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The Circadian Clock-Related Gene *pex* Regulates a Negative *cis* Element in the *kaiA* Promoter Region▽

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In the cyanobacterium *Synechococcus* sp. strain PCC 7942, a circadian clock-related gene, *pex*, was identified as the gene prolonging the period of the clock. A PadR domain, which is a newly classified transcription factor domain, and the X-ray crystal structure of the Pex protein suggest a role for Pex in transcriptional regulation in the circadian system. However, the regulatory target of the Pex protein is unknown. To determine the role of Pex, we monitored bioluminescence rhythms that reported the expression activity of the *kaiA* gene or the *kaiBC* operon in *pex* deficiency, *pex* constitutive expression, and the wild-type genotype. The expression of *kaiA* in the *pex*-deficient or constitutive expression genotype was 7 or 1/7 times that of the wild type, respectively, suggesting that *kaiA* is the target of negative regulation by Pex. In contrast, the expression of the *kaiBC* gene in the two *pex*-related genotypes was the same as that in the wild type, suggesting that Pex specifically regulates *kaiA* expression. We used primer extension analysis to map the transcription start site for the *kaiA* gene 66 bp upstream of the translation start codon. Mapping with deletion and base pair substitution of the *kaiA* upstream region revealed that a 5-bp sequence in this region was essential for the regulation of *kaiA*. The repression or constitutive expression of the *kaiA* transgene caused the prolongation or shortening of the circadian period, respectively, suggesting that the Pex protein changes the period via the negative regulation of *kaiA*.

The circadian clock, which drives many biological phenomena with a period of about 24 h, including gene expression, has been observed in prokaryotic cyanobacteria and in eukaryotic cells (5). Cyanobacteria exhibit robust circadian rhythms related to many of their biological activities (4, 7, 15, 19, 27, 29, 30). Molecular genetic techniques, e.g., efficient transformation and gene targeting by precise homologous recombination, have been used for studying the cyanobacterial *Synechococcus* sp. strain PCC 7942 (3, 6, 24). By integrating molecular genetic properties and a monitoring system for the in vivo gene expression of bioluminescence, we previously isolated several types of clock mutants that exhibited altered bioluminescence rhythms, including short- or long-period rhythms and even arrhythmias (16). We then cloned the causative gene cluster, composed of *kaiA* and *kaiBC*, for all the circadian mutants and named it *kaiABC* (9, 16). Positive and negative transcriptional autoregulation by the Kai proteins with a period of about 24 h was found, as observed in eukaryotic circadian clocks, but the Kai proteins seem to function robustly to sustain the transcription rhythm of the *kaiBC* operon over that of the genome, rather than to maintain the period of the clock (11, 18, 20). However, little is known about the regulation of this gene cluster.

A circadian clock-related gene, *pex* (for *p*eriod-*ex*tender) (17), was originally isolated as a genomic DNA region through which the C22a mutant harboring the *kaiC1* mutation in the *kaiC* locus (circadian period of 22 h) was suppressed (9, 16, 17). Cells with constitutive expression of *pex* showed a period prolongation phenotype (28 h). In contrast, the lack of *pex* shortened the period by about 1 h, suggesting its physiological function in regulating the clock (17). Database analyses of the domain structure classified Pex as a PadR family (pfam03551) protein. PadR in *Lactobacillus* binds to a promoter of its target gene (*padA*) and regulates the metabolism of an environmental toxin (2, 8). The *pex*-deficient strain of *Synechococcus* showed an abnormal phase angle of the clock (the acrophase of the *kaiBC* gene expression rhythm was about 3 h earlier than that of the wild type) under diurnal light-dark cycles, and *pex* mRNA and Pex protein increased in the dark period (31). X-ray crystal analysis of the Pex protein in *Synechococcus* showed a winged-helix protein, a structure commonly found in DNA-binding transcription factors, such as the multiple antibiotic resistance repressor (MarR) family proteins (1, 22). In addition, Pex specifically bound to an upstream DNA fragment of *kaiA* in vitro (1).

Here, we demonstrate that *kaiA* expression is significantly

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more abundant in *pex*-deficient cells than in wild-type cells, suggesting that Pex functions to repress *kaiA* expression. The bioluminescence reporter for *kaiA* was also used to find *cis* elements for Pex in the upstream region of *kaiA*. A 5-bp element (AGAGA) downstream from the *kaiA* transcription start site was essential to the negative regulation of *kaiA* by Pex. We were able to reproduce the period alteration that occurs in *pex*-related mutants by the exogenous up- or down-regulation of *kaiA* expression.

MATERIALS AND METHODS

Bacterial strains, medium, and cultures. We used wild-type *Synechococcus* sp. strain PCC 7942 and bioluminescence reporter strains for the *psbAI*, *kaiA*, and *kaiBC* genes (9, 15). In addition, the *psbAI* reporter strain lacking or constitutively expressing the *pex* gene (17) was used to obtain *kaiAp*::*luxAB* and *kaiBCp*::*luxAB* reporter strains harboring the *pex*-related genotype. A *kaiBC* reporter strain in which *kaiA* was inactivated by a nonsense mutation at the fourth codon (9) was used in a repression experiment for *kaiA* expression. The *Synechococcus* cultures for RNA analysis were grown in BG-11 liquid medium (26) under the standard light conditions previously described (17).

Monitoring of gene expression as bioluminescence. We used an automated photon-counting apparatus (9) with a photomultiplier tube to monitor the expression of *kaiA* or *kaiBC* gene bioluminescence. *Synechococcus* cells were grown for 3 to 4 days on solid BG-11 agar in 40-mm plastic dishes under standard conditions to give 30 to 60 colonies (0.2 mm in diameter). After a 12-h dark treatment for entrainment of the clock, the dishes were placed in the photon-counting apparatus. The bioluminescence intensity was normalized to the number of colonies. A representative rhythm among three to six replicates was found for each reporter strain.

