An allele of the *crm* gene blocks cyanobacterial circadian rhythms

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The SasA-RpaA two-component system constitutes a key output pathway of the cyanobacterial Kai circadian oscillator. To date, rhythm of phycobilisome associated (*rpaA*) is the only gene other than *kaiA*, *kaiB*, and *kaiC*, which encode the oscillator itself, whose mutation causes completely arrhythmic gene expression. Here we report a unique transposon insertion allele in a small ORF located immediately upstream of *rpaA* in *Synechococcus elongatus* PCC 7942 termed *crm* (for circadian rhythmicity modulator), which results in arrhythmic promoter activity but does not affect steady-state levels of RpaA. The *crm* ORF complements the defect when expressed in *trans*, but only if it can be translated, suggesting that *crm* encodes a small protein. The *crm1* insertion allele phenotypes are distinct from those of an *rpaA* null; *crm1* mutants are able to grow in a light:dark cycle and have no detectable oscillations of KaiC phosphorylation, whereas low-amplitude KaiC phosphorylation rhythms persist in the absence of RpaA. Levels of phosphorylated RpaA in vivo measured over time are significantly altered compared with WT in the *crm1* mutant as well as in the absence of KaiC. Taken together, these results are consistent with the hypothesis that the Crm polypeptide modulates a circadian-specific activity of RpaA.

chronobiology | transcription regulation

A circadian clock controls rhythms of gene expression in nearly all organisms. In the model unicellular cyanobacterium *Synechococcus elongatus* PCC 7942, the majority of genes show circadian expression (1). The core oscillator of the cyanobacterial circadian clock is composed of the KaiA, KaiB, and KaiC proteins (2). The phosphorylation state, ATPase activity, and dynamic conformation of KaiC all cycle with an approximate 24-h period (3, 4). Interaction of output proteins with KaiC conveys timing information to downstream effectors to influence gene expression patterns (5), chromosome compaction (6), and timing of cell division (7, 8). The histidine kinase Synechococcus adaptive sensor A (SasA) interacts physically with KaiC (9) and forms a two-component system with its cognate response regulator RpaA, a transcription factor of the winged-helix family. Together, SasA and RpaA are thought to comprise the major regulatory system of circadian output. RpaA is a regulator of global gene expression, and disruption of the *rpaA* (regulator of phycobilisome associated) gene renders gene expression from essentially all promoters arrhythmic (10). The phosphorylated form of RpaA is presumed to constitute its active state in promotion of gene expression (10, 11). RpaA also was recently shown to be a cognate response regulator for the input protein CikA, which displays phosphatase activity on RpaA in vitro (11).

Here we report the identification of a previously undescribed clock-related genetic element, designated *crm* (for circadian rhythmicity modulator), located immediately upstream of *rpaA*. The *crm* ORF encodes a predicted 62-residue polypeptide with no recognized functional domains. A transposon insertion allele of this gene, *crm1*, causes arrhythmicity from different classes of promoters without affecting RpaA protein levels, results in alteration of the RpaA phosphorylation state and KaiC protein levels, and eliminates the KaiC phosphorylation cycle. We propose that Crm functions as an allosteric modulator of clock-related RpaA activity.

Results

A Specific Allele of *crm* Disrupts Gene Expression Rhythms. A mutant strain that exhibits arrhythmic expression of a P_{kaiBC}::*luc* luciferase reporter, designated uni-gene set (UGS) mutant 18-B-10, was isolated from a transposon insertion library of *S. elongatus* (12) (Fig. 1*D*, *crm1*). The strain carries an insertion of a modified Tn*5* transposon at 358 bp upstream of the start codon of the *rpaA* gene (Fig. 1*A*). Because *rpaA* deletion is known to cause arrhythmia, we analyzed UGS mutant 18-B-10 for possible disruption of RpaA expression. Immunoblot analysis demonstrated the presence of RpaA at WT levels in the UGS mutant 18-B-10 background, however (Fig. 1*B*, *crm1*). Sequence analysis of the *S. elongatus* genome indicated an ORF at the transposon insertion site (Fig. 1*A*) that encodes a predicted 62-aa protein with an estimated molecular weight of 7.25 kDa and no recognized functional domains (Fig. 1*C*).

