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A Novel Allele of *kaiA* Shortens the Circadian Period and Strengthens Interaction of Oscillator Components in the Cyanobacterium *Synechococcus elongatus* PCC 7942▽

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The basic circadian oscillator of the unicellular fresh water cyanobacterium *Synechococcus elongatus* PCC 7942, the model organism for cyanobacterial circadian clocks, consists of only three protein components: KaiA, KaiB, and KaiC. These proteins, all of which are homomultimers, periodically interact to form large protein complexes with stoichiometries that depend on the phosphorylation state of KaiC. KaiA stimulates KaiC autophosphorylation through direct physical interactions. Screening a library of *S. elongatus* transposon mutants for circadian clock phenotypes uncovered an atypical short-period mutant that carries a *kaiA* insertion. Genetic and biochemical analyses showed that the short-period phenotype is caused by the truncation of KaiA by three amino acid residues at its C terminus. The disruption of a negative element upstream of the *kaiBC* promoter was another consequence of the insertion of the transposon; when not associated with a truncated *kaiA* allele, this mutation extended the circadian period. The circadian rhythm of KaiC phosphorylation was conserved in these mutants, but with some modifications in the rhythmic pattern of KaiC phosphorylation, such as the ratio of phosphorylated to unphosphorylated KaiC and the relative phase of the circadian phosphorylation peak. The results showed that there is no correlation between the phasing of the KaiC phosphorylation pattern and the rhythm of gene expression, measured as bioluminescence from luciferase reporter genes. The interaction between KaiC and the truncated KaiA was stronger than normal, as shown by fluorescence anisotropy analysis. Our data suggest that the KaiA-KaiC interaction and the circadian pattern of KaiC autophosphorylation are both important for determining the period, but not the relative phasing, of circadian rhythms in *S. elongatus*.

The sun rises at dawn and sets at dusk with a predictable daily pattern. Many living organisms show corresponding internal rhythms in their biological activities, including behavior, metabolism, and gene expression. These rhythms are generated by endogenous clocks, which anticipate the daily environmental changes to optimize the timing of physiological activities of an organism. Under constant conditions, these intrinsic rhythms show a self-sustained period of about (*circa*) a day (*dies*) and are thus called "circadian rhythms" (4). They can be entrained to the environmental cycle, like a clock being set to local time, through reception of light or temperature cues at appropriate environmental phases. For circadian activities, the free-running period of the rhythm is temperature compensated: that is, nearly constant over a physiological range of ambient temperatures (11, 12). Diverse eukaryotic organisms, such as fungi, insects, plants, birds, and mammals, including humans, have evolved to use this type of endogenous biological clock (2, 46). Cyanobacteria, a group of photosynthetic eubacteria, are the simplest organisms and the only prokaryotes known to possess a circadian clock (26). The majority of the studies of cyanobacterial circadian rhythms have been carried out using *Synechococcus elongatus* PCC 7942, a unicellular freshwater obligate photoautotroph (8–10).

The *kaiABC* locus encodes the circadian pacemaker of *S. elongatus*. Both the monocistronic *kaiA* transcript and the dicistronic *kaiBC* transcript display circadian cycling (15). The KaiB and KaiC protein levels are also robustly rhythmic, whereas KaiA accumulation varies only modestly, or imperceptibly, during the cycle (17, 20, 43). Mutations in any one of the *kai* genes cause a varied circadian period, ranging from 14 to 60 h (compared to 24 to 25 h in the wild type), or complete arrhythmicity (27). The period length of cyanobacterial circadian rhythms has been proposed to be determined by phosphorylation status and degradation rate of KaiC (33, 44). KaiA stimulates autophosphorylation of KaiC; this stimulation is attenuated by KaiB (19, 23, 42, 44). The KaiA dimer, KaiB tetramer, and KaiC hexamer compose large protein complexes in a circadian pattern, which is closely associated with the phosphorylation status of KaiC (13, 20, 21, 30, 31, 45). The circadian oscillation of KaiC phosphorylation can be regenerated in a test tube in vitro simply by mixing purified Kai proteins and ATP in the appropriate ratio (33), and the circadian period can be manipulated by changes in the relative

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TABLE 1. Cyanobacterial strains used in this study

Strain	Genetic background	Ectopic *kaiA*	Reporter gene(s)[a]	Source or reference
AMC541	WT		P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	8
AMC702	*kaiA* in-frame deletion		P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	7
AMC705	*kaiBC* in-frame deletion		P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	7
AMC1020	WT		P*psbAI*::*luxAB* (pAM1501 NS1 Sp^r); P*psbAI*::*luxCDE* (pAM1619 NS2 Km^r)	1
AMC1161	*kaiA* ΩKm insertion		P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	7
AMC1300	WT		P*kaiBC*::*luxAB* (pAM1887 NS1 Sp^r); P*psbAI*::*luxCDE* (pAM1619 NS2 Km^r)	Lab collection
AMC1483	*kaiA* *Mu*Cm insertion		P*psbAI*::*luxAB* (pAM1501 NS1 Sp^r); P*psbAI*::*luxCDE* (pAM1619 NS2 Km^r)	This study
AMC1484	*kaiA* *Mu*Cm insertion		P*kaiBC*::*luxAB* (pAM1887 NS1 Sp^r); P*psbAI*::*luxCDE* (pAM1619 NS2 Km^r)	This study
AMC1485	WT	P*kaiA*::*kaiA* (pAM2246 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1486	*kaiA* ΩKm insertion	P*kaiA*::*kaiA* (pAM2246 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1487	WT	P*kaiA*::*kaiA281* (pAM3434 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1488	*kaiA* ΩKm insertion	P*kaiA*::*kaiA281* (pAM3434 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1491	*kaiA* *Mu*Gm insertion		P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1492	*kaiA* ΩKm and *Mu*Gm insertions		P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1531	*kaiA* *Mu*Gm insertion	P*kaiA*::*kaiA* (pAM2246 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1532	*kaiA* *Mu*Gm insertion	P*kaiA*::*kaiA281* (pAM3434 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1533	*kaiA* ΩKm and *Mu*Gm insertion	P*kaiA*::*kaiA* (pAM2246 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study
AMC1534	*kaiA* ΩKm and *Mu*Gm insertion	P*kaiA*::*kaiA281* (pAM3434 NS1)	P*kaiBC*::*luc* (pAM2105 NS2 Cm^r)	This study

[a] Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin; Gm, gentamicin.

concentrations of the Kai proteins (21). Despite recent progress in elucidating the sequential phosphorylation of KaiC on Thr/Ser residues (35, 38) and monomer exchange among KaiC hexamers (16), as well as the importance of these events in circadian rhythm generation and synchronization, it is not fully understood how interactions among Kai proteins determine the phosphorylation status of KaiC and how the phosphorylation state is related to circadian period.