DNA sequencing and sequence analysis. DNA sequencing was carried out using a *Taq* DyeDeoxy terminator cycle sequencing kit and a model 373A DNA sequencing system (Applied Biosystems, Foster City, CA).

Northern blotting. Cultivation conditions and total RNA isolation were performed as described previously (9, 17). After sampling and RNA extraction, 5 μg of total RNA was subjected to electrophoresis on 1.0% agarose gel containing 1.0% formaldehyde, blotted onto positively charged nylon membranes, and hybridized with a digoxigenin (DIG)-labeled *kaiA* probe, as described previously (9, 17). Chemiluminescence images and the hybridization signal were obtained and quantified using a Fluor-S MultiImager (Bio-Rad, Hercules, CA).

Primer extension analysis. A primer extension experiment was performed using standard procedures (17, 28). The oligonucleotide used as a primer was 5′-CCGTCGATTCCACCCAAATGC-3′, which corresponds to nucleotides +89 to +109 of the *kaiA* gene. To make sequencing ladders, we carried out a sequencing reaction using the same primer. The labeling efficiency of the primer with [γ-^{32}P]ATP was >5,000 Ci/mmol.

Construction of plasmids for repression or induction of *kaiA* gene expression. To make control cells for the repression experiment with the *kaiA* gene, we constructed a pTS2*kaiA* plasmid. We amplified a 1-kb DNA segment that contained the promoter and open reading frame of *kaiA* from the *Synechococcus* genome by using PCR primers with a BglII linker sequence, i.e., 5′-CGAGATCTAAACAACAGCCCTCTATCATCTCAG-3′ (−92 to −68 of the *kaiA* gene; the BglII restriction site is underlined) and 5′-GAAGATCTAACAGGATAAAGAG-3′ (+958 to +971; the BglII restriction site is underlined), and digested this with the BglII restriction enzyme. The BglII digest was inserted into a unique BamHI site in neutral site II (NSII) (GenBank/EMBL/DDBJ database accession no. U44761) in the targeting vector pTS2K (M. Ishiura and S. Kutsuna, unpublished data). The amplified 1-kb *kaiA* fragment was also inserted into a unique BamHI site downstream of the *trc* promoter (*trcp*) in another NSII targeting vector, pTS2K*trcp*. We then selected the plasmid inserted into the *kaiA* fragment inverting to *trcp*. The obtained plasmid, pTS2*kaiA* or pTS2*kaiA*::*trcp*, was used for the transformation of a *kaiA*-inactivated strain of the *kaiBC* reporter.

For the constitutive expression of *kaiA*, we constructed plasmid pTS2*trcp*::*kaiA*-GTG. A 0.9-kb section of the open reading frame of *kaiA* was amplified using the primer sequences 5′-ATAGATCTTAAGACTCAGTCCTGACAGGAGCGACTGCG-3′ (+41 to +67; the BglII restriction site is underlined) and 5′-GAAGATCTAACAGGATAAAGAG-3′ (+958 to +971; the BglII restriction site is underlined) and digested with BglII. The digest was inserted into the BamHI site downstream of *trcp* in the pTS2K*trcp* plasmid. The obtained plasmid was introduced into a wild-type *kaiBC* reporter strain.

TABLE 1. Relative bioluminescence of deletion derivatives of *kaiA* reporters[a]

Gene or reporter	Upstream region	% Bioluminescence ± SD	No. of samples
kaiA	−457 to +402	100 ± 9	3
D1	−92 to +402	28 ± 5	4
D2	−66 to +402	45 ± 6	4
D3	−44 to +402	53 ± 3	4
D4	−20 to +402	1	3
D5	+6 to +402	1	3
D6	+25 to +402	2	3
D7	−457 to +68	59 ± 8	4
D8	−457 to +49	68 ± 8	4
D9	−457 to +33	102 ± 21	4
D10	−457 to +23	385 ± 25	4
D11	−457 to +11	441 ± 18	4
D12	−457 to −16	11 ± 1	4

[a] The reporters were recombined in the NSII genomic region.