Unexpectedly, however, substitution of the small ORF with an antibiotic resistance cassette did not result in arrhythmic *kaiBC* promoter activity (Fig. S1*A*), indicating that the circadian phenotype of UGS mutant 18-B-10 is allele-specific. The new ORF was named *crm* (for circadian rhythmicity modulator), and the Tn*5* insertion allele was designated *crm1*. Recreation of the *crm1* mutation in *psbAI* and *purF* reporter strains also caused arrhythmic expression from those promoters (Fig. 1 *F* and *G*). These genes and *kaiBC* have been assigned to different circadian output categories (13), suggesting that the *crm1* allele exerts a global influence on gene expression rhythms.

Because an antisense RNA runs through the site of *crm1* insertion (14) (Fig. 1*A*), we investigated whether the arrhythmic phenotype results from disruption of the *crm* ORF or of the antisense RNA. Quantification of the antisense transcript on either side of the *crm1* insertion by quantitative RT-PCR (qRT-PCR) showed normal levels (Table S1). Moreover, circadian bioluminescence rhythms driven from the *kaiBC* promoter were restored when a WT *crm* gene was expressed in *trans* in the *crm1* background, demonstrating complementation of arrhythmia (Fig. 1*D*). Additional experiments showed that the start codon of *crm* is necessary for complementation, suggesting the creation of a Crm protein (Fig. S1*B*). Addition of an affinity tag to the ectopically expressed allele abolished complementation, complicating confirmation of the protein product.

***crm1* Phenotypes Are Distinct from Those of an *rpaA* Null Mutant.** Several phenotypes distinguish a *crm1* mutant from an RpaA-deficient strain. The overall level of expression from the P_{kaiBC}::*luc* reporter is notably higher in a *crm1* mutant background than in an *rpaA* null background (Fig. 1*D*). RpaA-deficient mutants do not form colonies under alternating 12-h light:dark (LD)

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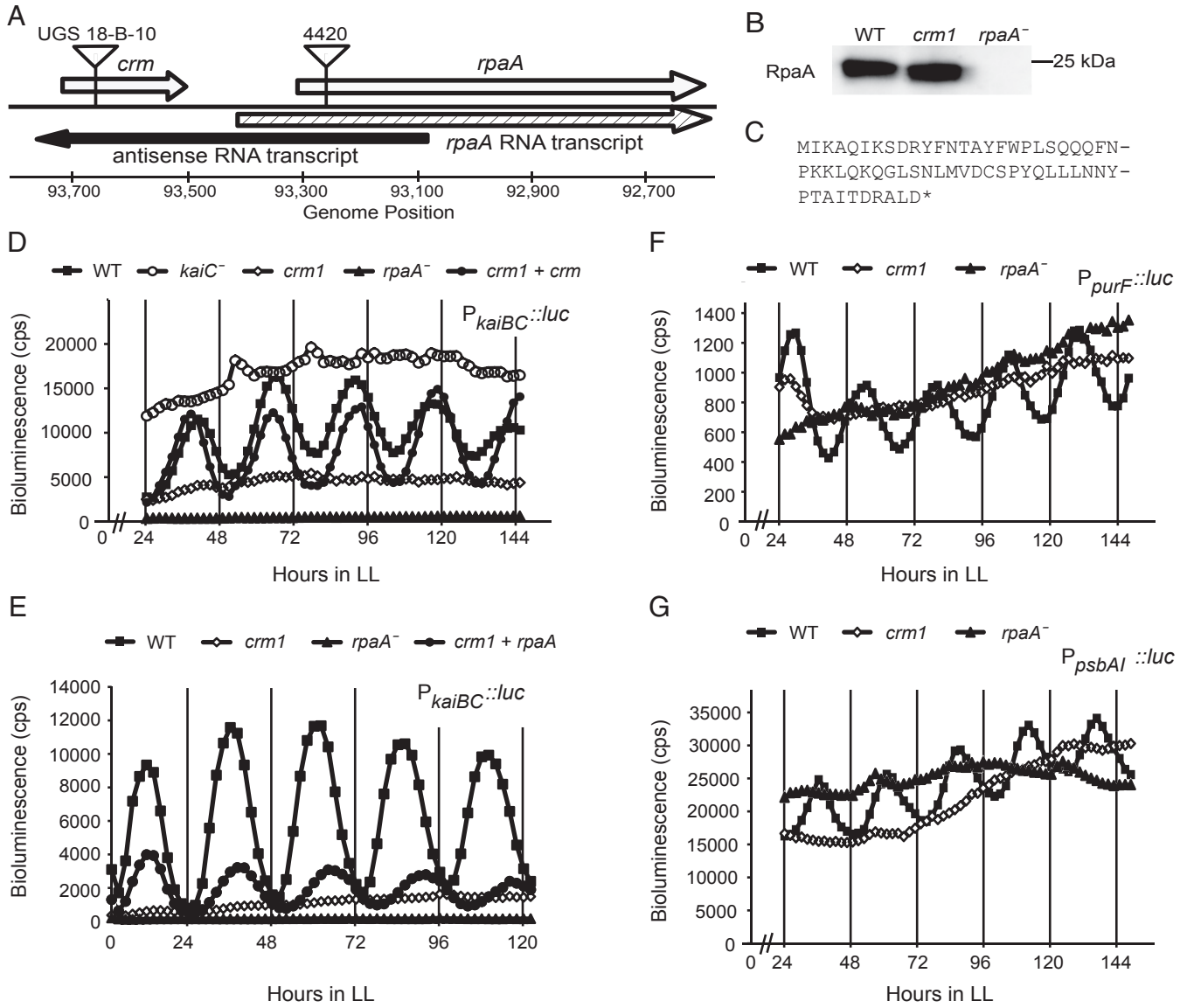