KaiA contains a pseudo-receiver domain in its amino-terminal half that is proposed to receive environmental cues, probably transmitted along circadian input pathways, and a carboxyl-terminal domain responsible for directly enhancing the autokinase activity of KaiC (19, 22, 23, 42, 44). A previous study showed that almost all *kaiA* point mutants displayed either arrhythmia or a long-period phenotype (34). Here, we report a novel mutation in *kaiA* that causes an atypical short-period phenotype. This insertional mutant was identified in a screen of an *S. elongatus* transposon mutant library that represents an insertion in essentially every gene and carries a luciferase reporter gene to track circadian rhythms of bioluminescence (14). One mutant, which has a shortened free-running period and elevated KaiB and KaiC protein levels, carries a transposon insertion near the C-terminal end of the *kaiA* coding region. Further genetic and biochemical analyses confirmed that the short-period phenotype in this mutant was due to the truncation of KaiA and that the truncated KaiA variant interacts more strongly with the KaiC C-terminal peptide than does the wild-type KaiA protein. Elevation of KaiB and KaiC protein accumulation resulted from disruption of a negative element upstream of the *kaiBC* promoter via the insertion of the transposon. Changes in the circadian pattern of KaiC phosphorylation state did not affect the phasing of bioluminescence rhythms.

MATERIALS AND METHODS

Cyanobacterial strains, media, and culture conditions. The cyanobacterial strains used in this study are summarized in Table 1. All wild-type (WT) reporter and mutant strains were created in *Synechococcus elongatus* PCC 7942. Cyanobacterial strains were grown in BG-11 medium (37) under continuous light (LL) conditions (~50 to 70 μE/m^{2}s) at 30°C with appropriate antibiotics. Two different versions of luciferase reporter genes were used: (i) firefly luciferase (*luc*) in the neutral site II (NS2) locus and (ii) *Vibrio harveyi* luciferase (*luxAB*) in the neutral site I (NS1) locus and the aldehyde substrate synthesis genes *luxCDE* in NS2 (1). Neutral sites are segments of *S. elongatus* genomic DNA, carried by cloning vectors, which mediate homologous recombination with the *S. elongatus* chromosome and can be interrupted with no apparent phenotypes (1).

Plasmid construction. The plasmids used in this report are described in Table 2. Unless otherwise stated, plasmids were constructed in *Escherichia coli* strain DH10B (Invitrogen, Carlsbad, CA). Plasmid pAM2246 (7) carries a copy of WT *kaiA* with its native promoter region in an NS1 vector. The WT *kaiA* was then converted to *kaiA281* using the QuikChange mutagenesis method (3) to engineer a stop codon, which resulted in pAM3434. Plasmid pAM3582 was constructed by inserting a PCR-amplified fragment, containing the original mini*Mu*Cm (GeneJumper kit; Invitrogen, Carlsbad, CA) transposon 7G3-GG5 (14) inserted at the 3′ end of the coding region of *kaiA* along with its flanking sequences, into cloning vector pLitmus 29 (New England Biolabs, Beverly, MA). The *cat* gene in the center of *Mu*Cm was then substituted for by the gentamicin resistance gene, *aacA*, from pAM3515 (pHP45ΩGm) to create pAM3613. The coding sequence

TABLE 2. Bacterial plasmids used in this study

Plasmid	Characteristic(s)	Antibiotic resistance[a]	Source or reference
pAM2246	P*kaiA*::*kaiA* (NS1)	Sp^r Sm^r	7
pAM3434	P*kaiA*::*kaiA281* (NS1)	Sp^r Sm^r	This study
pAM3515	pHP45ΩGm	Gm^r Ap^r	This study
pAM3582	*kaiA*::*Mu*Cm	Gm^r Ap^r	This study
pAM3613	*kaiA*::*Mu*Gm	Gm^r Ap^r	This study
pAM3633	pET-32a(+)-*kaiA*	Ap^r	40, 41
pAM3630	pET-32a(+)-*kaiA281*	Ap^r	This study

[a] Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; Sm, streptomycin; Ap, ampicillin.

TABLE 3. Circadian clock period of WT and mutated *kaiA* strains

PCC 7942 strain	Original *kaiA* locus[a]	Ectopic *kaiA* (NS1)[b]	KaiB (KaiC) protein level	Mean period ± SEM (h)	*n*
AMC1020	WT		Normal	24.40 ± 0.05	39
AMC1483	281 (*Mu*Cm)		↑[c]	23.32 ± 0.05	57
AMC1300	WT		Normal	25.35 ± 0.04	41
AMC1484	281 (*Mu*Cm)		↑	24.42 ± 0.03	48
AMC541	WT		Normal	25.03 ± 0.02	54
AMC1161	Null (ΩKm)			AR[d]	20
AMC1485	WT	WT	↑	24.12 ± 0.03	25
AMC1487	WT	281	↑	23.81 ± 0.03	26
AMC1486	Null (ΩKm)	WT	Normal	25.02 ± 0.03	28
AMC1488	Null (ΩKm)	281	Normal	24.49 ± 0.05	23
AMC1491	281 (*Mu*Gm)		↑	24.51 ± 0.05	23
AMC1492	Null (ΩKm *Mu*Gm)			AR	24
AMC1531	281 (*Mu*Gm)	WT	↑	24.48 ± 0.10	17
AMC1532	281 (*Mu*Gm)	281	↑	23.81 ± 0.08	16
AMC1533	Null (ΩKm *Mu*Gm)	WT	↑	25.62 ± 0.04	25
AMC1534	Null (ΩKm *Mu*Gm)	281	↑	NA[e]	16

[a] WT, WT P*kaiA*::*kaiA* allele; 281, truncated P*kaiA*::*kaiA281* allele; *Mu*Cm, original *Mu* insertion at the 3′ end of the *kaiA* coding region; ΩKm, the omega cassette inserted at the 5′ half of *kaiA* for construction of a null strain; *Mu*Gm, the reconstructed Gm version of the original *Mu* insertion.
[b] P*kaiA*::*kaiA* or P*kaiA*::*kaiA281* alleles introduced in NS1.
[c] ↑, increase.
[d] AR, arrhythmicity.
[e] NA, not available: unstable traces, phase advanced, and low amplitude (Fig. 3A).

of *kaiA* was inserted into pET-32a(+) (Novagen, EMD, San Diego, CA) at HindIII/EcoRV sites to build pAM3633. The last 3 amino acid (aa) residues of *kaiA* in pAM3633 were then deleted using the QuikChange strategy to construct pAM3630.

