downstream of the *kaiC* gene. NUC43 was then transformed with the ligated fusion plasmids. *Synechococcus* carrying the PCR mutations were selected with spectinomycin and screened for rhythmic profiles.

Bioluminescence rhythm assay. Transformants were cultured on solid BG-11 plates and bioluminescence rhythms were then monitored with a cooled-CCD camera system (Kondo *et al.*, 1994). In total, ~4000 clones were screened and the period for each clone was calculated. As a more precise assay, bioluminescence profiles of selected mutants were monitored using a photomultiplier tube (PMT)-based system (Ishiura *et al.*, 1998). *Synechococcus* mutants were grown on solid BG-11 plates for 2–3 days in LL and then subjected to darkness for 12 h to synchronize the clock. After the 12 h darkness period, a small dish containing n-decanal (which had been dissolved in vacuum pump oil at a concentration of 3 %, v/v) was placed on each plate and bioluminescence was monitored under LL at the standard conditions ($46 \mu\text{mol m}^{-2} \text{s}^{-1}$ from white fluorescent lamps at 30 °C). In this report, amplitudes of the rhythm were classified into four types (high amplitude, medium amplitude, low amplitude and arrhythmic) by peak-to-trough ratio of bioluminescence intensity.

DNA sequence of mutants. We directly sequenced mutant *kaiA* genes after *in vitro* amplification of *kaiA* segments by PCR. *Synechococcus* cells were mixed in a tube with 0.1 mm zircon/silica beads and lysed by shaking with a Multi-Beads Shocker (Yasui Kikai). Genomic DNA was extracted by phenol extraction and purified by ethanol precipitation. Segments carrying *kaiA* were amplified by PCR using the following pair of primers: 818(3)up (5'-ACCGGCCACGTA-GGGCTGTCC-3') (–409 to –389 of *kaiA*) and 715(1)low (described above). DNA sequence was then analysed with a DNA sequence kit and an ABI PRISM model 310 sequencer.

RESULTS AND DISCUSSION

Efficient mutagenesis by error-prone PCR

In this study, we obtained ~4000 *Synechococcus* clones carrying a PCR copy of the *kaiA* gene, which were screened with a luciferase reporter for individual circadian rhythms. The luciferase reporter, driven by the *kaiBC* operon promoter, can indicate transcriptional activity of *kaiBC*, a central step in the circadian pacemaker of cyanobacteria. The profile of this reporter in the wild-type strain reveals a robust rhythm with a period of 25 h (under current screening conditions) with a sharp expression peak at the subjective dusk and

almost completely shut off at the trough phase (Ishiura *et al.*, 1998). Thus, high-amplitude clones with periods close to that of wild-type (24–26 h) presumably carry wild-type *kaiA* (i.e. a correct PCR product), a silent mutation (resulting in no change at the amino acid level) or insignificant amino acid alterations. As seen in Table 1, these clones amount to ~87% of the total. Nevertheless, we obtained ~400 mutants in this study, or about 12% of the total number of clones screened. These mutant clones showed distinct abnormalities in circadian profile, including long periods, low amplitudes and arrhythmia. Sufficient numbers of mutants were obtained to allow statistical analysis of the mutations. Whilst it is difficult to estimate the number of indistinguishable, covert mutations among the clones with normal phenotypes, the error-prone PCR mutation rate can be estimated to be even higher than 12 %, indicating that PCR mutagenesis is much more efficient than chemical mutagenesis.

As screening of circadian rhythm inevitably requires normal clone growth, we were only able to screen for regularly growing mutant clones on agar plates. Thus, it is possible that some clones with growth-inhibiting mutations had been lost prior to screening, which would in turn disturb our statistical analysis. However, this is unlikely to be the case here because *Synechococcus* growth does not change even with the complete deletion of *kaiA* (Ishiura *et al.*, 1998). If fact, we did not notice any change in growth rate in a wide spectrum of mutant phenotypes obtained in this study. Note also that the complete deletion of *kaiA* induced complete arrhythmia and severe lowering of *kaiBC* expression (Ishiura *et al.*, 1998).