Fig. 1. Transposon insertion in the *crm* gene affects circadian rhythmicity, but not *rpaA* expression. (*A*) Diagram of genomic region containing *crm* and *rpaA* genes. Arrows indicate direction of transcription. Positions of insertions are noted. (*B*) Immunoblots of whole-cell extracts of WT, *crm1*, and *rpaA* null (4420 insertion) strains (10 μg total protein/lane) probed with RpaA antiserum. The *crm1* mutation did not alter levels of the RpaA protein. (*C*) Amino acid sequence of *crm* ORF. (*D–G*) Bioluminescence rhythms. After entrainment to a 12:12 LD cycle, bioluminescence traces were captured over 6 d under LL. *crm1* causes arrhythmic expression from the *kaiBC*::*luc* reporter. *rpaA*$^-$ strains are arrhythmic and demonstrate only a barely detectable level of *kaiBC* reporter expression, whereas *kaiC*$^-$ strains display high-baseline arrhythmic expression. The *crm1* mutant transformed with the *crm* coding sequence in neutral site I complements arrhythmic expression from *kaiBC*::*luc* in *trans*. Representative traces are shown. cps, counts per second. (*E*) Ectopic expression of an extra copy of *rpaA* in a *crm1* background partially restores rhythmicity, displaying a low-amplitude, long-period rhythm compared with WT. Average bioluminescence levels of each strain are shown. (*F* and *G*) The *crm1* allele abolishes rhythmic expression from the *purF* (*F*) and *psbAI* (*G*) promoters. Bioluminescence in *crm1* strains is consistently near WT peak level as measured from the class II *purF* promoter, in contrast to WT trough level as measured from the class I promoters *kaiBC* and *psbAI*. Representative traces are shown.

cycles (10), but a *crm1* mutant grew normally in LD (Fig. 2). The kinase CikA is a major sensor for the input pathway of the circadian clock and acts as a phosphatase for RpaA (11, 15, 16). In WT cells, ZsGreen-tagged CikA exhibits unipolar localization (17); in a subset of clones in which *rpaA* is disrupted, CikA is mislocalized and diffuse (Fig. S2). CikA remains localized to the pole in all cells of a *crm1* background (Fig. S2), reinforcing the difference between this defect and that of an *rpaA* null.

KaiC Levels Are Reduced and Phosphorylation Cycling Is Impaired in *crm1* Mutants. Structural changes corresponding to ordered cycling of the phosphorylation state of KaiC are crucial for interactions with clock output effectors (18, 19). Disruption of *rpaA* has been reported to result in attenuated expression from the *kaiBC* promoter and to abolish cyclic phosphorylation of the core oscillator protein KaiC, which appears to be constitutively phosphorylated in an *rpaA* null strain under constant light (LL) conditions (10).