Deletion mapping of the negative element in the *kaiA* upstream region. From the 0.8-kb SmaI-XhoI segment, which carried the promoter of *kaiA*, we constructed a series of deletion derivatives carrying upstream regions of various lengths (Table 1). To make a deletion derivative (nucleotides −92 to +402), we used a DraI-XhoI restriction fragment of the *kaiA* region. Appropriate segments for the other derivatives were synthesized by PCR using the following oligonucleotides: upper primers 5′-GAAGGCCTAACTTTTGAGAACTGT-3′ (−66 to −51 of *kaiA*; the StuI restriction site is underlined here and in the following sequences), 5′-GAAGGCCTGTGGACAAAGCGATC-3′ (−44 to −30), 5′-GAAGGCCTTGAGCTGCAGTGCTA-3′ (−20 to −6), 5′-GAAGGCCTAATTTTTCCTTTGTCC-3′ (+6 to +21), and 5′-GAAGGCCTATCTGTCTGCAGACT-3′ (+30 to +44) and the lower primer (5′-GTGGTTGGCCCCCATCAGCAT-3′ (+481 to +501), which corresponded to a sequence downstream of the XhoI site in the *kaiA* gene. The PCR products were digested with StuI and XhoI. The digests were inserted between the unique sites EcoRV and XhoI upstream of *luxAB* in the pTS2Slux plasmid (17). Oligonucleotides 5′-CGGGAGCTCTACAGTAATCGACTCC-3′, which corresponded to a sequence upstream of the SmaI site, and 5′-AAACGCTCGAGACGCAGTCGCTCCTGT-3′ (+53 to +68; the XhoI site is underlined) were used in the PCR. The amplified DNA was digested with SmaI and XhoI and inserted between the unique sites in pTS2Slux. By PCR using the SK primer as the upper one (5′-TCTAGAACTAGTGGATC-3′ [Toyobo, Osaka, Japan]), which corresponded to the plasmid sequence upstream of the SmaI site of the *kaiA* promoter region fused to *luxAB* in the *kaiA* expression reporter construct with the genomic segment NSII, and using six primers as lower ones (5′-CAGGCCTGACTGAGTCTGCAGAC-3′ [+34 to +49], 5′-CAGGCCTGACTGAGTCTGCAGAC-3′, 5′-TAGGCCTAGATTAATCTCTGGAC-3′ [+18 to +33], 5′-TAGGCCTCTGGACAAAGGAAAAA-3′ [+8 to +23], 5′-CAGGCCTAAAATTTAATTTAGCC-3′ [−5 to +11], and 5′-AAGGCCTGCTCATGAGGCCGCG-3′ [−30 to −16]; StuI sites are underlined), the downstream deletion fragments of the *kaiA* upstream region with a vector portion were amplified from the *kaiA* expression reporter construct for NSII. These products were digested with BamHI and StuI and inserted between the unique sites BamHI and EcoRV in pTS2Slux. The deletion of the *kaiA* upstream region was checked by sequencing with the M13 reverse primer (Toyobo, Osaka, Japan). We introduced the obtained derivatives into wild-type *Synechococcus* and measured the bioluminescence quantitatively after the selection of the transformed cells with spectinomycin (40 μg ml^{-1}), as described previously (9).

For the preparation of deletion derivatives of the *kaiA* reporter in the targeting site NSI, we inserted the deletion fragments of the *kaiA* upstream region into the upstream region of *luxAB* in pTS1CluxΔ. The obtained plasmids were targeted into NSI, which has *psbAp*::*luxAB* reporter DNA in the genome of the bioluminescence reporter strain AMC149 (15). The selection of the transformation was performed on BG-11 agar with a chloramphenicol concentration of 7.5 μg ml^{-1}.

Mutation analysis of the negative element in the promoter region of *kaiA*. We used PCR to line up mutated D9 reporters with base pair substitutions. The SK primer (Toyobo), which corresponds to the upstream part of *kaiA* in the plasmid pTS1CluxΔ, and mutant primers with the StuI linker were used to amplify the mutation fragments, as performed for the upper primer. The sequences of the

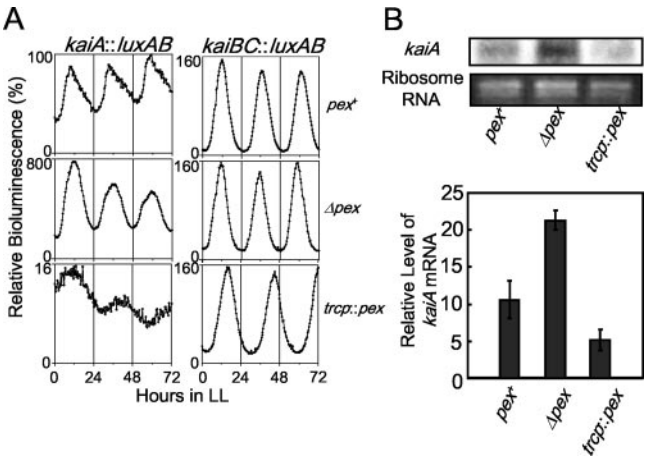

FIG. 1. Expression of *kaiABC* in the circadian period mutants. The expression of *kaiA* and *kaiBC* was analyzed in strains carrying the *pex*$^+$, Δ*pex*, and *trcp*::*pex* genotypes. (A) Panels represent the bioluminescence rhythms of the expression activity of the upstream region of *kaiA* or *kaiBC* (*kaiA*::*luxAB* or *kaiBC*::*luxAB*, respectively). The ordinate shows the percentage of relative bioluminescence; the abscissa shows the hours in continuous light after 12 h of darkness. The reporter gene fusions were recombined in the genomic region NSI. To activate *trcp*::*pex* gene fusion in a *trcp*::*pex* strain, we used the inducer 1 mM IPTG. (B) The upper panel shows results of Northern blotting of *kaiA* mRNA in the three genotypes after 6 h in constant light (*kaiA* mRNA) and used total RNA (rRNA). Five micrograms of total RNA was applied. The fluorescence image of the gel stained with ethidium bromide confirmed that an equal amount of the total RNA existed in each sample by referring the 23S and 16S ribosomal RNAs among the genotypes. For the lower panel, the relative level was calculated by comparing the amount of *kaiA* mRNA in each genotype to the total value of the three genotypes. The mean ± standard error (error bars) ($n = 3$) of the calculated relative level of each genotype is shown in the graph. *kaiA* mRNA was detected with a DIG-labeled *kaiA* probe by using PCR-based DIG-dUTP (DIG DNA labeling mix; Roche Diagnostics, Mannheim, Germany) incorporated into the PCR product of the *kaiA* coding region. *Synechococcus* cultures were grown under standard light conditions until the optical density at 730 nm reached 0.2. Three independent sets of experiments were conducted. LL, continuous light condition of 50 μmol m^{-2} s^{-1}.

lower mutation primers were as follows: for M1, 5′-TAGGCCTAGATTAATC TCTCCTCAAAGGAAAAATTTAATT-3′; for M2, 5′-TAGGCCTAGTAATT TCTCTGGACAAAGGAAAAA-3′; and for M3, (5′-TAGGCCTAGATTAAT CACAGGACAAAGGAAAAT-3′ (mutations are underlined). The PCR products were digested with BamHI and StuI and then cloned into the BamHI-SmaI multicloning sequence of the plasmid.