Bioluminescence assay and data analysis. The measurement of bioluminescence from the reporter strains was performed as described previously (7, 14, 29). The acquired bioluminescence data were processed and graphed by the Import and Analysis (IandA) Excel macro set (S. A. Kay Laboratory, The Scripps Research Institute, La Jolla, CA). To calculate the circadian periods of these strains, the data sets processed by IandA were then exported to BRASS (Biological Rhythms Analysis Software System; A. J. Millar laboratory, University of Edinburgh, Scotland, United Kingdom), which is an Excel interface for FFT-NLLS (a Fast Fourier Transform statistical suite of programs; Martin Straume). The measurement of each strain was conducted in at least three independent experiments with similar results. It was not technically possible to assay all strains simultaneously, so strains were grouped to ensure that the most relevant genotypes were assayed together; AMC541 was included with every run, and all data from this strain were pooled to ensure reproducibility among assays (Table 3). For each strain sample, the data from at least four circadian cycles in LL were used to calculate the period. The standard error of the mean (SEM) was calculated in Excel (Microsoft, Redmond, WA). An unpaired *t* test that incorporates the mean, SEM, and *n* was performed to compare mutant or complemented strains with corresponding WT strains.

Immunoblot analysis. Protein sample preparation and immunoblot analysis for single-time-point experiments were performed as described previously (7) with slight modifications. To facilitate sample collecting in a 24-h period, aliquots of cultures (optical density at 750 nm of 0.6 to 1.0) for time course analysis were first entrained in opposite phases at the same time with at least two cycles of 12-h/12-h of either light/dark (LD) or dark/light (DL) and then released to LL conditions (~50 $\mu E/m^2 s$). Samples were taken either every 4 h for 12 to 24 h or every 1 h for 4 h, depending on the experimental design.

Peptide purification and fluorescein labeling. Segments of the *S. elongatus kaiC* gene that encode the desired peptide sequences were cloned in a pET-32a(+) vector (Novagen, EMD, San Diego, CA), thereby creating thioredoxin-poly(His)-peptide fusions (22, 36, 40). *Escherichia coli* BL21(DE3) (Novagen, EMD, San Diego, CA) was transformed with the resulting plasmids and grown in Luria broth. Expression of peptide fusion constructs was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; Calbiochem, EMD, San Diego, CA) to a final concentration of 1 mM. The cells were harvested after 4 h, resuspended in a buffer containing 50 mM NaCl and 20 mM Tris-HCl (pH 7.4), and lysed. Cell lysates were separated by centrifugation, and the fusion peptide was purified from the supernatant fraction by metal affinity chromatography. The fusion peptide was labeled with fluorescein at the N terminus by the manufacturer's protocol (Molecular Probes, Eugene, OR). The sample was buffer exchanged to 50 mM NaCl–20 mM Tris-HCl (pH 7.4), and the peptide was cleaved from the affinity tag by using enterokinase (Novagen, EMD, San Diego, CA). Cleavage by this enzyme results in the addition of three non-KaiC-derived residues (AMC) at the N terminus of the peptide. Peptides were isolated by reverse-phase chromatography and lyophilized. Peptide identity and purity were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy, and quantification was carried out by measuring the UV absorbance of fluorescein.

Fluorescence anisotropy-based binding experiments. Fluorescence anisotropy experiments were carried out with an ISS PC1 photon-counting spectrofluorometer (ISS, Champaign, IL) with fluorescein-labeled KaiC peptide at 30°C. The peptide concentration in all cases was fixed at 100 nM in 20 mM Tris-HCl, 150 mM NaCl, and 0.5 mM EDTA (pH 8.0). An initial volume of 1.8 ml of fluorescein-labeled KaiC peptide was used. Up to 2.2 ml of 0.3 mM KaiA-protein stocks was added to the fluorescein-labeled KaiC peptide solution up to a total concentration of ~200 μM protein. Fluorescence anisotropies were measured as a function of the concentrations of KaiA.

RESULTS

Identification of an atypical short-period *kaiA* mutant. Known *kaiA* mutations in *S. elongatus* cause either arrhythmia or a long-period phenotype (34). When we screened mutants that carry *Mu*Cm insertional mutations in a segment of the *S. elongatus* genome that is present on cosmid 7G3 for circadian clock phenotypes, we found an unusual short-period mutant allele of *kaiA*. The insertion of *Mu*Cm (7G3-GG5) in *kaiA* shortened the free-running period length of the circadian bioluminescence rhythm by about 1 h (AMC1483 and AMC1484, respectively) (Fig. 1B and Table 3) in reporter strains that carry bacterial luciferase genes under the control of an *S. elongatus* promoter: P*psbAI*::*luxAB* in AMC1020 and P*kaiBC*::*luxAB* in AMC1300 (Table 1). Note that the WT free-running period of the bioluminescence rhythm in AMC1300 is around 25 h, 1 h longer than that of AMC1020 (~24 h) under the screening conditions used (see Discussion and reference 5). Regardless of the genetic background, the *kaiA* insertional mutant strains displayed the same degree of period shortening and showed no discernible growth phenotype compared to their corresponding WT strains.