KaiA mutations extend the period

As shown in period histograms (Fig. 2A and B), the most striking feature of *kaiA* mutagenesis is an almost complete lack of short-period mutations. We found 301 long period mutants (period >27 h) and 92 arrhythmic mutants, whereas only one mutant showed a short-period phenotype and the oscillation rapidly damped in this mutant. Based on these statistical results, it can be concluded that mutations in *kaiA* generally extend the period, whereas periods can be both extended and shortened by mutations in *kaiC* (Ishiura *et al.*, 1998) and *kaiB* (our unpublished data). This tendency also holds true for the four *kaiA* mutants obtained by chemical (EMS) mutagenesis (Ishiura *et al.*, 1998). The one exceptional mutant (F224S) that showed a short period phenotype of 22 h (Fig. 3C) is of particular interest; the biochemical properties of this mutant KaiA protein must be analysed to shed further light on the mechanism underlying the cyanobacterial oscillator.

Some KaiA mutants had period lengths as long as 50 h and many arrhythmic mutants were also found. However, when limited to high-amplitude mutants (peak-to-trough ratio >10), the longest period was about 35 h, a 40% extension of the 25 h wild-type period (Fig. 2A). Parameters of the rhythmicity other than period length

Table 1. Summary of PCR-based mutagenesis of *kaiA*

Class of mutant*	No. colonies		No. mutants	Percentage of mutants
	Total	Period > 27 h†		
High amplitude	3648	162	162	4.1
Low amplitude	226	139	226	5.7
Arrhythmic	92	–	92	2.3
Total	3966	–	480	12.1

* Based on amplitude of rhythm, each rhythmic phenotype was classified into three classes. Amplitude is defined as peak-to-trough ratio of bioluminescence intensity. High amplitude, peak-to-trough > 10; low amplitude, 10 > peak-to-trough > 3; arrhythmic, peak-to-trough < 3.

† Numbers of clones which have a period longer than 27 h.

were not altered in mutants shown in Fig. 3A; the waveform of these mutants is similar to that of wild-type, bioluminescence drops to completely shut off levels at the trough phase and the peak bioluminescence intensity levels were changed only slightly (–20% to +10% of wild-type expression). In addition, the phase of the rhythm reset by 12 h darkness was also not altered by the mutations (Fig. 3A). Thus, these KaiA mutations apparently changed the period length selectively, implying a direct role for KaiA in period determination.

Medium-amplitude mutants also sustained robust rhythmicity with period lengths of up to 40 h. As shown in Fig. 3B, the peak *kaiBC* expression was again little changed (–20% to +10%), whilst 10–30% of peak *kaiBC* expression levels remained at trough phases. Thus, as with the high-amplitude group (Fig. 3A), period extension up to 40% can be induced without a large change in *kaiBC* expression. These observations imply that the role of the KaiA protein is to control the period length of the circadian oscillation without activation of *kaiBC* expression. Direct examination of the biochemical activity of KaiA should be performed to address the molecular mechanism for this periodicity modulation. The phase of rhythm, once reset by 12 h darkness, was again not altered by these mutations (Fig. 3B).

KaiA mutations lower *kaiBC* expression

The periods of lower amplitude mutants spanned an even wider range than those in the high-amplitude group, with period lengths as long as 50 h (Fig. 2B). Severe low-amplitude mutants (peak-to-trough < 3) and arrhythmic mutants are shown in Fig. 3C. Low-amplitude mutants in Fig. 3C still sustain stable rhythms with 24–50 h periods, whereas *PkaiBC* activity was considerably elevated at the trough phase and *kaiBC* expression at peak phase was lowered by 30–50%. Exceptionally, *kaiBC* expression was elevated in the A245D mutant but this mutant failed to extend the period length. Mutants with very long periods are shown in Fig. 3D. Note that *kaiBC* expression was

significantly lowered in these very long-period mutants. Thus, the long periods of these mutations were induced not only as a direct effect of altered KaiA biochemistry, but also as an indirect effect of lowering *kaiBC* expression.