RESULTS

Pex as a negative regulator of a circadian clock gene, *kaiA*. Previously, using a bioluminescence reporter for the photosynthesis gene *psbAI*, we determined that the free-running period of the cells of *pex*-deficient (Δ*pex*) or *pex* constitutive expression (*trcp*::*pex*, with the inducer 1 mM isopropyl-β-D-thiogalactopyranoside [IPTG]) genotype was about 1 h shorter or about 3 h longer, respectively, than that of the wild-type *pex* locus (*pex*$^+$) (17), suggesting that changes in *pex* expression may affect certain aspects of the clock gene cluster *kaiABC*. To find this type of abnormality in the period mutants, we also used the bioluminescence reporter genes (*luxAB*). Approximately 0.8-kb upstream regions of *kaiA*, including its open reading frame (~0.3 kb), or *kaiBC* (~0.9 kb) were fused to *luxAB*. The obtained constructs were introduced into the cells with Δ*pex* or *trcp*::*pex*. We then examined the cells obtained for bioluminescence. Compared with bioluminescence rhythms in *pex*$^+$, we confirmed that the timing of the rhythms in Δ*pex* and *trcp*::*pex* was advanced or delayed in every cycle, leading to shortened or lengthened circadian periods, respectively (Fig. 1A). The effects on the period were the same as that of a clock-regulated bioluminescence reporter for the *psbAI* gene (17). The bioluminescence level for *kaiA* expression in Δ*pex* or *trcp*::*pex* was 7 times (mean relative level ± standard deviations = 731.6% ± 37.8%; $n = 6$) or <1/7 times (15.3% ± 2.7%; $n = 5$), respectively, that in *pex*$^+$ (100 ± 9.2%; $n = 6$) over the time course of the rhythm (Fig. 1A). However, the bioluminescence of the *kaiBC* expression level was the same in the three genotypes (*pex*$^+$, 157.8% ± 8.5%, $n = 6$; Δ*pex*, 138.4% ± 10.5%, $n = 6$; *trcp*::*pex*, 162.2% ± 8.5%, $n = 4$) (Fig. 1A). These results suggest that the period mutants have abnormal accumulations of *kaiA* mRNA.

Northern blotting confirmed the level of *kaiA* mRNA accumulation in the three genotypes of the cells (Fig. 1B, upper panel). The cells of *pex*$^+$, Δ*pex*, and *trcp*::*pex* were examined under standard light conditions following 12 h of darkness to reset the clock. After 6 h in continuous light, we collected the cultures and examined *kaiA* mRNA. The accumulation of *kaiA* mRNA in Δ*pex* or *trcp*::*pex* cells was significantly (about two times) higher or lower, respectively, than that in *pex*$^+$ (Fig. 1B, lower panel). Thus, the deficiency or constitutive expression of *pex* caused an increase or decrease in the accumulation of *kaiA* mRNA, respectively, suggesting that Pex negatively regulates *kaiA* expression through the 0.8-kb *kaiA* upstream region.

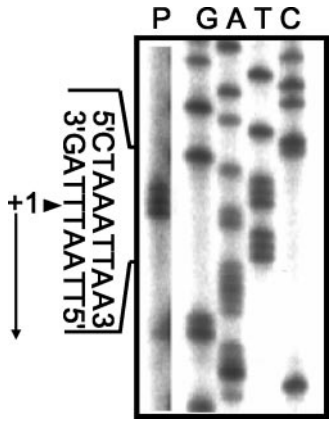

FIG. 2. Transcription start site of *kaiA* determined using the primer extension method. Thirty micrograms of total RNA was hybridized to a primer with the 5′ end labeled with γ-^{32}P and reverse transcribed with reverse transcriptase. The product was analyzed on a sequencing gel (lane P). A sequencing ladder (lanes G, A, T, and C) was obtained by sequencing reactions in which the same primer was used as a sequencing primer. The arrowhead indicates the 5′ termini of *kaiA* mRNA; the arrow indicates the direction of transcription. To clear the bands obtained in lane P, the area is shown at a lower threshold than the sequence ladder.

Transcription start site of *kaiA*. We analyzed the 5′ terminus of *kaiA* mRNA by using the primer extension method (Fig. 2). There were three 5′ termini, at nucleotides 64, 65, and 66 upstream of the translation initiation codon of *kaiA*. We mapped the 5′ terminus of *kaiA* mRNA at nucleotide 66, the

TABLE 2. Relative bioluminescence of *kaiA* reporters in *pex*$^+$ and Δ*pex*[a]

Gene or reporter	Relative bioluminescence for[b]		% *pex*$^+$	Position
	pex$^+$	Δ*pex*		
kaiA	100 ± 3	469 ± 43	469	−457–+402
D3	40 ± 4	103 ± 6	258	−44–+402
D8	51 ± 9	183 ± 17	359	−457–+49
D9	74 ± 9	349 ± 17	471	−457–+33
D10	314 ± 49	346 ± 46	110	−457–+23
D11	297 ± 17	297 ± 9	100	−457–+11

[a] The reporters were recombined in the NSI genomic region.
[b] Values are the means ± standard deviations of bioluminescence (n = 4).

potentially stable one of *kaiA*. Thus, it is probably the start site for the transcription of the *kaiA* gene. Hereafter, the position of nucleotide 66 is referred to as +1.