Sequencing of the *Mu*Cm insertion site revealed its location

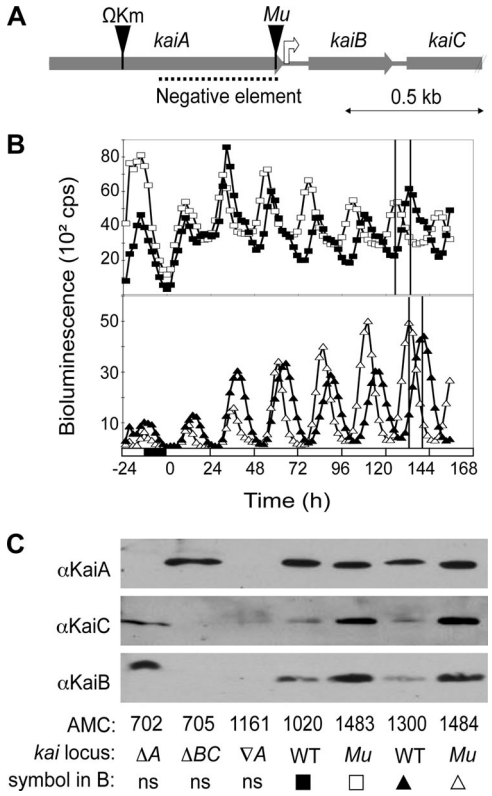

FIG. 1. An atypical short-period *kaiA* insertional mutant. (A) Schematic representation of the *kai* locus showing the relative positions of insertions: an omega cassette (ΩKm) and *Mu*Cm transposon 7G3-GG5 (*Mu*). ΩKm was inserted in the N-terminal coding region of *kaiA* to create AMC1161, a *kaiA* null mutant. The *Mu* mutant encodes KaiA281, truncated by three residues, and disrupts a negative control element upstream of the *kaiBC* promoter. A bent arrow shows the position of the *kaiBC* promoter. The dashed line under the C-terminal coding region of *kaiA* indicates the *kaiBC* upstream negative element. (B) Representative bioluminescence traces from the WT (closed symbols) and the *Mu* mutant (open symbols) in two different reporter backgrounds: P*psbAI*::*luxAB* (AMC1020 [squares]) and P*kaiBC*::*luxAB* (AMC1300 [triangles]). Peaks of circadian rhythms are indicated by vertical lines. On the *x* axis, the blank bars represent light conditions, and the black bar represents 12 h of darkness. (C) Immunoblot analysis of Kai proteins in WT and *Mu* insertional mutant strains. Total soluble protein (~20 μg) was loaded in each well. AMC702 and AMC705 carry in-frame deletions of *kaiA* (ΔA) or *kaiBC* (ΔBC), respectively; AMC1161 carries an insertional null allele of *kaiA* (∇A). αKaiA, αKaiC, and αKaiB, anti-KaiA, -C, and -B antibodies, respectively; *Mu*, the 7G3-GG5 *Mu*Cm insertion strains (AMC1483 and AMC1484); ns, not shown.

at the C-terminal coding region of *kaiA*. The insertion of *Mu*Cm separates the last 12 nucleotides, encoding the three terminal amino acid residues RET plus the stop codon, from the rest of the coding region of *kaiA* (Fig. 1A). The end sequence of the transposon forms a new stop codon for a truncated *kaiA*, which would encode a protein with 281 aa instead of the 284 aa in WT KaiA. Immunoblot analysis of KaiA protein levels confirmed the expression of a KaiA variant comparable to the WT KaiA protein in size and amount, with slightly faster mobility on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (Fig. 1C).

There is a negative regulatory element that lies in the C-terminal-half of the *kaiA* coding region and affects *kaiBC* expression (28) (Fig. 1A). The disruption of this element is known to significantly increase the expression from the *kaiBC* promoter. Immunoblot analysis confirmed elevated KaiB and KaiC abundance in the insertional mutant strains (Fig. 1C). A different *kaiA* insertional knockout strain, AMC1161, was used as a negative control. This strain was generated by inserting a kanamycin-resistant version of the Ω cassette into a BamHI site in the N-terminal coding region of *kaiA* (7), such that the negative regulatory element of P*kaiBC* would not be affected (Fig. 1A). As KaiA protein stimulates P*kaiBC* expression (15), disruption of *kaiA* in AMC1161 caused a decrease in KaiB and KaiC protein levels such that only trace amounts of these two proteins could be detected (Fig. 1C).

In short, the 7G3-GG5 *Mu*Cm insertion not only truncated KaiA at its carboxyl terminus but also disrupted a negative element and resulted in noticeably elevated levels of KaiB and KaiC protein (Fig. 1C). Thus, the shortened period of the *Mu*Cm mutant could be due to either the truncation of KaiA, the disruption of the negative element, or both.

The short-period phenotype is due to the truncation of KaiA. To separate these two possible causes for the clock phenotype, two different mutant strains were made, each mimicking one of the two consequences of the *Mu*Cm insertion. Both are based on the AMC1161 background, in which the native *kaiA* locus carries a disrupting insertion in the N-terminal coding region (*kaiA* null) and a copy of the firefly luciferase gene, under the control of the *kaiBC* promoter, serves as a reporter of gene expression. In one mutant, a truncated allele of *kaiA* (*kaiA281*) was added ectopically and the negative element at the native *kaiA* locus remained intact. In the other, the negative element was disrupted in the AMC1161 background at the same position as the 7G3-GG5 transposon mutant, while a copy of WT *kaiA* was provided ectopically. We predicted that the former would show the effect of truncated KaiA alone, whereas the latter would recapitulate the effects of elevated KaiB and KaiC levels only.

As shown in Table 3 and Fig. 2A, the introduction of an ectopic copy of WT *kaiA* restores circadian rhythmicity to the AMC1161 background (AMC1486) with a WT period of ~25 h. The *kaiA281* allele also restored rhythmicity to the *kaiA* null strain background (AMC1488), but with a significantly shorter period (~24.5 h). The protein levels of KaiA and KaiB in these two complemented strains were not noticeably different from those of the WT strain (Fig. 2B). Thus, the truncation of KaiA alone is not likely the cause of elevated KaiB and KaiC protein levels in the original *Mu* mutant. The presence of two copies of WT *kaiA* shortened the period by almost 1 h (AMC1485). The period was shortened even more when a copy of *kaiA281* and a WT copy of *kaiA* coexisted in the same strain (AMC1487) (Table 3 and Fig. 2A). KaiA is known to stimulate *kaiBC* expression (15). When two copies of the *kaiA*/*kaiA281* allele were present, we indeed saw notably higher KaiB protein levels in these strains (Fig. 2B), which suggests that the truncated KaiA281 protein retains the function to positively regulate *kaiBC* expression. In conclusion, these results clearly show that the truncation of *kaiA* could cause a short-period phenotype, although it was not as marked as in the original transposon mutant strain.