To assess whether the *crm1* affects KaiC similarly to an *rpaA* disruption, we compared levels of KaiC in whole-cell extracts and examined in vivo phosphorylation cycling of KaiC in *crm1* and an *rpaA* null strain over 48 h using Phos-tag gels and immunoblotting. Steady-state levels of KaiC were reduced by approximately two-thirds in both *crm1* and *rpaA* null backgrounds

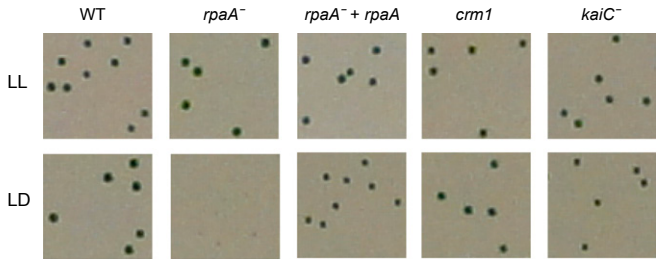

Fig. 2. Phenotypes of *crm1* mutants differ from those of an *rpaA* mutant. Growth of *crm1* strain is not impaired in an LD cycle. WT, *crm1*, *kaiC*⁻, *rpaA*⁻, and *rpaA*-complemented strains were plated for single colonies and maintained in either 100 μmol $m^{-2}s^{-1}$ LL (*Upper*) or a 12:12 LD cycle (*Lower*) over 4 d. The *crm1* mutant does not exhibit the growth impairment of an *rpaA* strain under the LD regimen.

compared with WT (Fig. 3*A*). Although both phosphorylated and nonphosphorylated KaiC were present in *crm1* mutants at all time points, no cycling was detected (Fig. 3*C*). Surprisingly, although KaiC phosphorylation was sometimes constitutively elevated in an *rpaA* null background, rhythmic phosphorylation of KaiC was detected in replicate experiments (Fig. 3*D*).

Phosphorylation Cycling of RpaA Is Perturbed in Arrhythmic *crm1* and *kaiC* Strains. Because phosphorylated RpaA is postulated to reflect RpaA activity (10, 11), we investigated whether *crm1* affects the in vivo phosphorylation state of RpaA in circadian and diurnal cycles. In WT cells, RpaA phosphorylation peaks at subjective dusk (8-12 h after light onset) and ebbs at subjective dawn (11, 20). To examine the influence of a dark cue on the RpaA phosphorylation pattern, we compared replicate WT samples that were entrained to a 12-h LD cycle (12:12 LD) and either left in the light or transferred to the dark at subjective dusk and sampled every 3 h after last light. The LD set was transferred to the dark at 11 h after lights on, so that the 12-h, 15-h, and 18-h samples from the two sets were from the same circadian times but experienced different LD cues. Samples were detected by separation on Phos-tag gels and immunoblotting with RpaA antiserum. Although exposure to darkness for 1 h slightly reduced the ratio of phosphorylated RpaA to nonphosphorylated RpaA at zeitgeber time (ZT) 12 (Fig. 4*A*), the overall pattern of phosphorylation was not substantially different under LL and LD, indicating that the RpaA phosphorylation cycle is largely under circadian clock control and is only mildly affected by a diurnal cycle.

In entrained cultures, phosphorylated RpaA was present at higher levels in *crm1* samples than in WT near subjective dawn (LL2), and did not show the marked increase at subjective dusk (LL9) characteristic of WT (Fig. 4*B*). These results support a mechanism in which the *crm1* allele affects rhythmic gene transcription via modulation of RpaA activity. Unexpectedly, substantial levels of phosphorylated RpaA were detected in the absence of KaiC at both subjective dawn (L 2 h) and dusk (L 9 h) (Fig. 4*B*), demonstrating that a functional clock is not required for RpaA phosphorylation, but suggesting that regulation of