Deletion analysis of the upstream region of the *kaiA* gene. To find the postulated regulatory element(s) in the *kaiA* upstream region, we constructed deletion derivatives of the *kaiA* reporter and introduced them into wild-type cells. These reporter genes were recombined into a genome region, NSII. We then compared the bioluminescence rhythms of the colonies obtained. The peak bioluminescence on the second day was applied to calculate the expression activity. The deletions from −457 to −44 (reporters D1, D2, and D3) showed expression activity (Table 1), but further deletions (reporters D4, D5, and D6) diminished the activity. Therefore, sequences in the region of −44 and lower are essential to the promoter per se, consistent with the putative transcription start site. In addition, the −457 to −44 region might function to positively regulate the promoter. Although D1, D2, and D3 reporter strains showed expression activity, the deletion of a downstream region (+49 to +402 [D7 and D8]) decreased the activity. In contrast, the deletions from +11 to +49 (D9, D10, and D11) tended to increase the activity, whereas further deletions (+11 to −16 [D12]) decreased the activity significantly. This result implies that a negative regulatory element(s) occurs in the DNA (+11 to +33). Therefore, the upper region (−457 to −44) and lower region (+11 to +49) were roughly mapped as positive and negative regulatory regions, respectively. We deduced that the D3-D11 overlapping regions (−44 to +11) are the minimum promoter regions.

Analysis of the negative regulatory element upstream of *kaiA* using *pex*-deficient mutants. We examined whether the predicted negative regulatory element was related to the postulated function of Pex. We measured the bioluminescence of the deletion derivatives in *pex*$^+$ or Δ*pex* cells (Table 2). Compared to *pex*$^+$, the expression activities of D3, D8, and D9 in Δ*pex* cells were 2.6, 3.6, and 4.7 times that of their expression in *pex*$^+$ cells. The activities of further deletions, i.e., D10 and D11, in Δ*pex* cells were the same as in *pex*$^+$ cells. Thus, the reporters D3, D8, and D9 had an element negatively regulated by Pex, but others did not. In addition, the difference between D9 and D10 (i.e., the 10 bp +23 to +33) is essential to regulation.

Mapping the sequence for *pex*-related negative regulation. We further scanned regions of *kaiA* to find the putative *cis*-element for Pex. We made base pair substitutions in segment D9 fused to the *luxAB* reporter gene (Fig. 3A) and examined the obtained reporter cells of the derivatives. Derivatives M1 and M2 exhibited the same expression activity as did the original, D9 (Fig. 3A), suggesting that the two substitutions apparently have no effect on the postulated negative regulation. In contrast, the activity in the reporter of M3 was three times that of D9 but the same as that of D10 (Table 2).

The M1 to M3 reporters were assayed in Δ*pex* cells, and the bioluminescence in these mutant cells was greater than three times that of *pex* cells. However, the M3 reporter bioluminescence levels in cells of *pex*$^+$ and Δ*pex* were similar (Fig. 3B). Therefore, the 5 bp between M1 and M2, i.e., AGAGA, likely constitutes the negative element.

Effects of repression or induction of the *kaiA* gene. Since our molecular genetic approach suggested that the circadian period was in inverse proportion with the expression level of *kaiA*, we hypothesized that the period could respond to *kaiA* gene expression. To evaluate this hypothesis, we examined the period of the oscillator in cells in which *kaiA* gene expression was interrupted or constitutively expressed. To regulate the expression level of the gene, we made inducible constructs for the suppression or induction of *kaiA* gene expression in the

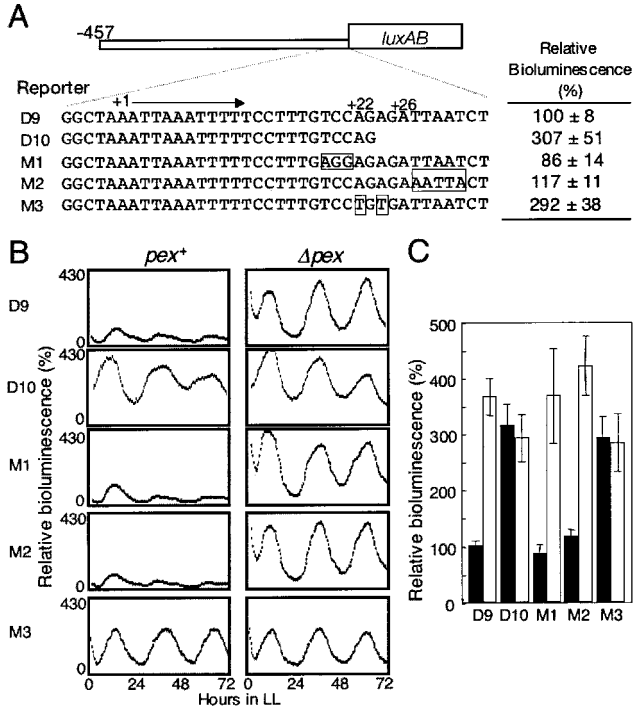

FIG. 3. Mutation analysis of the *kaiA* promoter region fused to *luxAB*. (A) The *kaiA* reporter base pair substitution and relative bioluminescence. The reporter gene functioned at genomic site NSI. Boxes with −457 and *luxAB* represent the D9-type *kaiA* reporter. A series of mutations of the *pex*-related regulatory region in the *kaiA* reporter are superimposed. The transcription start site of *kaiA* is shown as +1, with an arrow. The mutations of segments are boxed. The peak in the mean relative bioluminescence, indicating the expression activity of *kaiA*, was on the second day (± standard deviation; $n >$ 3). (B) Representative bioluminescence rhythms of the base-pair-substituted D9 reporters in *pex*$^+$ and Δ*pex* cells. (C) Means ± standard deviations (n = 3) relative bioluminescence of each reporter in *pex*$^+$ and Δ*pex* cells. Error bars indicate standard deviations. Filled bars, *pex*$^+$; open bars, Δ*pex*. LL, see the legend to Fig. 1.

