Expression of the Circadian Clock–Related Gene *pex* in *Cyanobacteria* Increases in Darkness and Is Required to Delay the Clock

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Abstract The time measurement system of the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 is analogous to the circadian clock of eukaryotic cells. Circadian clock–related genes have been identified in this strain. The clock-related gene *pex* is thought to maintain the normal clock period because constitutive transcription or deficiency of this gene causes respectively longer (~28 h) or shorter (~24 h) circadian periods than that of the wild type (~25 h). Here, the authors report other properties of *pex* in the circadian system. Levels of *pex* mRNA increased significantly in a 12-h exposure to darkness. Western blotting with a GST-Pex antibody revealed a 13.5-kDa protein band in wild-type cells that were incubated in the dark, while this protein was not detected in *pex*-deficient mutant cells. Therefore, the molecular weight of the Pex protein appears to be 13.5 kDa in vivo. The PadR domain, which is conserved among DNA-binding transcription factors in lactobacilli, was found in Pex. In the *pex* mutant, several 12-h light/12-h dark cycles reset the phase of the clock by 3 h earlier (phase advance) compared to wild-type cells. The degree of the advance in the *pex* mutant was proportional to the number of exposed light–dark cycles. In addition, ectopic induction of *pex* with an inducible *Escherichia coli* promoter, *Ptrc*, delayed the phase in the examined recombinant cells by 2.5 h (phase delay) compared to control cells. These results suggest that the dark-responsive gene expression of *pex* delays the circadian clock under daily light–dark cycles.

Key words circadian clock, cyanobacteria, input, *pex*, *padR*, phase delay

Organisms ranging from cyanobacteria to plants and animals have evolved circadian clocks to adapt to 24-h environmental changes (Bünning, 1973). Environmental light conditions entrain the circadian clock by modulating the phase and period of the clock (Aschoff, 1981). Several photoreceptor proteins that perceive light for entraining the clock have been identified using molecular genetic approaches (Crosthwaite et al., 1997; Somers et al., 1998). In a cell, it is postulated that these photoreceptors cause biochemical change, such as light-responsive gene expression, by which the clock is reset. This serial

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phototransduction (light-input pathway) remains to be elucidated, although genes relevant to the putative pathway have been identified (McWatters et al., 2000; Doi et al., 2001; Heintzen et al., 2001).

Cyanobacteria are the simplest organisms that display circadian rhythms. In the strain *Synechococcus elongatus* PCC 7942, a luciferase has been used as a reporter for the expression of the clock-regulated gene *psbAI* (Kondo et al., 1993). More than 50 mutants of bioluminescence rhythms have been established, which include rhythms with long or short periods, as well as arrhythmic or low-amplitude rhythm phenotypes.

To find the causative gene(s) of these mutants, wild-type genomic DNA regions ~10 kilobase pairs (kb) in length were introduced into these mutant cells randomly, and the recombinant colonies obtained were then examined for their bioluminescence rhythms. Several colonies displayed bioluminescence rhythms similar to that of the wild type (Kondo et al., 1994; Ishiura et al., 1998). One genomic DNA region was cloned, and the complementation activity for those mutants was confirmed. Sequencing analysis of this DNA region mapped mutations of nucleotides in all the mutant genomes to within 3 genes in tandem, which were named *kaiA*, *kaiB*, and *kaiC* (Ishiura et al., 1998). Therefore, these 3 genes cause the abnormal rhythms. There are 2 promoters in *kaiABC*, and *kaiA* and *kaiBC* mRNAs are transcribed independently. Both promoters are under circadian-feedback regulation, as shown in eukaryotic cells (Ishiura et al., 1998). Compared to other clock-regulated genes, the *kaiBC* operon appears to be predominantly regulated by the clock (Liu et al., 1995; Nakahira et al., 2004).

Before constructing the genomic DNA library used to isolate *kaiABC*, we introduced another type of plasmid DNA library that was constructed from ~4-kb random DNA fragments from the wild-type genome into the cells of the clock mutants listed above to determine their causative gene(s) (Kondo et al., 1994). A clone with an apparently normal circadian rhythm was isolated from the library-introduced colonies of the rhythm mutant SP22, whose circadian period is 22 h. Using the plasmid rescue method, ~2 kb of wild-type DNA was recovered from the genomic DNA of this isolated clone. After introducing the obtained genomic DNA into SP22 mutant cells, its bioluminescence rhythm resembled that of the wild type.

However, this DNA region did not allow other mutants to recover rhythms similar to that of the wild type. Therefore, we predicted the presence of an SP22 mutation in the 2-kb genomic DNA region. If this were the case, a point mutation of SP22 should be found in this region of its genome. Several sequencing analyses failed to detect a mutation in this region, suggesting that a kind of suppression occurs in SP22 with the introduction of the DNA region, rather than the typical complementation of the SP22 mutation by its innate gene (in fact, the point mutation of SP22 has been mapped onto the *kaiC* locus, as the *kaiC1* allele; Ishiura et al., 1998).