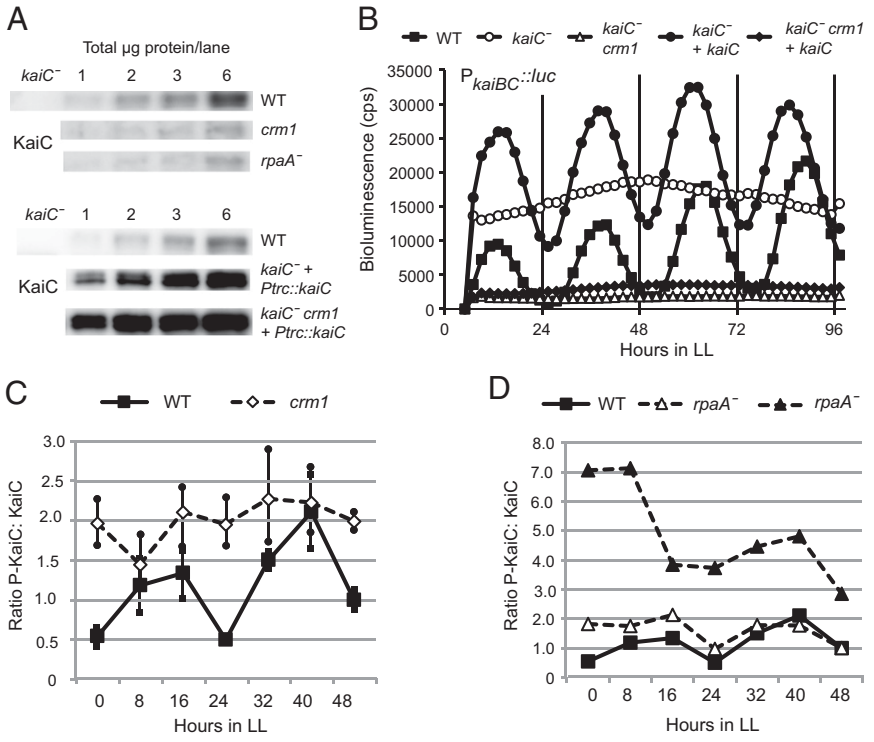

Fig. 3. Quantitation of in vivo KaiC levels and phosphorylation rhythm in *crm1* and *rpaA* deletion mutants. (*A*) (*Upper*) Immunoblots of whole-cell extracts of WT, *crm1*, *kaiC*⁻, and *rpaA*⁻ strains (1, 2, 3, and 6 μg total protein/lane) sampled at 9 h after onset of light and probed with KaiC antiserum. Steady-state levels of KaiC were reduced by approximately two-thirds in both *crm1* and *rpaA*⁻ mutants. (*Lower*) Immunoblots of whole-cell extracts of WT and strains expressing KaiC from uninduced trc promoter in *kaiC*⁻ and *kaiC*⁻ *crm1* backgrounds. (*B*) Arrhythmic gene reporter expression in *crm1* background is not due to low KaiC levels. Shown are representative bioluminescence traces of strains with KaiC expressed ectopically from a neutral site. Expression from *kaiBC*::*luc* reporter in a *kaiC*⁻ *crm1* background remains low and arrhythmic with ectopically expressed KaiC, whereas ectopic KaiC expression restores rhythms in a *kaiC* deletion background. (*C* and *D*) Densitometric analysis of immunoblots of whole-cell extracts from strains entrained to a 12:12 LD regime and sampled every 8 h in LL for 48 h. (*C*) Densitometric analysis showing average ± SD values of two blots of *crm1* and two WT samples. The *crm1* strain is characterized by constitutively elevated KaiC phosphorylation and no discernible overall rhythm over 48 h. (*D*) KaiC phosphorylation is elevated in *rpaA*⁻ cells, but the rhythm of phosphorylation remains detectable. Quantitation of two separate blots of *rpaA*⁻ samples is shown compared with the WT trace shown in *C*.