Next we checked whether the disruption of the negative

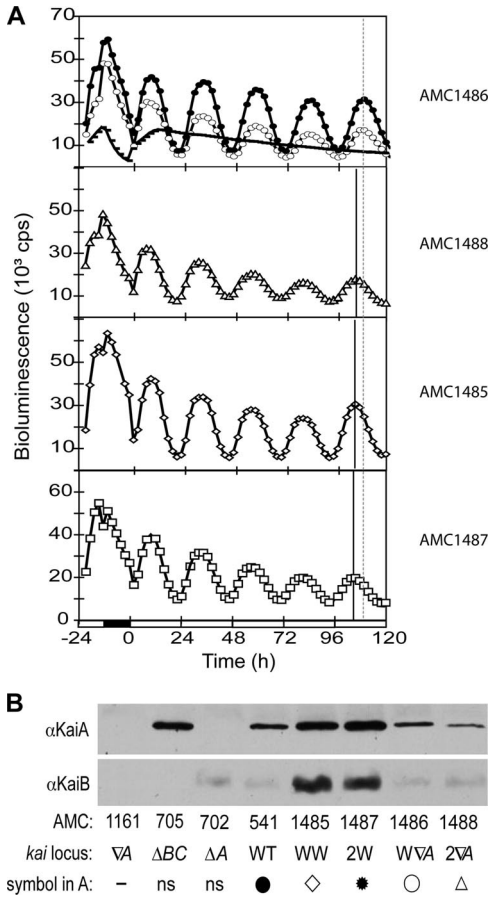

FIG. 2. Truncation of KaiA by three residues at its carboxyl terminus causes a short period. (A) Representative bioluminescence traces from the WT strain (AMC541 [closed circles]), the insertional *kaiA* null strain (AMC1161 [dashed lines]), and complemented strains (open symbols) in the reporter background of P*kaiBC*::*luc*. The WT and null strains are shown only in the top panel. Open circles, an ectopic copy of WT *kaiA* in the AMC1161 background (AMC1486); open triangles, an ectopic copy of truncated *kaiA* (*kaiA281*) in AMC1161 (AMC1488); open diamonds, an ectopic copy of WT *kaiA* in AMC541 (AMC1485); open squares, an ectopic copy of *kaiA281* in AMC541 (AMC1487). The last peak of WT circadian rhythms is indicated by a vertical dotted line, while that of complemented strains is positioned with a vertical black line. Axes are as for Fig. 1. (B) Immunoblot analysis of KaiA and KaiB proteins performed as for Fig. 1. KaiC consistently behaved like KaiB and is not shown here. AMC702, AMC705, and AMC1161 are described in the legend to Fig. 1. αKaiA and αKaiB, anti-KaiA and -B antibodies, respectively; WT, wild-type *kaiA*; WW, and 2W, an ectopic *kaiA* or *kaiA281* allele, respectively, in a WT background; W∇A and 2∇A indicate an ectopic wild-type *kaiA* or *kaiA281* allele, respectively, in a *kaiA* insertional null background; ns, not shown.

element also contributes to the shortened period in the *Mu*Cm mutant strain. For compatibility of antibiotic resistance genes, the original *Mu*Cm mutant was reconstructed with a gentamicin resistance variant (*Mu*Gm). The insertion of *Mu*Gm into *kaiA* at the same position as in the original *Mu*Cm strain shortened the period by about 0.5 h in the P*kaiBC*::luc background (AMC1491). Cells remained arrhythmic after the insertion of *Mu*Gm into *kaiA* in the *kaiA* insertional null background (AMC1492) (Table 3 and Fig. 3A). The KaiB protein

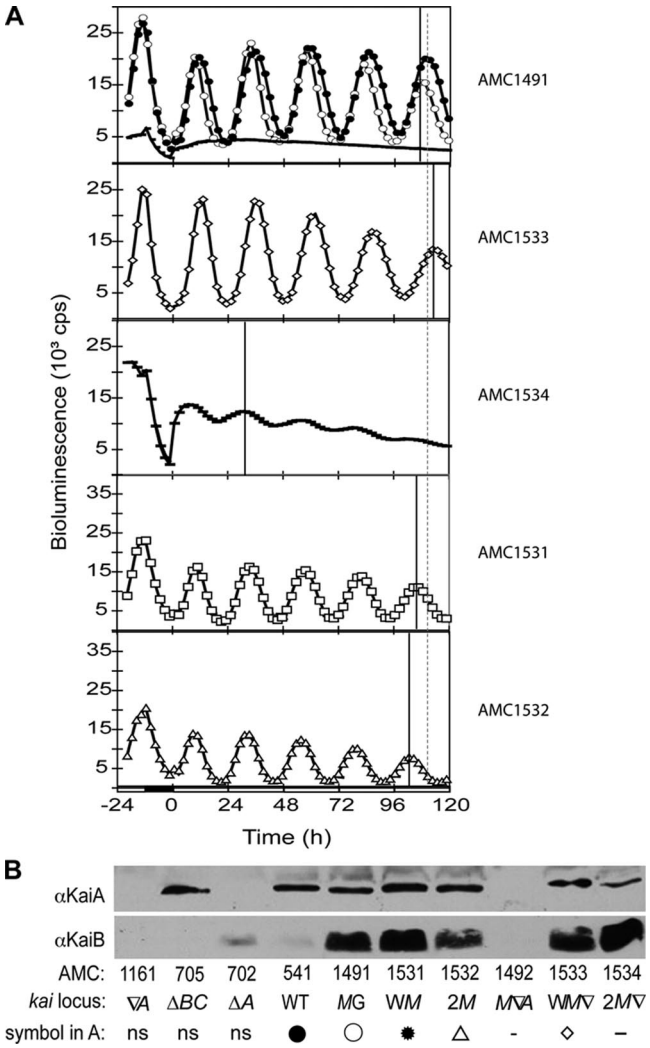

FIG. 3. Disruption of the upstream negative element of the *kaiBC* promoter increases KaiB protein level and slows the clock. (A) Representative bioluminescence traces from the P*kaiBC*::*luc* reporter background. The following two reference strains are shown only in the top panel: closed circles, WT background (AMC541); short horizontal bars, *kaiBC* negative element disrupted and insertional *kaiA* null (AMC1492). From the top pane: open circles, reconstructed *Mu*Gm mutant (AMC1491); open diamonds (AMC1533) and long horizontal bars (AMC1534) carry an ectopic WT and a *kaiA281* allele of *kaiA*, respectively, in an AMC1492 background; open squares (AMC1531) and open triangles (AMC1532) carry an ectopic WT and a *kaiA281* allele of *kaiA*, respectively, in an AMC1491 background. The last peak of WT circadian rhythms is indicated by a vertical dotted line, while that of complemented strains is positioned with a vertical black line. There is an exception in the third pane, where circadian rhythms damped after two cycles in LL in the complemented strain, AMC1534. Instead, two vertical lines were drawn to indicate the second peak in LL for AMC541 and AMC1534. Axes are as described for Fig. 1. (B) Immunoblot analysis of KaiA and KaiB proteins performed as for Fig. 1. AMC541, AMC702, AMC705, and AMC1161 strains are described in the legends to Fig. 1 and 2. αKaiA and αKaiB, anti-KaiA and -B antibodies, respectively; WT, wild-type *kaiA*; *M*G, *Mu*Gm in a WT background; W*M* and 2*M*, an ectopic *kaiA* or *kaiA281* allele in a *Mu*Gm background, respectively; *M*∇*A*, *Mu*Gm in the *kaiA* insertional null (AMC1161) background; W*M*∇ and 2*M*∇, an ectopic WT or *kaiA281* allele in the *M*∇*A* background, respectively; ns, not shown.