To reveal the cause of the suppression, several deletion derivatives of the plasmid were examined for putative suppression of SP22 activity. Part of the genomic DNA region that encodes an apparent 148–amino acid protein with unknown function was essential for the SP22 suppression (Kutsuna et al., 1998). Using homologous recombination-based gene manipulation, this putative gene in the wild-type cell was replaced by an antibiotics-resistant gene to obtain a mutant deficient in the gene. The obtained gene-deficient mutant cell showed an abnormal bioluminescence rhythm with a period 1 h shorter than that of the wild type, suggesting that the putative gene functions to lengthen the period of the clock. In fact, with constitutive transcription of the gene using an *Escherichia coli trc*-promoter, the period was lengthened by 3 h. This gene was named *pex* after its physiological activity, which causes simple lengthening of the rhythm (period prolongation) in wild-type cells (Kutsuna et al., 1998).

Here, we report an increase in *pex* expression in darkness and a *pex* mutant phenotype in the phase of the oscillator. In addition, we compared the PadR domain (pfam03551) in the midportion of the amino acid sequence of the Pex protein. The PadR protein was originally isolated from the lactobacillus *Pediococcus pentosaceus* and was characterized as a negative regulator of the *padA* gene, which encodes phenolic acid decarboxylase to metabolize environmental toxins, such as *p*-coumaric acid (Barthelmebs et al., 2000). Then, we discuss the role of *pex* in light input in the cyanobacterial circadian system.

MATERIALS AND METHODS

Bacterial Strains

Since the expression of the *kaiBC* operon is an important process of the cyanobacterial clock (Ishiura et al., 1998; Kutsuna et al., 2005), the bioluminescence reporter cell NUC42 (*kaiBC*::*luxAB*) was used for monitoring *kaiBC* expression in *S. elongatus*

PCC 7942 as the wild-type cell in this study (Kutsuna et al., 2005). Using natural transformation activity-based homologous recombination in the genome, the *pex* gene in this cell was exchanged for a spectinomycin-resistant gene cassette Ω of DNA pDpex, as previously demonstrated in the *psbAI* reporter (Kutsuna et al., 1998). The absence of the *pex* gene in the obtained mutant was confirmed by Southern blotting with the digoxigenin (DIG; Roche, Basel, Switzerland)–labeled *pex* open reading frame (ORF) region. To obtain a *pex*-restored reporter cell from the *pex* mutant, the mutant was transformed with pPEX-474 (Kutsuna et al., 1998) plasmid harboring the *pex* gene in a 1-kb *Bam*HI genomic DNA segment. In the resulting cell, *pex* was transcribed from a specific site of the genome, neutral site II, using homologous recombination between the endogenous DNA and that in the pPEX-474.

Northern Blotting Analysis

The optical density (OD_{730}) of bacterial cultures was maintained between 0.2 and 0.4. Total RNA was isolated as previously described (Kutsuna et al., 1998). Five micrograms of total RNA was electrophoresed on a 1.0% agarose gel containing 1.0% formaldehyde, blotted onto a positively charged nylon membrane, and hybridized with DIG-labeled *pex* probe, as previously described (Kutsuna et al., 1998). Chemiluminescence and the hybridization signal were obtained and quantified using a Fluor-S MultiImager (Bio-Rad, Hercules, CA).

Anti-Pex Antiserum and Western Blotting Analysis

Pex protein was expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein. Purified GST-Pex protein was used as the antigen to obtain antisera for Pex protein. A 10-week-old female New Zealand White rabbit was immunized with ~1.1 mg of protein. The cyanobacterial cells were disrupted by sonication with Bioruptor in Tris-buffered saline (TBS) at 5 °C for 10 min (cycle conditions: 1 min of sonication and 1 min of resting). After centrifugation, the supernatant containing 15 μg of total protein extract was electrophoresed through a 20% sodium dodecyl sulfate (SDS) acrylamide gel and blotted onto a polyvinylidene difluoride membrane. The membrane was blocked in phosphate-buffered saline–Tween (0.3%; PBS–T) with 10% skim milk. The prepared anti-GST-Pex antiserum was diluted 200-fold in PBS–T and used as primary antibody for 1 h. After washing the membrane in PBS–T, horseradish peroxidase–linked antirabbit Ig was diluted in PBS–T (1/1500) and used as the secondary antibody (Amersham Biosciences, Buckinghamshire, UK). These procedures were performed at 5 °C. The chemiluminescence substrate was Immun-Star HRP Luminol/Enhancer, used with the Fluor-S MultiImager chemiluminescence imaging system (Bio-Rad).

Colony Formation and Bioluminescence Monitoring

In cyanobacteria, photosynthesis and its light-absorbing apparatus affect circadian resetting (Katayama et al., 2003). Therefore, it is important to conduct experiments with colonies of the same size when examining the effect of external light because of the dependence of light penetration on colony thickness.