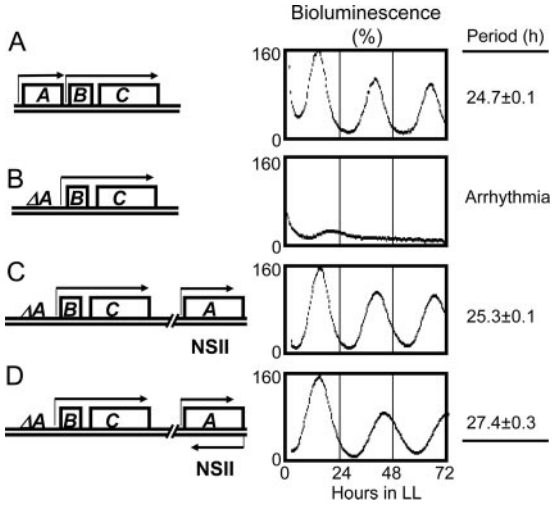

FIG. 4. Effect of repression of *kaiA* gene expression in the NSII genome region. The genomic regions of *kaiABC* and NSII in the *kaiBC* reporter strains are depicted in the left panels. The right panels show reporter bioluminescence profiles. Boxes show the functional *kai* gene on the genome. Δ*A*, the *kaiA* gene with nonsense mutation; NSII, a targeting site for *kaiA*; arrow, direction of gene transcription. "Period" is the mean circadian period ± standard deviation ($n = 6$). Panels A through D represent the *kai* genes and the rhythms of the reporter strains of *kaiABC*$^+$ (A), the inactivated *kaiA* gene (B), the inactivated *kaiA* and *kaiA*$^+$ genes (C), and *kaiA*::*trcp* (D). The *trcp* is located downstream of the recombined *kaiA* in an inverted direction. Ten-millimolar IPTG inducer was used for antisense transcription from *trcp*. LL, see the legend to Fig. 1.

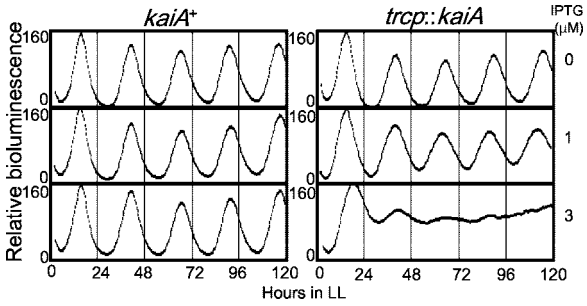

FIG. 5. Effect of the induction of *kaiA* in the NSII genome region. Bioluminescence rhythm of a *kaiBC* expression reporter in *kaiA*$^+$ and *trcp*::*kaiA* genotypes with or without the inducer IPTG. Without IPTG, the reporter cells of *trcp*::*kaiA* showed a rhythm similar to that of *kaiA*$^+$. The mean period was 24.9 ± 0.1 h (± standard deviation) ($n = 6$). The addition of 1 μM IPTG to induce the transcription of *trcp*::*kaiA* in NSII resulted in a further short-period phenotype (24.2 ± 0.2 h; $n = 6$). The mean period of the rhythm in *kaiA*$^+$ with 0 to 3 μM IPTG was 25.2 ± 0.1 h ($n = 6$). LL, see the legend to Fig. 1.

genome. The construct was recombined into a specific site of the genome, NSII, in which DNA insertion per se did not affect the fundamental cell activity or circadian rhythm. And at NSII, the inducible promoter *trc* used here had functioned in *Synechococcus* (6, 9, 17). To control the down-regulation of *kaiA* expression, we used the transcription-translation interference technique, using antisense transcription in *Synechococcus* (25). First, we confirmed the complementation activity of *kaiA* DNA in NSII using a *kaiA*-inactivated arrhythmic mutant (Fig. 4B). The mutant was transformed with an NSII-targeting plasmid harboring a native *kaiA* gene (Fig. 4C), and the obtained transformed cell exhibited circadian rhythm similar to that in the control reporter cells (Fig. 4A). Thus, *kaiA* could function in NSII. Based on *kaiA* activity in NSII, we examined the effect of interference of the *kaiA* transgene expression by using an inverted *trc* promoter at the 3′ side of the gene (*kaiA*::*trcp*). We then introduced the plasmid pTS2*kaiA*::*trcp* into a *kaiA*-inactivated reporter cell. The obtained cell, with the addition of 10 mM IPTG, had a period of circadian rhythm extended to 27 h (Fig. 4D), suggesting that antisense transcription changed the period because it decreased *kaiA* transcription or translation.

We also examined the effect of constitutive expression of *kaiA* by making a reporter strain harboring a *trcp*::*kaiA* fusion gene, through which *kaiA* mRNA was produced (Fig. 5). Although cells with *trcp*::*kaiA* exhibited a rhythm period (24.9 h) similar to that of the wild type (25.2 h) on an agar plate without IPTG, it exhibited a 1-h shorter rhythm period (24.2 h) than that of *kaiA*$^+$ (25.2 h), consistent with the period in the Δ*pex* genotype. The addition of 3 μM IPTG resulted in a lowered amplitude of the rhythm. However, the peaks of the rhythms with low amplitude clearly advanced every cycle because of their shortened period. Therefore, the constitutive expression of *kaiA* shortens the period of the circadian rhythm in a dose-dependent manner. Our results are consistent with the abnormal level of *kaiA* expression observed in *pex* genotype-related period mutants.