Arrhythmic mutants displayed various profiles. Some mutants showed normal peak and troughs during the first cycle, but were then damped through elevation of the trough level. Other mutants were arrhythmic from the beginning of LL. An important feature of these mutants is that *PkaiBC* activity was significantly lowered; in general, the level of *PkaiBC* reporter expression was lower than 10% of wild-type. As KaiA is known to enhance *kaiBC* expression (Ishiura *et al.*, 1998), it is likely that the mutant KaiA proteins in this group could not sustain rhythmicity because they fail to activate *kaiBC* expression.

In this study, we have quantitatively examined the *PkaiBC* activity of KaiA mutants by normalizing bioluminescence data with colony size and number. From these assays, we found that most KaiA mutations lowered *PkaiBC* activity to varying degrees. As random mutation generally lowers the native function, these results would be consistent with a role for KaiA in enhancing *PkaiBC* activity (Ishiura *et al.*, 1998). On the other hand, it appears that the level of *kaiBC* expression contributes little to period determination, but is important in maintaining robustness of the circadian rhythm.

Note also that the phase of the rhythm reset by 12 h darkness was changed in some low-amplitude mutants (Fig. 3C; D136V, D136Y and A245D). This suggests that KaiA is also involved in the phase-resetting mechanism of the cyanobacterial clock.

Clustering of mutations on the Kai map

We subsequently sequenced 45 mutants and found that 39 mutants have a single point mutation while five mutants overlapped with other mutants. Each point mutation was mapped to a residue in KaiA. As illus-

Fig. 2. Period distribution of *kaiA* mutants. Bioluminescence rhythms for clones that carry error-prone PCR copies of *kaiA* were assayed with a CCD camera system. The distribution of 3874 clone periods is shown in a histogram of 1 h steps. On the basis of peak-to-trough ratio of bioluminescence, these rhythms were classified as high amplitude and low amplitude/arrhythmic. (A) Histogram of the periods of 3648 high-amplitude clones (peak-to-trough ratio > 10). (B) Histogram of the periods of 226 medium- and low-amplitude clones (peak-to-trough ratio < 10).

trated in Fig. 4, we were able to identify in KaiA an evident cluster of period mutations (residues 239–245) and another weak one of period mutations (residues 113–119). This suggests that cluster regions of KaiA are important in controlling the period length of the circadian oscillation, since most mutations that mapped to these regions have long period phenotypes (arrhythmia-causing mutations have not been found in these regions so far). The regions of KaiA that sustain rhythm by enhancing *kaiBC* expression are likely located elsewhere, because the mutations in arrhythmic mutants

mapped to other parts of KaiA. These mutants (C53S, V76A, F178S, F224S, E274K) are also very interesting because these residues are essential for enhancing *kaiBC* expression. To make more detailed and reliable assessments of the different regions of KaiA, more mutations need to be identified.

Three very-long-period mutants (phenotype similar to those shown in Fig. 3D) carried double mutations in the *kaiA* sequence. Three other mutations that substantially affect KaiA all induced arrhythmicity. These included a missing start codon (GTG→GCG), a stop codon insertion (Q117) and a frame-shift at residue 143. The severe phenotype resulting from these mutations is also compatible with a crucial role for KaiA in sustaining the cyanobacterial circadian rhythm. Such a function could be interaction(s) of KaiA with other clock proteins (especially with KaiC, Iwasaki *et al.*, 1999; Taniguchi *et al.*, 2001), or alteration of phosphorylation of KaiC by KaiA (T. Nishiwaki, Y. Kitayama, H. Iwasaki & T. Kondo and H. Iwasaki, Y. Kitayama & T. Kondo, unpublished data).