We prepared colonies 0.2 mm in diameter under continuous light, as reported previously (Ishiura et al., 1998). *S. elongatus* was cultured under constant light for 108 h to generate colonies 0.2 mm in diameter. The cell clock was then reset by 12 h in darkness. The lid of a microcentrifuge tube containing 30 μL of vacuum pump oil with 3% bioluminescence substrate (n-decanal; Wako, Osaka, Japan) was placed on solid agar medium with the obtained colonies (Ishiura et al., 1998). Bioluminescence was monitored under continuous light using an automated system equipped with photon-multiplier tubes. Colonies were also cultivated under 12-h light/12-h dark cycles. Since the colony formation rate is inversely proportional to the number of light–dark cycles, we adjusted the length of the continuous light period before examining the light–dark cycles, except for the 8- to 11-day cycles. Cells cultivated in cycles of 8, 9, 10, or 11 days formed colonies at least 0.2 mm in diameter and required no additional growth time in light. In the experiment with the 6- and 4-day cycles, the lengths of the light periods before the cycles were 36 and 60 h, respectively. Before the 2- and 3-day cycles, cells were cultivated under constant light for 84 h. Bioluminescence was examined under continuous light.

Ectopic Expression of *pex* on Addition of an Inducer

The *pex* mutant was transformed with a plasmid, pTS2C*Ptrc*::*pex*, in which the inducible *E. coli trc*-promoter transcribed the *pex*. This plasmid inserts

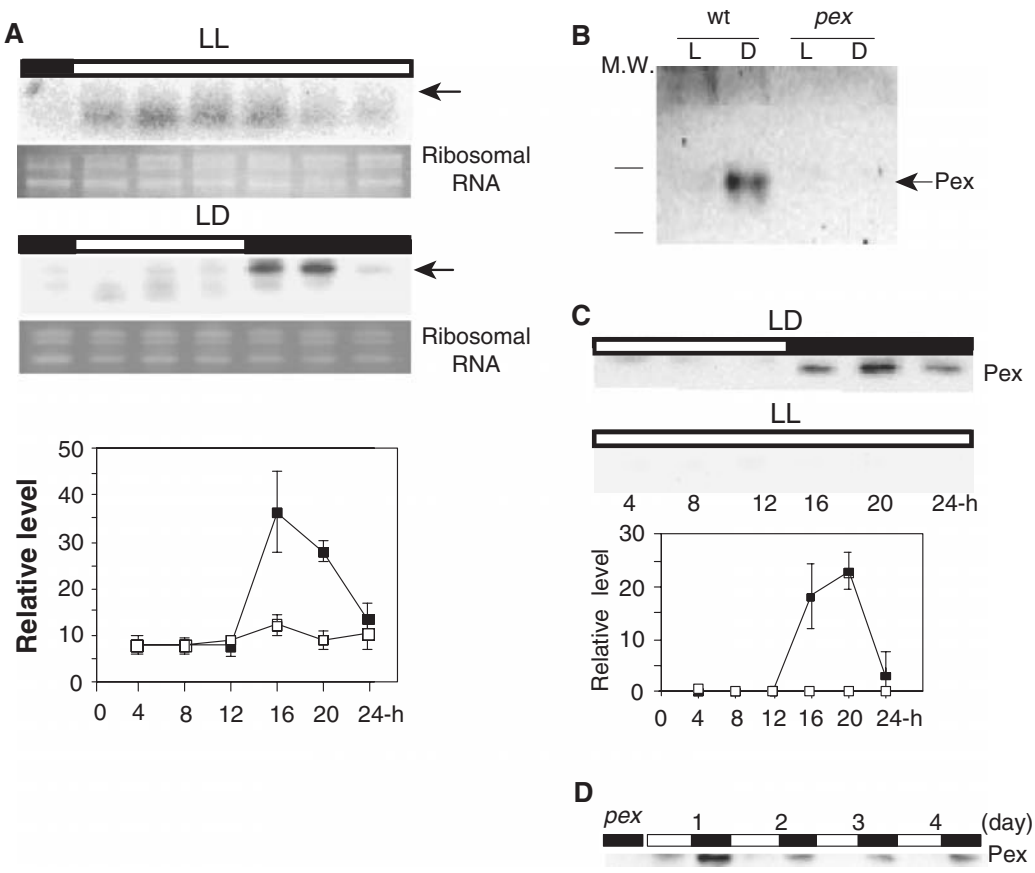

Figure 1. ***pex*** **expression in continuous light and in 12-h light/12-h dark cycles. (A) Northern analysis of** ***pex*** **mRNA transcripts in LL and in 12-h LD cycles. Northern blotting with digoxigenin (DIG)–labeled RNA probe for** ***pex*** **mRNA. Five micrograms of total RNA was loaded in each lane of the agarose gel. Arrows indicate** ***pex*** **mRNA. Ribosomal RNA stained with ethidium bromide is shown below each blotting membrane. Densitometric analysis of** ***pex*** **transcript levels (mean ± SE,** $n = 3$**) is shown below. Open squares,** ***pex*** **mRNA accumulation profile in continuous light. Solid squares,** ***pex*** **mRNA accumulation profile in the light-dark cycle. (B) Western blotting with anti-GST-Pex antiserum detecting Pex protein in** ***Synechococcus elongatus*** **cell extracts: wt, wild-type cell;** ***pex***, ***pex*****-deficient mutant; L and D, cell extract in light and dark, respectively; a band of Pex protein (13.5 kDa) was detected in the** ***S. elongatus*** **cell extract; M.W., positions of 6- and 14-kDa molecular weight markers. (C) Pex protein accumulation profiles under LL and LD cycle at 4-h intervals and its densitometric analysis (mean ± SE,** $n = 3$**). Fifteen micrograms of total protein in the cell lysate was loaded in each lane of a 20% sodium dodecyl sulfate (SDS) acrylamide gel. Open squares, Pex levels in continuous light; solid squares, Pex levels in the light–dark cycle. (A-D) Open bars, light period; filled bars, dark period. (D) Western blotting of Pex protein expression in LD cycles.**