level was greatly elevated in the reconstructed *Mu*Gm mutant strain, as predicted for disruption of the negative element. In the absence of KaiA, only a trace amount of KaiB was detected even when the negative element was also eliminated in the double-insertional mutant strain (AMC1492) (Fig. 3B). A copy of WT *kaiA* or *kaiA281* was then introduced ectopically into these two strains, respectively. In the double-insertional mutant strain background, which has no KaiA and elevated KaiB and KaiC, the introduction of a copy of WT *kaiA* restored the rhythmicity with a longer period, ~25.6 h (AMC1533) (Fig. 3A and Table 3). However, the introduction of a copy of *kaiA281* in the same background, potentially mimicking the original *Mu* insertional strains, could not restore a stable WT circadian rhythm in the cells (AMC1534). The circadian traces of these cells showed reduced amplitude, advanced phase, and fast damping phenotypes (Fig. 3A, the third pane). The period may also be shortened, but could not be calculated accurately from the damped cycles. The addition of an ectopic copy of WT *kaiA* did not significantly change the period in the AMC1491 background (*kaiA281*, disrupted negative element), whereas an extra copy of *kaiA281* in the same strain shortened the period (AMC1531 and AMC1532, respectively) (Fig. 3A and Table 3). Immunoblot data showed the expected elevation in KaiB protein level when the negative element was disrupted in the presence of an ectopic copy of KaiA or KaiA281 (Fig. 3B). Based on these results, we can conclude that the disruption of the negative element along with normal expression level of KaiA significantly elevates *kaiBC* expression, which results in an extended circadian period.

Together, these data indicate that the short period caused by the insertion of the *Mu* transposon in *kaiA* is due to the truncation of KaiA. The *kaiA281* allele, without disrupting basic KaiA function as a central oscillator component, shortens the circadian period by ~0.5 to 1.0 h, depending on the background of the reporter strain. On the contrary, disruption of the negative regulatory element extends the period. Because the phosphorylation status of KaiC is thought to be the major factor that determines the period length of cyanobacterial circadian clock (33, 44), we speculated that the phosphorylation pattern of KaiC in the mutant strains would be different from that of WT strains.

KaiC autophosphorylation pattern is altered by KaiA281. A time course analysis was performed to check the circadian pattern of KaiC phosphorylation in these strains. Synchronized *S. elongatus* cultures were first sampled every 4 h under LL conditions (see Materials and Methods). As shown in Fig. 4A, phosphorylated KaiC levels in the WT strain (AMC541) peaked at around 8 to 12 h (LL8-12), while unphosphorylated KaiC peaked at LL0/24. Both *kaiA*- and *kaiA281*-complemented strains (AMC1486 and AMC1488, respectively) displayed an increased ratio of phosphorylated KaiC to unphosphorylated KaiC at LL0-4 and LL20-24. Samples taken at 1-h intervals clearly showed that the level of phosphorylated KaiC in these strains was elevated during these time points. In complemented strains, a notable amount of phosphorylated KaiC was seen even at LL0 and LL24, when mainly unphosphorylated KaiC was present in the WT strain (Fig. 4B). However, the overall circadian patterns of unphosphorylated KaiC in either complemented strains or the WT strain were very similar. There was also no difference in the KaiC phosphorylation

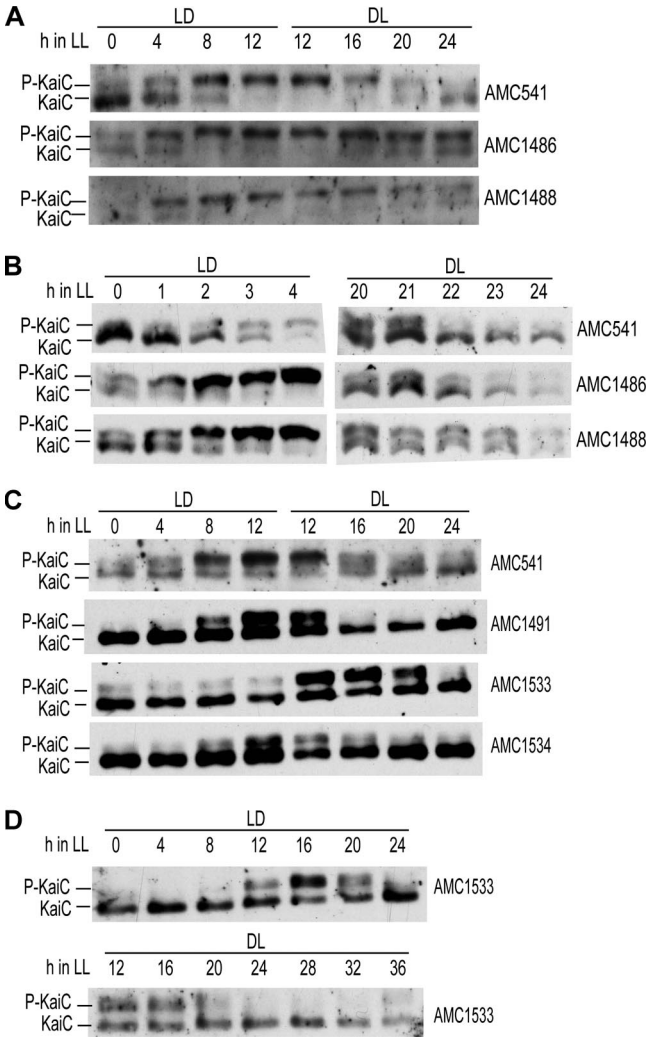

FIG. 4. KaiC autophosphorylation pattern in cyanobacterial strains. (A) Time course immunoblot analysis of *S. elongatus* strains AMC541 (WT), AMC1486 (WT *kaiA*-complemented strain), and AMC1488 (*kaiA281*-complemented strain). Shown are results from 4-h time point samples taken over 12 h. (B) One-hour time point samples taken over 4 h for strains in panel A. (C) Time course immunoblot analysis of *S. elongatus* strains AMC541, AMC1491 (*Mu*Gm), AMC1533 (WT *kaiA*-complemented and negative element-disrupted strain), and AMC1534 (*kaiA281*-complemented and negative element-disrupted strain). Shown are results from 4-h time point samples taken over 12 h. (D) Four-hour time point samples taken over 24 h for AMC1533. LD (light/dark) and DL (dark/light) indicate opposite entrainment conditions. The number of hours released into LL for each time point is shown on top of each lane. P-KaiC, phosphorylated KaiC; KaiC, unphosphorylated KaiC. Total soluble protein (~40 μg) was loaded in each well.

patterns between the *kaiA281*-complemented strain (AMC1488) and the WT *kaiA*-complemented strain (AMC1486), even though they were slightly different in their circadian period of bioluminescence.