DISCUSSION

In the *Synechococcus* circadian oscillator, the clock protein KaiC exhibits phosphorylated or dephosphorylated forms in a circadian manner under conditions of no transcription-translation (32). This cycle requires physical interaction(s) with KaiA and KaiB (12, 14); in vitro, the three proteins plus ATP establish a reaction of KaiC with a period of about 24 h (21). In this reaction, KaiA promotes phosphorylation (11), suggesting that KaiA has a role in regulating the circadian period within a short range. Consistent with this explanation, most of the period mutants harboring a mutation in the *kaiA* locus exhibited longer periods of the rhythm than did the wild-type strain (23).

We found stronger *kaiA* expression and more significant accumulation of *kaiA* mRNA in the Δ*pex* strain than in the *pex*$^+$ strain (Fig. 1). This result suggests that the short-period phenotype in the Δ*pex* strain is caused by an increase in *kaiA* expression and the acceleration of phosphorylation of the KaiC protein. Further analysis to quantify *kaiA* expression levels should be carried out to confirm this. A short period in the strain constitutively expressing the *kaiA* transgene (*trcp*::*kaiA*), using a *trc* promoter at an intermediate concentration of the inducer IPTG (Fig. 5), provided further support for this conclusion. Another circadian resetting-related mutant, *ldpA* (*l*ight-*d*ependent *p*eriod) (10, 13), named after one of its mutant phenotypes for the loss of the period response to light intensity in free-running conditions, showed the same circadian period as that in Δ*pex* cells. KaiA is more abundant in the *ldpA* mutant than in the wild type, but *ldpA* is a different gene from *pex* and encodes a protein with Fe_4S_4 motifs that sense the cellular redox state (10). Thus, deficiency in the *pex*

or *ldpA* gene causes similar abnormalities in *kaiA* expression and the period of the rhythm.

Our primer extension and in vivo bioluminescence reporter analyses indicated the transcription start site and the negative regulatory region upstream the *kaiA* gene. Within this region, a 5-bp sequence (AGAGA) (Fig. 3A) was essential for negative regulation by Pex. Together with our previous in vitro data, in which Pex specifically bound to the double-strand DNA of the *kaiA* regulatory region (1), it is plausible that Pex directly binds to this region and functions as a repressor of the *kaiA* gene in vivo.

Using the cyanobacterial genome database Cyanobase (Kazusa DNA Research Institute, Chiba, Japan), we estimated the 5-bp essential sequence of *kaiA* in other cyanobacterial species whose genomes have *pex* orthologs to understand the conservation and significance of the regulation. A nitrogen-fixing multicellular cyanobacterium (*Anabaena* sp. strain PCC 7120), a thermophilic cyanobacterium (*Thermosynechococcus elongatus* BP-1), and a marine cyanobacterium (*Synechococcus* sp. strain WH8102) have a 5-bp AGACA motif at 73, 64, and 67 bp, respectively, upstream of each *kaiA* translation initiation codon, whereas *Synechococcus* sp. strain PCC 7942 had the 5-bp motif 41 bp upstream of the gene. Therefore, the 5-bp motif and adjacent region of *kaiA* in the three cyanobacteria might be the binding site of Pex for negative regulation of the gene.

The clock of the Δ*pex* strain subjected to diurnal light-dark cycles shows a phase advance of about 3 h compared to that of the *pex*$^+$ strain (31). Thus, Pex affects a specific step in the oscillator. We assume that the negative regulation of *kaiA* by Pex is related to the delay function in the oscillator under a light-dark cycle. If this assumption is correct, *kaiA* expression should decrease in light-dark conditions.

X-ray diffraction analysis of the Pex crystal structure showed that it is a winged-helix dimer protein (1). A representative winged-helix repressor in *Synechococcus*, SmtB, derepresses the transcription of the *smtA* gene in response to the heavy metal cadmium, and the dimer binds to several sites in the *smtA* promoter region. Therefore, it will be important to demonstrate the existence of the Pex dimer in vivo.

In summary, Pex was required for the negative regulation of *kaiA* and the circadian period was dependent on the *kaiA* expression level. These in vivo results and the demonstrated in vitro binding between Pex and upstream DNA of *kaiA* (1) demonstrate that Pex is a direct *kaiA* regulator in cyanobacteria that maintains the circadian period.