the inducible *pex* gene into the neutral site II of the genome. A kanamycin resistance marker is useful for selecting transformed cells (Kutsuna et al., 1998). The obtained cells were used to form colonies on nitrocellulose membrane (Millipore, Billerica, MA) on 4 mL of BG-11 solid medium under continuous light over 5 days with isopropyl-β-D-thiogalactoside (IPTG). After resetting the clock with 12 h of dark, the IPTG was removed by placing the membrane on the surface of liquid BG-11 twice, as described previously (Ishiura et al., 1998). The washed membrane was placed on fresh solid BG-11 medium, and bioluminescence was monitored.

RESULTS

Increase in *pex* Gene Products in Darkness

To gain insight into the role of *pex* in the circadian system, *pex* mRNA and Pex protein levels were determined under LL or 12-h LD cycles. In LL, the *pex* mRNA levels were constitutively low and showed weak circadian variation. In contrast, *pex* mRNA accumulation was significantly greater in darkness (Fig. 1A), with levels up to 4 times those observed in constant light. The increase in *pex* mRNA continued until hour 20 in the LD cycle (zeitgeber time 20,

ZT20), after which the detectable levels decreased substantially. In addition, the accumulation profile in *pex* mRNA in continuous darkness for 48 h did not show a secondary peak (data not shown).

Pex Protein

Cyanobacterial cultures of wild-type and *pex*-deficient mutant strains whose *pex* gene was replaced with an antibiotic resistance gene using a homologous recombination method were grown in continuous light and then subjected to 6 h of darkness. Cells were collected just before and after the 6 h of darkness. Soluble proteins extracted from the collected cells were fractionated through a 20% SDS polyacrylamide gel. Using the antiserum raised against the Pex protein, a 13.5-kDa band was detected in the wild-type lysate extracted from the cultures subjected to darkness (Fig. 1B, wt D). After 12 h of darkness, the 13.5-kDa protein decreased below the detectable level. To confirm whether the protein was Pex, we made a *pex* mutant by introducing a DNA able to remove the *pex* gene from the cyanobacterial genome into the cell (Bacterial Strains in Materials and Methods) and examining the resulting *pex* mutant cell culture. In the lysate of *pex* mutant cells, no specific band was detected (Fig. 1B; *pex* L and D), suggesting that the 13.5-kDa protein is the Pex protein in *S. elongatus*.

Pex accumulation was determined at 4-h intervals in LL or LD cycles. Consistent with the *pex* mRNA profile, Pex protein was abundant from ZT16 to 20 (Fig. 1C) and still detectable at ZT24. This suggests that Pex accumulation is mostly due to transcription/posttranscription activities of the *pex* gene. This Pex accumulation was observed in every dark period over 4 days (Fig. 1D).

PadR Domain in the Predicted Pex Protein

An analysis of the amino acid sequence of the Pex protein using the programs BLAST and Conserved Domain Search (National Center for Biotechnology Information [NCBI], Bethesda, MD) implied that the Pex protein harbored a PadR domain (pfam03551), which has also been found in PadR proteins in lactobacilli (Barthelmebs et al., 2000; Gury et al., 2004). We compared the cyanobacterial Pex proteins (Cyanobase; Kazusa DNA Research Institute, Chiba, Japan) and lactobacillus PadR proteins in terms of the location of the PadR domain (Fig. 2A). The 2 PadR proteins have the domains in the N-terminal portion, whereas the domain is central in the Pex proteins. The PadR domains of Pex in *S. elongatus* and PadR in *Lactobacillus plantarum* were compared (Fig. 2B) since the PadR of *L. plantarum* binds directly to its *padA* promoter DNA fragment in vitro (Gury et al., 2004). The numbers of amino acid residues were consistent in both, and 11 amino acids were conserved over the domain. However, no significant similarity was found on direct comparison of the 2 PadR domains.

Resetting the Clock in the *pex* Mutant

The free-running period is ~1 h shorter in the *pex*-deficient mutant cell than in the wild-type cell. Conversely, constitutive transcription of the gene lengthens the period by 3 h in a dose-dependent manner (Kutsuna et al., 1998), suggesting that *pex* function is related to the clock. In addition to these findings, since *pex* mRNA and Pex protein increased in darkness (Fig. 1), we expected a potential role of *pex* in putative phototransduction to the central oscillator. The wild type and *pex* mutants were examined using bioluminescence monitoring with the *kaiBC*::*luxAB* reporter gene fusion because of its prominent sharp shape and the robustness of its rhythm due to exclusive circadian feedback regulation of the promoter (Nakahira et al., 2004). Therefore, the properties of this reporter would be ideal for monitoring the oscillator, especially for the first circadian cycle (i.e., the day after the 12 h of darkness used to reset the clock), because use of the clock reporter would avoid perturbations in monitoring the rhythm, such as masking effects of the circadian rhythm (Aschoff, 1999). Then, the *psbAI* promoter of the *luxAB* bioluminescence gene was replaced with the *kaiBC* promoter in a *pex*-deficient mutant or wild-type cell using natural transformation activity-based gene exchange with a DNA capable of the promoter exchange.