We then checked the phosphorylation pattern of KaiC in strains in which the negative element upstream of *kaiBC* is disrupted. As in the reconstructed *Mu*Gm mutant (AMC1491), the total level of KaiC protein was elevated throughout the free-running circadian cycle in *kaiA* null double-insertional

mutant strains complemented with either *kaiA* (AMC1533) or *kaiA281* (AMC1534) (Fig. 4C). AMC1491 and AMC1534 showed similar patterns, such that the elevated KaiC was mainly in the unphosphorylated state. The circadian profile of accumulation of phosphorylated KaiC was altered in these two strains compared to that of the WT strain: the rise of phosphorylated KaiC was delayed at least 4 h. Although phosphorylated KaiC still peaked around LL8-12 as in the WT strain, it then quickly diminished and was barely detectable at LL16, when the WT strain still had abundant phosphorylated KaiC (Fig. 4C). The rise of phosphorylated KaiC in AMC1533 was further delayed until LL12 and peaked at LL12-16 (Fig. 4C). It appeared that the KaiC phosphorylation cycle was shifted 4 h later in this strain compared to the other strains. This observation was confirmed in samples taken in a continuous 24-h sampling period (Fig. 4D).

From the results described above, we can conclude that the truncation of KaiA does not significantly alter its ability to stimulate autophosphorylation of KaiC and that transcomplemented KaiA or KaiA281 increases phosphorylated KaiC at times when it is low in WT strains (LL0-4 and LL20-24) (Fig. 4A and B). On the other hand, the KaiC phosphorylation pattern in strains with elevated KaiB and KaiC is different from that of the WT strain in some aspects, such as the phase of peak phosphorylation. Nevertheless, the overall cycle of KaiC phosphorylation is retained. Thus, there is no direct phase correlation between the phosphorylation pattern of KaiC and the bioluminescence rhythms produced from the reporter genes.

The interaction between KaiA and KaiC is strengthened in KaiA281. The changes in the phosphorylation pattern of KaiC and the period of bioluminescence cycles in mutant strains suggest altered interactions between KaiA and KaiC. We performed fluorescence anisotropy-based binding assays to determine whether there is a change in the interaction between KaiA and a KaiC-derived peptide, referred to as the CII ATP binding domain (CIIABD), in the KaiA281 variant. CIIABD is the C-terminal peptide of KaiC and specifically binds KaiA (22, 36, 40). KaiA281 exhibited a tighter interaction with the KaiC peptide (Fig. 5) than did WT KaiA. The KaiA281 values were more similar to those of the C-terminal domain of KaiA, KaiA180C, which is known to bind CIIABD more tightly and stimulate autokinase activity of KaiC more than does full-length KaiA (22, 42). The results suggest that the short-period gene expression phenotype of *kaiA281* results from the strengthened KaiA-KaiC interaction.

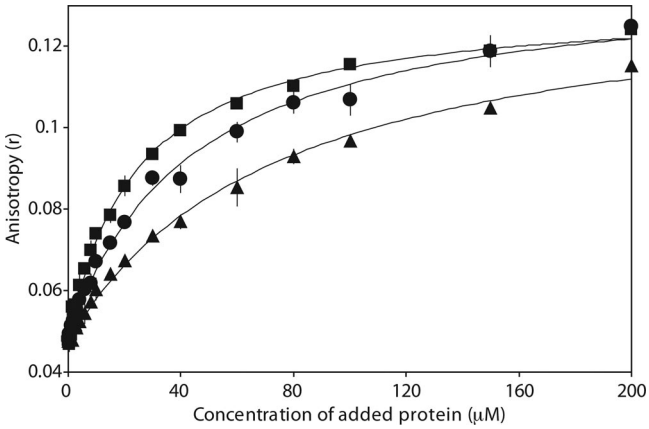

FIG. 5. Fluorescence anisotropy data from 6-iodoacetamido-fluorescein-labeled KaiC peptides as a function of KaiA180C (squares), WT KaiA (triangles), and KaiA281 (circles). The KaiC peptide consists of the C-terminal 32 residues of *S. elongatus* KaiC. All fluorescence anisotropy values were measured at 30°C.

DISCUSSION

Among circadian clock period mutants, both short- and long-period alleles have been observed for *kaiC*; however, only short-period mutants have been identified with *kaiB*, and usually long-period phenotypes result from mutation of *kaiA* (15, 34). This phenomenon is probably related to the ability of the mutant proteins to affect the autokinase activity of KaiC and the greater probability of loss-of-function rather than gain-of-function mutants. A long-period *kaiA* mutant, KaiA2 (also called A30a), which carries an R249H missense point mutation, has a 30-h period (15). This mutant has a reduced KaiC autokinase rate and accumulates unphosphorylated KaiC (19). Period mutations in *kaiA* or *kaiC* have also been shown to alter the interaction between these two proteins in yeast, even though the results were somewhat contradictory (39).

If there is a direct correlation between KaiC phosphorylation status and the circadian period, the *kaiA281* short-period mutant might be expected to show more accumulated phosphorylated KaiC. The time course analysis of KaiC protein samples, however, did not show notable differences in accumulation of phosphorylated KaiC between a *kaiA281*-complemented *kaiA* insertional null strain and its corresponding WT complementation strain (Fig. 4A and B). Because the period difference between these two strains is less than 1 h, a change in KaiC phosphorylation status might be present that is not revealed with the resolution of the time course. A KaiC autokinase assay is a more quantitative way to check the difference between KaiA281 and WT KaiA in stimulating KaiC autophosphorylation. Preliminary data showed that when KaiB was absent, the sample containing purified KaiA281 did show slightly increased stimulation of KaiC autophosphorylation activity compared with full-length KaiA (data not shown). These data are consistent with the fact that long-period KaiA mutants, including A30a/KaiA2, have a shorter lifetime of phosphorylated KaiC but do not differ much from WT strains in the turnover rate of unphosphorylated KaiC (44). The prolonged KaiC phosphorylation state in the presence of KaiA281 might also reflect differences in the degradation rate of KaiC in vivo.

The fluorescence anisotropy data indicate that the loss of the three KaiA C-terminal residues alters the interaction between KaiA and KaiC (Fig. 5). KaiA binds specifically to the C-terminal domain of KaiC possibly at two interfaces: CIIABD peptide and the ATP binding pocket (36, 40). Both *kaiA2* and *kaiA281* mutants carry mutations that affect the C-terminal domain of KaiA, which is responsible for interaction with KaiC and stimulation of KaiC autophosphorylation (41, 42). The R249H substitution encoded by *kaiA2* is in the KaiA-KaiC interaction interface and very close to the ATP binding pocket of KaiC; in contrast, the last three residues of KaiA map at the edge of one of the dimer interfaces (45). Thus, deletion of these residues probably results in minor conformational