Function of KaiA

By examining *kaiBC* expression of many KaiA mutants, we found that the KaiA protein could extend the period to some extent without lowering *kaiBC* expression. KaiA may interact with other clock proteins (especially with KaiC, Iwasaki *et al.*, 1999; Taniguchi *et al.*, 2001), possibly altering the phosphorylation of KaiC (Nishiwaki *et al.*, 2000) or forming hetero-complexes of clock proteins (Iwasaki *et al.*, 1999). KaiA function is not yet well understood; analysis of the mutants identified in this research and, in particular, identification of regions in which they cluster, will lead to further elucidation of its biochemical mechanism of action. It should be noted that mutations can enhance or diminish protein activity (e.g. enzymic or binding activity), although it is assumed generally that most random mutations lower protein activity. In contrast to mutations in KaiB or KaiC, the vast majority of KaiA mutations extended the period and only rarely shortened it. The statistical observations made in this study lead us to conclude that the biochemical function of the KaiA protein is to move the clock operation forward.

On the other hand, in KaiA mutants with severe phenotypes, *kaiBC* expression was lowered by 40–50% in very-long-period mutants and by 90% in arrhythmic KaiA mutants compared to wild-type. As our previous study (Ishiura *et al.*, 1998) revealed that a function of KaiA was to activate *kaiBC* transcription and that *kaiBC* expression was severely lowered in a *kaiA*[−] strain, our observations for severe KaiA mutants are compatible with our previous study (Ishiura *et al.*, 1998). Clearly, arrhythmic mutants obtained in this study were very important for dissection of these actions. Interestingly, the site of arrhythmic or severe low-amplitude mutants was not included in the region in which moderate-period mutants clustered. These results

Fig. 3. Circadian bioluminescence profiles of *kaiA* mutants. Bioluminescence rhythms of representative mutants. After specific mutations were identified, rhythms were tracked with a PMT-based system to assay the rhythmic profile precisely. Mutants were cultured on a small dish containing BG-11 solid medium. After being cultured under LL for 3 days, clones were subject to 12 h darkness to reset the rhythm, then monitored using the PMT system in LL for bioluminescence. The temperature was kept at 30 °C throughout the study. Bioluminescence intensity of each trace is normalized to that of the wild-type (NUC42) rhythm. (A) Wild-type (NUC42) and high-amplitude (peak-to-trough ratio >10) group; (B) medium-amplitude group (10 > peak-to-trough ratio >3); (C) low-amplitude or arrhythmic mutants (peak-to-trough ratio <3). Vertical axes are normalized to maximum bioluminescence. Bioluminescence intensities are shown on a relative scale (percentage of wild-type); (D) clones with very long periods. Vertical axes are normalized to maximum bioluminescence. These profiles were those obtained from screening with the CCD camera system. Bioluminescence of each mutant is normalized to that of the wild-type. Mutations in *kaiA* of these mutants have not yet been identified.

Fig. 4. Mapping of *kaiA* mutations onto the KaiA amino acid sequence. Point mutations, as identified by direct sequencing of each mutant DNA, were mapped onto the KaiA protein. Mutations resulting in multiple substitution or frame-shifts are not included. Phenotypes are listed in three columns. HA, MA, LA and AR in Type column represent high amplitude, medium amplitude, low amplitude and arrhythmic, respectively. The period of the rhythm in hours and *PkaiBC* activity (percentage of wild-type) are also listed. NA, not examined. Numbers in parentheses for Q113R, Q117L, E239G and D242G indicate duplicated clones identified by sequencing. Mutants obtained by chemical (EMS) mutagenesis are also shown on the left side of the figure. Column *PkaiBC* lists the maximum bioluminescence intensity attained by each mutant during an assay period of 3–4 days (normalized to that of the wild-type).

also suggest the dual action of KaiA in the circadian time-keeping process in cyanobacteria.

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