The obtained *kaiBC* reporter cells of the *pex*-deficient mutant and wild-type cell were cultured in continuous light to obtain colonies of the standard diameter (0.2 mm). The circadian clock of each colony was synchronized by 12 h of darkness, and the bioluminescence from the *kaiBC* reporter was measured under continuous light (Fig. 3A).

No difference was observed in the timing of the first peak of the bioluminescence rhythms between the wild-type and *pex* mutant cells (difference = 0 ± 0.33, SD, $n = 4$), suggesting that circadian resetting was normal in the *pex* mutant cultivated in LL. In contrast, the second peak of the bioluminescence rhythm of the

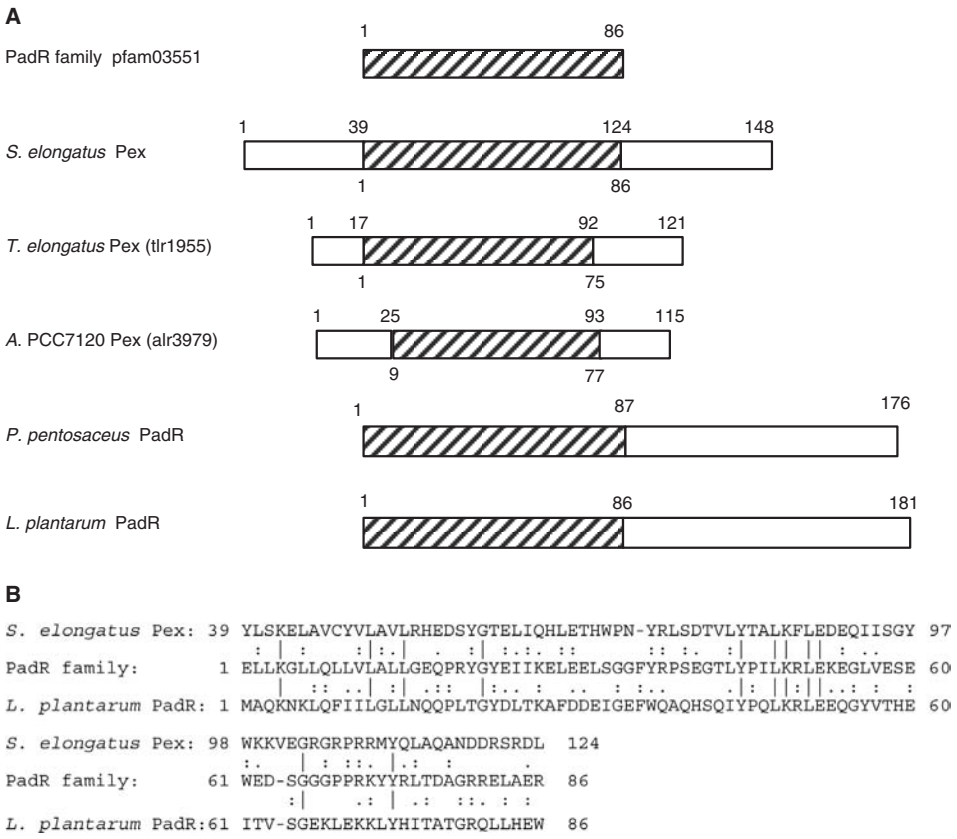

Figure 2. Comparison of Pex homologs and their PadR domains. (A) Pex proteins in cyanobacteria (*Synechococcus elongatus* PCC 7942, *Thermosynechococcus elongatus* BP-1, and *Anabaena* sp. PCC 7120) and reported PadR proteins in 2 lactobacilli (*Pediococcus pentosaceus* and *Lactobacillus plantarum*). The Pex protein coding genes in the genomes of *T. elongatus* and *Anabaena* PCC 7120 are denoted in parentheses. These proteins are shown relative to their PadR domains. Open bars, putative full length of the proteins, with the numbers of the amino acids at each end of the PadR domain (hatched box). (B) Alignments of the deduced amino acid sequences of PadR domains of *S. elongatus* and *L. plantarum* after pairwise comparison using the BLAST search program using the consensus PadR sequence pfam03551 (PadR family). Consensus amino acid residues among the 3 PadR domains are connected with vertical lines. Identical residues between pfam03551 and each PadR domain are denoted with a colon. A dot indicates amino acids similar to those of pfam03551. Pex-pfam03551: Identities = 31/78 (39%), Positives = 41/78 (51%); PadR-pfam03551: Identities = 32/82 (39%), Positives = 47/82 (57%).