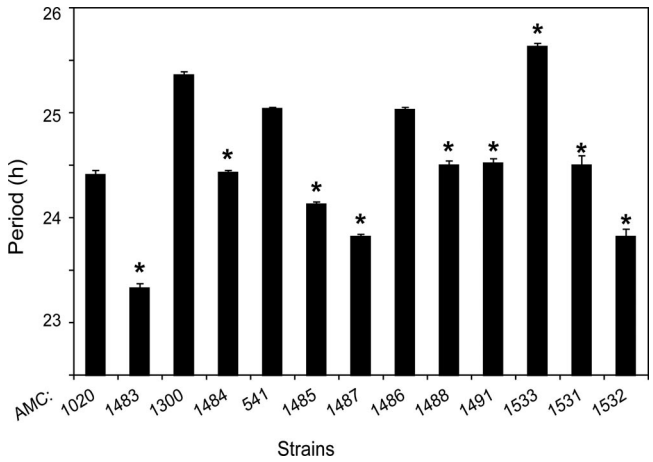

FIG. 6. Column chart of circadian period of cyanobacterial strains in Table 1. The *x* axis shows results for cyanobacterial strains, and the *y* axis shows the circadian period in hours. ⋆, $P < 0.0001$ by unpaired *t* test compared with corresponding WT strains.

change of the KaiA dimer, which in turn strengthens the interaction between KaiA and KaiC and enhances its ability to stimulate the autokinase activity of KaiC. KaiA281 may not only interact more strongly with KaiC but also affect the binding of KaiB to KaiC and hence the dephosphorylation of KaiC, because the binding of KaiB to KaiC is enhanced in the presence of KaiA (18).

The original *Mu*Cm *kaiA* insertion, 7G3-GG5, shortens the period in both P*psbAI*::*luxAB* and P*kaiBC*::*luxAB* reporter strains by almost 1 h. The period difference between the reconstructed *Mu*Gm *kaiA* insertion mutant and its corresponding WT strain carrying a P*kaiBC*::*luc* reporter is diminished to around 0.5 h. The two complemented *kaiA* strains, one with WT *kaiA* and the other with *kaiA281*, also show a period difference of ~0.5 h with each other (Table 3 and Fig. 6). These differences may reflect the variations in the promoter activity and/or posttranscriptional regulation of different reporter constructs. We have observed previously that the period length differs among some WT reporter strains (5).

The short-period *kaiA281* allele does not lose its basic function as a central oscillator component. Our results suggest that *kaiA281* functions normally in enhancing *kaiBC* promoter activity and sustaining the circadian oscillation (Table 3 and Fig. 2). Nevertheless, KaiA281 is different from WT KaiA in some other aspects, as it could not completely complement AMC1492, in which *kaiA* and the negative element are both disrupted by insertion, when provided ectopically (Fig. 3A). There might be a position effect of *kaiA* alleles (*cis* versus *trans*); note that even the WT allele did not complement with a normal period in this elevated KaiB/KaiC situation. Ectopic *kaiA* or *kaiA281* was introduced into the NS1 locus, which is located almost 180° from the native *kai* locus on the circular chromosome. In all cyanobacterial species that carry *kaiA*, this gene is always located immediately upstream of *kaiBC*. In addition, the negative element of P*kaiBC* is located entirely within the coding region of *kaiA* in *S. elongatus*. This structural conservation of the *kai* locus is likely to have significance in clock function. The ectopically expressed *kaiA* or *kaiA281* functions similarly to its *cis* counterpart in AMC1486, AMC1488, and AMC1533, in which the oscillator is not otherwise compromised. It is known that a promoter with peak expression 180° out of phase to that of P*kaiBC*, such as P*purF* (7), or even the heterologous *E. coli* promoter P*trc* expressed from a neutral site (32, 44), can successfully replace the original *kaiBC* promoter to sustain a functional circadian clock in *S. elongatus*. Thus, there may be a "buffering mechanism" in cyanobacterial clocks to tolerate certain internal alterations, which has also been observed in a corresponding mammalian system (6). Circadian rhythms in cyanobacteria can be sustained by either the phosphorylation oscillation or the transcriptional-translational feedback regulation of KaiC (24, 32). In the strain AMC1534, which mimics the original transposon insertional mutant, the combined effect of disrupting the negative element and a truncated KaiA expressed in *trans* may simply compromise the timing mechanism beyond its capacity for robustness. Although they show clear rhythmicity, complemented strains AMC1486 and AMC1488 are different from WT strain AMC541 in their circadian patterns of KaiC phosphorylation. The increased amount of phosphorylated KaiC at the early and late stages of a circadian cycle (Fig. 4A and B) may be another consequence of the ectopic expression of *kaiA* or *kaiA281*. This pattern is not seen in AMC1533, probably because of the presence of an excess amount of unphosphorylated KaiC (Fig. 4C).

The ratio of KaiA to KaiC seems to be important to sustain a normal circadian period (21). Here we show that when the KaiA/KaiC ratio is high due to an extra copy of *kaiA*, more KaiB (and coexpressed KaiC, which is not shown) accumulates and circadian period is shortened (as seen in AMC1485 and AMC1487) (Table 3 and Fig. 2B). Conversely, when the ratio is lowered due to the disruption of the *kaiBC* upstream negative element, there is more KaiB (and KaiC) expressed and a longer period (as seen in AMC1533) (Table 3 and Fig. 3B). We propose that this correlation is generally true in the cyanobacterial circadian clock. The *kaiA281* allele also displays a dominant effect: even with a WT *kaiA* allele present, a strain that encodes *kaiA281* always has a shorter circadian period than the WT strain on which it is based (Table 3 and Fig. 6). The disruption of the negative element upstream of *kaiBC* promoter alone extends the period. In the presence of a *kaiA281* allele, however, the long period is masked by the short-period phenotype, as shown in the *Mu* insertional strains (Table 3).

No correlation between the peak time of accumulation of phosphorylated KaiC and rhythmic bioluminescence traces can be deduced from our results. Strain AMC1534, mimicking the original 7G3-GG5 *Mu*Cm insertional strain, is phase advanced in its bioluminescence rhythms, but not in the KaiC phosphorylation pattern. On the contrary, AMC1533 shows a normal phase in bioluminescence traces but is phase delayed (~4 h) in time course immunoblot analysis (Fig. 4C and D). Previously, a point mutation of *kaiC* (C1265T) was shown to cause the loss of the rhythmic KaiC accumulation and reduced circadian oscillation in phosphorylation. However, this mutant, called *pr1*, still possesses robust bioluminescence rhythms with a WT period, albeit 3 to 4 h phase advanced (25). Thus, there must be other mechanisms for phase regulation—probably through a circadian output pathway. Nonetheless, the overall circadian rhythms of the KaiC phosphorylation cycles are conserved in

these strains. Because the period difference among all the strains in this study (except AMC1534) is less than 1 h, the KaiC phosphorylation and dephosphorylation cycle recurs after approximately 24 h despite variations in phosphorylation pattern during the cycle.

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