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REFERENCES

1. **Arita, K., H. Hashimoto, K. Igari, M. Akaboshi, S. Kutsuna, M. Sato, and T. Shimizu.** 2007. Structural and biochemical characterization of a cyanobacterium circadian clock-modifier protein. J. Biol. Chem. **282:**1128–1135.
2. **Barthelmebs, L., B. Lecomte, C. Divies, and J. F. Cavin.** 2000. Inducible metabolism of phenolic acids in *Pediococcus pentosaceus* is encoded by an autoregulated operon which involves a new class of negative transcriptional regulator. J. Bacteriol. **182:**6724–6731.
3. **Bustos, S. A., and S. S. Golden.** 1991. Expression of the *psbDII* gene in *Synechococcus* sp. strain PCC 7942 requires sequences downstream of the transcription start site. J. Bacteriol. **173:**7525–7533.
4. **Chen, T., T.-L. Chen, L.-M. Hung, and T. C. Hung.** 1991. Circadian rhythm in amino acid uptake by *Synechococcus* sp. RF-1. Plant Physiol. **97:**55–59.
5. **Dunlap, J. C., J. J. Loros, and P. J. DeCoursey (ed.).** 2004. Chronobiology: biological timekeeping. Sinauer, Sunderland, MA.
6. **Geerts, D., A. Bovy, G. D. Vrieze, M. Borrias, and P. Weisbeek.** 1995. Inducible expression of heterologous genes targeted to a chromosomal platform in the cyanobacterium *Synechococcus* sp. PCC 7942. Microbiology **141:**831–841.
7. **Grobblelarr, N., T. C. Huang, H. Y. Lin, and T. J. Chow.** 1986. Dinitrogen-fixing endogenous rhythm in *Synechococcus* RF-1. FEMS Microbiol. Lett. **37:**173–177.
8. **Gury, J., L. Barthelmebs, N. P. Tran, C. Divies, and J. F. Cavin.** 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding *padA* gene of *Lactobacillus plantarum*. Appl. Environ. Microbiol. **70:**146–153.
9. **Ishiura, M., S. Kutsuna, S. Aoki, H. Iwasaki, C. R. Andersson, A. Tanabe, S. S. Golden, C. H. Johnson, and T. Kondo.** 1998. Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. Science **281:**1519–1523.
10. **Ivleva, N. B., M. R. Bramlett, P. A. Lindahl, and S. S. Golden.** 2005. LdpA: a component of the circadian clock senses redox state of the cell. EMBO J. **24:**1202–1210.
11. **Iwasaki, H., T. Nishiwaki, Y. Kitayama, M. Nakajima, and T. Kondo.** 2002. KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. Proc. Natl. Acad. Sci. USA **99:**15788–15793.
12. **Kageyama, H., T. Kondo, and H. Iwasaki.** 2003. Circadian formation of clock protein complexes by KaiA, KaiB, KaiC, and SasA in cyanobacteria. J. Biol. Chem. **278:**2388–2395.
13. **Katayama, M., T. Kondo, J. Xiong, and S. S. Golden.** 2003. *ldpA* encodes an iron-sulfur protein involved in light-dependent modulation of the circadian period in the cyanobacterium *Synechococcus elongatus* PCC 7942. J. Bacteriol. **185:**1415–1422.
14. **Kitayama, Y., H. Iwasaki, T. Nishiwaki, and T. Kondo.** 2003. KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system. EMBO J. **22:**2127–2134.
15. **Kondo, T., C. A. Strayer, R. D. Kulkarni, W. Taylor, M. Ishiura, S. S. Golden, and C. H. Johnson.** 1993. Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc. Natl. Acad. Sci. USA **90:**5672–5676.
16. **Kondo, T., N. F. Tsinoremas, S. S. Golden, C. H. Johnson, S. Kutsuna, and M. Ishiura.** 1994. Circadian clock mutants of cyanobacteria. Science **266:** 1233–1236.
17. **Kutsuna, S., T. Kondo, S. Aoki, and M. Ishiura.** 1998. A period-extender gene, *pex*, that extends the period of the circadian clock in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J. Bacteriol. **180:**2167–2174.
18. **Kutsuna, S., Y. Nakahira, M. Katayama, M. Ishiura, and T. Kondo.** 2005. Transcriptional regulation of the circadian clock operon *kaiBC* by upstream regions in cyanobacteria. Mol. Microbiol. **57:**1474–1484.
19. **Mitsui, A., S. Kumazawa, A. Takahashi, H. Ikemoto, and T. Arai.** 1986. Strategy by which nitrogen fixing unicellular cyanobacteria grow photoautotrophically. Nature **323:**720–722.
20. **Nakahira, Y., M. Katayama, H. Miyashita, S. Kutsuna, H. Iwasaki, T. Oyama, and T. Kondo.** 2004. Global gene repression by KaiC as a master process of prokaryotic circadian system. Proc. Natl. Acad. Sci. USA **101:**881–884.
21. **Nakajima, M., K. Imai, H. Ito, T. Nishiwaki, Y. Murayama, H. Iwasaki, T. Oyama, and T. Kondo.** 2005. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation *in vitro*. Science **308:**414–415.
22. **Nikaido, H.** 1998. Multiple antibiotic resistance and efflux. Curr. Opin. Microbiol. **5:**516–523.
23. **Nishimura, H., Y. Nakahira, K. Imai, A. Tsuruhara, H. Kondo, H. Hayashi, M. Hirai, H. Saito, and T. Kondo.** 2002. Mutations in KaiA, a clock protein, extend the period of circadian rhythm in the cyanobacterium *Synechococcus elongatus* PCC 7942. Microbiology **148:**2903–2909.
24. **Porter, R. D.** 1988. DNA transformation. Methods Enzymol. **167:**703–712.
25. **Ramasubramanian, T. S., F. Pu, and J. W. Golden.** 1995. Isolation of the *Anabaena* sp. strain PCC 7120 *sigA* gene in a transcriptional-interference selection. J. Bacteriol. **177:**6676–6678.
26. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
27. **Ronnenberg, T., and E. J. Carpenter.** 1993. Daily rhythm of O_2-evolution in the cyanobacterium *Trichodesmium thiebautii* under natural and constant conditions. Mar. Biol. **117:**693–697.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

29. **Stal, L. J., and W. E. Krumbein.** 1985. Nitrogenase activity in the non-heterocystous cyanobacterium *Oscillatoria* sp. grown under alternating light-dark cycles. Arch. Microbiol. **143:**67–71.
30. **Sweeney, B. M., and M. B. Borgese.** 1989. A circadian rhythm in cell division in a prokaryote, the cyanobacterium *Synechococcus* WH7803. J. Phycol. **25:**183–186.
31. **Takai, N., S. Ikeuchi, K. Manabe, and S. Kutsuna.** 2006. Expression of the circadian-clock-related gene *pex* in cyanobacteria increases in darkness and is required to delay the clock. J. Biol. Rhythms **21:**1–10.
32. **Tomita, J., M. Nakajima, T. Kondo, and H. Iwasaki.** 2005. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. Science **307:**251–254.