pex mutant cell occurred 1 h earlier than in the wild type (0.95 ± 0.39 h, SD, $n = 4$), which seemed to reflect the 1-h shorter circadian period of its oscillator (Kutsuna et al., 1998). Cells of the wild type and *pex* mutant were cultivated in LD cycles since we expected that LD cycling would increase the unknown effect of the Pex protein (Fig. 1C). Eight days of LD alterations resulted in colonies with the standard size of 0.2 mm in diameter for reporter cells of the wild type and *pex* mutant. When the eighth cycle of darkness was complete, these oscillators were monitored as bioluminescence rhythms under continuous light. In contrast to the result with continuous light cultivation, all the bioluminescence peaks of the *pex* mutant occurred significantly earlier than those in the wild type. Even in the first circadian cycle, the peak in the *pex* mutant appeared 3 h earlier than that in the wild type (3.0 ± 0.2 h, SD, $n = 4$; Fig. 3A). In addition, the second circadian peak appeared earlier in the *pex* mutant than in the wild type (4.2 ± 0.4 h, SD, $n = 4$). Since the free-running period was 1 h shorter in the *pex* mutant than in the wild type (data not shown), the oscillator in the *pex* mutant should advance by ~3 h. Therefore, this conditional phase advance corresponded well with that based on the first peak (Fig. 3). Estimation of clock movement based on the second cycle appears to avoid the possible effect of masking on the first cycle of the circadian rhythm. However, under this experimental condition, the first peak reflected certain phases of the oscillator per se and could conveniently be applied as the representative index of circadian resetting. Culture

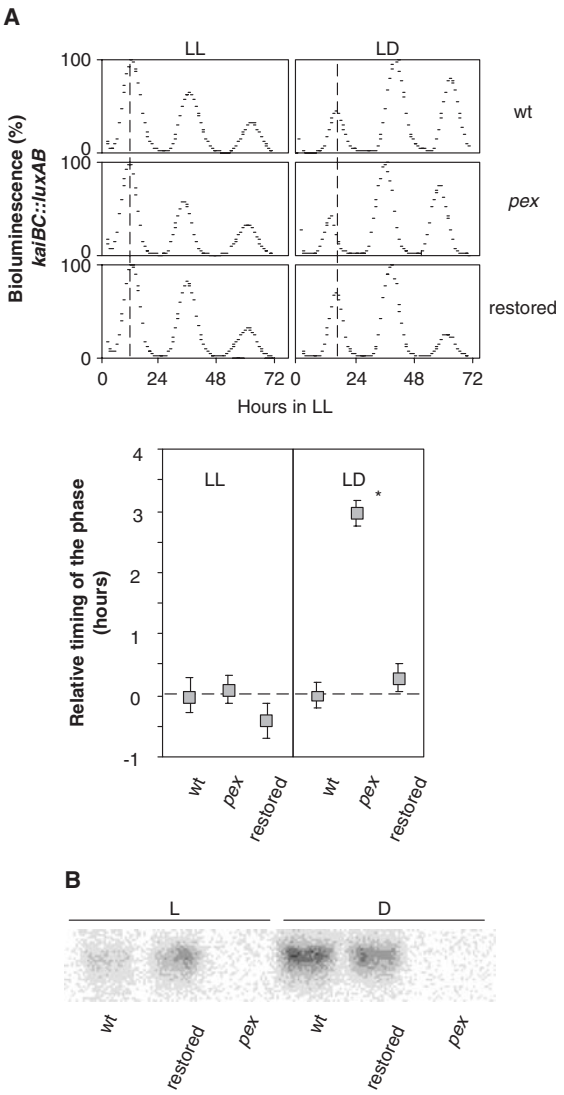

Figure 3. Input phenotype of the *pex* mutant after LD cycles. (A) Bioluminescence monitoring of the expression of the clock operon *kaiBC*. The bioluminescence rhythm in wild-type (wt), *pex*-deficient mutant (*pex*), and *pex*-restored (restored) cells. The circadian oscillators of the colonies subjected to LL or 12-h LD cycles were entrained by a 12-h dark period. Thereafter, bioluminescence of the colonies was monitored under constant light. Broken lines indicate the timing of the first peak of the bioluminescence in wild-type cells. The timing of the peak in the *pex* mutant (or the *pex*-restored) cell relative to that in the wild type is plotted on the ordinate in the lower panels (mean ± SE, $n = 4$). *$p < 0.001$ (t test). Positive and negative values indicate phase advance and phase delay of the clock as estimated from the timing of the first peak. (B) Northern blot analysis of the *pex* transcript. L, hour 6 in a 12-h light period (ZT6); D, hour 8 in a 12-h dark period (ZT20).

in LD cycles did not change the periods of the oscillators (data not shown). Therefore, *pex* seems to be required to delay the clock. In addition, no difference was observed in the color and size of the colonies, suggesting that the abnormality in the *pex* mutant subjected to LD cycles was specific to the clock.

To confirm whether this abnormality in the oscillator was due to the lack of *pex*, we examined whether the 1 kb of DNA containing the *pex* gene complemented the abnormality of the *pex* mutant. We created a *pex*-restored cell in which the *pex* DNA was recombined in the *pex* mutant genome and measured the bioluminescence rhythm. The phase of the oscillator in the obtained *pex*-restored cell was nearly identical to that of the wild type (Fig. 3A). In addition, the presence of *pex* mRNA in the *pex*-restored cell was confirmed by Northern blotting (Fig. 3B). Therefore, the abnormality of the *pex* mutant cultivated in the LD cycles was due to the absence of *pex*.

Degree of the Phase-Advance Phenotype against LD Cycles

To determine the correlation between the phase-related phenotype in the *pex* mutant and the increase in Pex in daily periods of darkness, the *kaiBC*::*luxAB* reporter cells of the wild type and *pex* mutant were subjected to serial LD cycles. The bioluminescence was then monitored under continuous light. After 2 LD cycles, an intermediate phase advance of ~1.5 h was observed in the *pex* mutant (Fig. 4). After 3 to 4 days with LD cycles, the phase of the *pex* mutant resulted in a maximum phase advance of 3 h. These results suggest that the degree of the phase advance in the mutant tended to increase in proportion to the number of days under LD. Considering this result together with the accumulation of Pex in darkness (Figs. 1 C and D), the increased Pex in the dark seemed to fine-tune the clock by the second and third accumulation of Pex, whereas it could not explain why no phase delay was observed after the first dark resetting. Furthermore, after 9 to 11 cycles of the daily LD change, the phase advance in the *pex* mutant reporter was also observed in the resulting larger colonies, which were 0.2 to 0.5 mm in diameter (Fig. 4). These results suggest that the phase of the clock in the *pex* mutant cell had advanced by ~3 h compared to that in wild-type cells under LD conditions.

Dose Dependency of the Phase Delay

If dark-accumulated Pex delays the phase of the oscillator, we postulated that the ectopic induction of *pex* causes this delay. We tested this assumption using

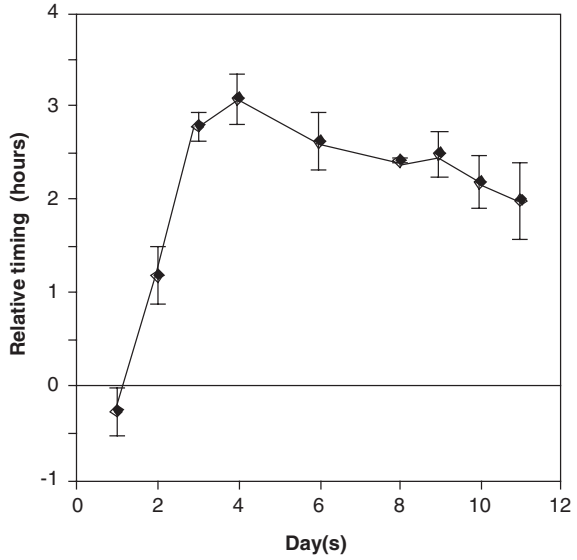

Figure 4. Relative phase of the clock as estimated by the peak in the bioluminescence rhythm of the *pex* mutant reporter after the given number of days in the LD cycles. The *kaiBC* reporter cells of the wild type and *pex* mutant were incubated under constant light for an adequate amount of time (see Materials and Methods) and then subjected to a given number of days under LD cycles (12-h light/12-h dark). The bioluminescence of the obtained colonies was monitored under constant light. The relative timing of the first peak of the rhythm in the *pex* mutant compared to that of the wild type is shown as in Figure 2 (means ± SE, $n = 3$).

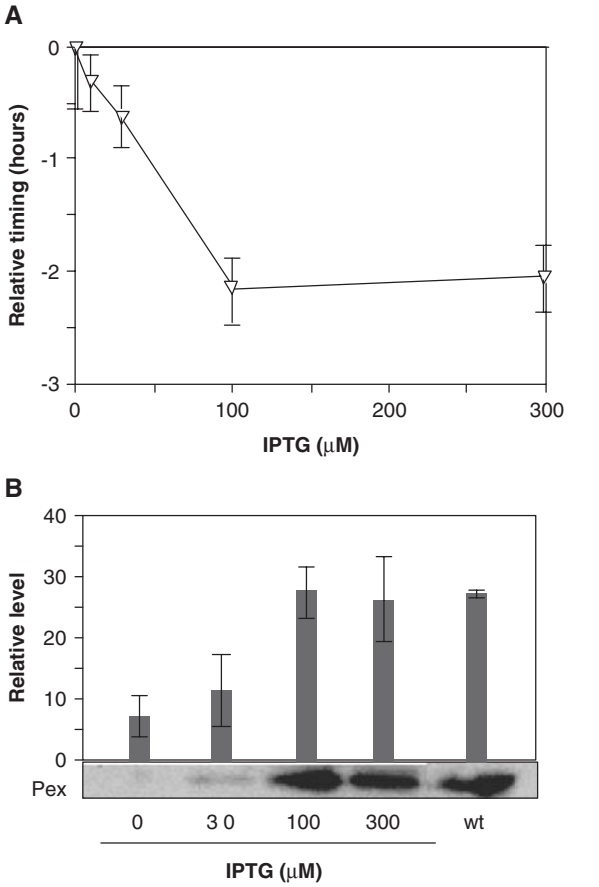

Figure 5. Analyses based on the ectopic induction of *pex*. The inducible *pex* gene *Ptrc*::*pex* was introduced into the bioluminescence reporter cell of the *pex* mutant. The obtained *pex*-inducible cell was cultivated on a nitrocellulose membrane filter with the inducer isopropyl-β-D-thiogalactoside (IPTG) for 5 to 6 days. After 12 h of darkness to entrain the clock, the IPTG was removed and the bioluminescence rhythm was monitored. (A) The relative timing of the first peaks of the bioluminescence rhythm among the *pex*-induced reporter cells was plotted against the applied concentration of IPTG (mean ± SE, $n = 3$). (B) Immunoblotting analysis of Pex accumulation in *pex*-inducible cells with IPTG application. The level of Pex protein was examined in the *pex*-inducible cells prepared for bioluminescence monitoring, as quantified in Figure 1. As a reference, an extract of the wild-type culture treated in darkness for 6 h was used (wt).

a *pex*-inducible cell, whose genome harbored a gene fusion of an inducible promoter, *Ptrc*, and *pex* instead of its original genome. We induced *pex* gene expression by adding the induction substrate IPTG at concentrations of 0 to 300 μM for 5 days under constant light. After 12 h in darkness to reset the clock, IPTG was removed to stop the induction, as observed in the native cell. The bioluminescence rhythms of the cells were then monitored. Compared to cells without added IPTG, the phase in the *pex*-induced cells was delayed by ~2 h (Fig. 5A). The degree of the phase delay was dose dependent until it reached 100 μM IPTG, when the maximum delay occurred. A similar phase delay was observed when the IPTG exceeded 100 μM. The degree of the phase delay was consistent with the accumulated levels of induced Pex (Fig. 5B). Therefore, this strongly suggests that Pex produces phase-delay activity in a dose-dependent manner.

DISCUSSION

The increase in *pex* transcripts in darkness might be due to repression by a repressor responding to light and its derepression under darkness. In this postulated process, the DNA element for repressor binding would function to downregulate the *pex* promoter. Therefore, the transcription activity of the full-length *pex* promoter monitored using the luciferase reporter gene would be weaker than that of the *pex* basal promoter, which consists of –35-10 boxes for binding RNA polymerase in vivo. For the activation process involving an activator, the transcription activity of the full-length *pex* promoter would be stronger than that of the basal promoter.

Our anti-GST-Pex antiserum detected the Pex protein, and the molecular weight of the detected protein (13.5 kDa; Fig. 1B) was smaller than that deduced from the *pex* ORF (17.1 kDa). However, the antiserum detected no protein in the *pex*-deficient mutant. In addition, ectopic expression of the *pex* gene produced a protein of the same molecular weight (13.5 kDa), suggesting that *pex* expresses the 13.5-kDa protein in vivo. Therefore, an internal potential translation start codon or unknown posttranslational modification might function in Pex. Interestingly, Pex has a newly identified PadR domain in its central portion; in contrast, the PadR proteins of *P. pentosaceus* and *L. plantarum* contain the domain in their N-terminal portions (Fig. 2A). We hypothesize that the functional form of Pex has a PadR-like arrangement. This might mean that the N-terminal site of Pex is near the PadR domain. Consistent with this assumption, the Pex observed in vivo was close to the calculated molecular weight (13 kDa), starting from the 39th amino acid (tyrosine, shown as Y), which corresponded to the first residue within the PadR domain (Fig. 2). In addition, the Pex protein expressed from the *pex* gene fused to the DNA sequence of an epitope tag, cMyc, at its 3′-end was detected using Western blotting with the anti-cMyc antibody as a slightly smaller protein than the deduced value (Kutsuna et al., 1998). In the future, we might be able to determine the N-terminal amino acid residues of Pex-cMyc in *S. elongatus* by purifying it with the cMyc antibody. The DNA binding activity of the Pex protein should be examined because of its weak homology to PadR.

The cyanobacterial circadian light resetting appears to be initiated by the photosynthetic apparatus (Katayama et al., 2003; Ivleva et al., 2005; Okamoto and Ashihara-Inouye, unpublished data), in contrast to eukaryotic input, which uses specific photoreceptors such as cryptochrome (Zordan et al., 2001), WC-1, and WC-2 (Froehlich et al., 2002; He et al., 2002) for blue light and plant phytochrome for red light (Somers et al., 1998). The free-running period of the *pex* mutant resembles that of several input mutants previously identified in this organism, such as *cikA* (22 h) and *ldpA* (23 h). Interestingly, *ldpA*-deficient cells show abnormal accumulation of clock-related proteins (decrease in CikA and increase in KaiA, a positive element of the circadian feedback loop; Ivleva et al., 2005), suggesting the regulatory mechanism for the input. To understand its function at the molecular level, it may be important to quantify these gene products in the *pex* mutant. DNA microarrays of *S. elongatus* could be used to screen for related genes whose transcription (or posttranscription) is regulated by Pex.

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