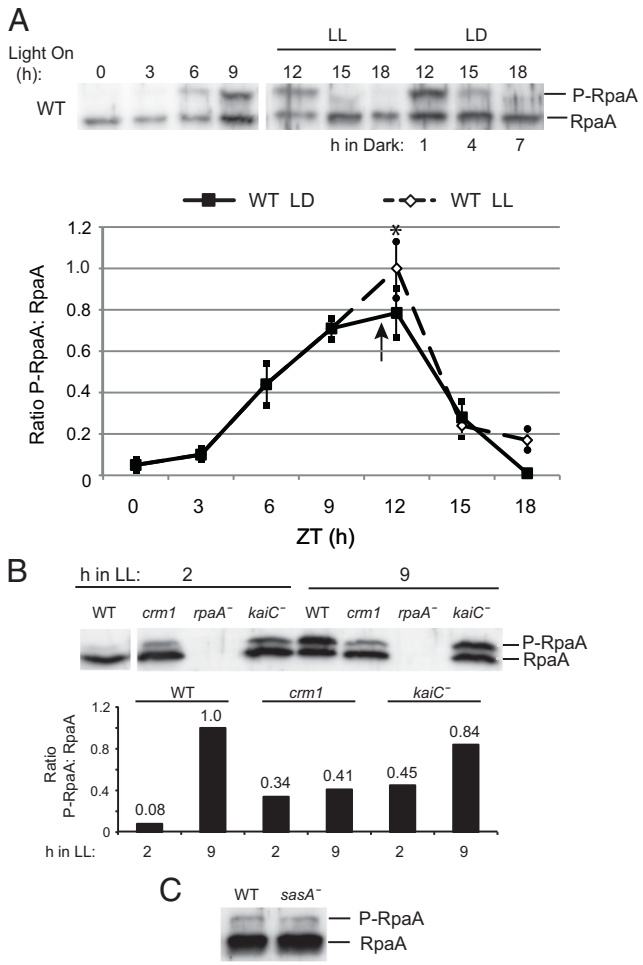

Fig. 4. The in vivo RpaA phosphorylation cycle is minimally sensitive to a dark cue and is perturbed in arrhythmic strains. (*A*) Time points from representative immunoblots (*Upper*) and densitometric analysis showing average ± SD values from multiple immunoblots (*Lower*) demonstrating overall levels of RpaA phosphorylation in WT cells entrained to a 12:12 LD cycle and sampled every 3 h over 18 h. Samples were either maintained in LL or collected after entering dark at 11 h after onset of light. The overall RpaA phosphorylation level in LD is significantly lower after 1 h in dark compared with the 12-h LL sample, but the overall phosphorylation cycle remains substantially the same under both LL and LD conditions. The arrow indicates onset of darkness (at ZT = 11 h) for LD samples. (*B*) Representative immunoblot (*Upper*) and quantification (*Lower*) of the P-RpaA:RpaA ratio of whole-cell extracts (10 μg total protein/lane) sampled at 2 h and 9 h after onset of light after entrainment to a 12:12 LD cycle and probed with RpaA antiserum. A high proportion of RpaA is phosphorylated in the WT strain at 9 h after entering light, whereas the majority of RpaA appears nonphosphorylated at 2 h. The P-RpaA:RpaA ratio does not change substantially in *crm1* cells sampled at 2 h compared with 9 h. In a *kaiC* deletion strain, P-RpaA:RpaA remains at intermediate levels compared with WT at both time points. (*C*) Immunoblot of nonentrained WT and *sasA* deletion strain probed with RpaA antiserum. P-RpaA is detectable in the absence of SasA in vivo.

RpaA dephosphorylation requires the core clock. Surprisingly, phospho-RpaA was detected in a *sasA* null background (Fig. 4*C*), indicating that other kinases are able to substitute for SasA in vivo for phospho-transfer to RpaA.

Discussion

The timing information from the circadian oscillator in cyanobacteria is proposed to be conveyed through at least three output pathways, one of which is the two-component SasA-RpaA system (20). According to recent models, CikA and LabA act as negative effectors and converge on RpaA, which functions as the master circadian regulator of transcription (20), and circadian regulation of RpaA activity is the result of opposing kinase activity of SasA and phosphatase activity of CikA (11). Deletion of *rpaA* has pleiotropic effects on transcript levels and physiology in *S. elongatus*. Overall transcript levels are dramatically reduced, and cells cannot survive multiple LD cycles (10). In contrast, *crm1* or *kaiC* mutants grow robustly in LD, and reporter levels suggest transcripts within the normal range, although closer to trough levels when Crm is disrupted as a *crm1* allele. These data suggest that Crm has more specific effects on circadian aspects of gene expression compared with RpaA. As such, a *crm1* background provides an arrhythmic but otherwise fit strain in which to assess the roles of circadian gene expression.

In light of the drastic effects of *crm1* on promoter rhythmicity and complementation of the mutant phenotype with the *crm* ORF expressed in *trans,* the lack of a discernible phenotype in a *crm* deletion strain is puzzling. The Tn*5* insertion may produce a "poison" product that interferes with protein–protein interactions key to generating circadian rhythms; complementation suggests that even a small amount of WT Crm is sufficient to restore normal regulation, and, similarly, expression of an extra copy of *rpaA* in a *crm1* background partially restores rhythmic expression from the *kaiBC* promoter (Fig. 1*E*). Lack of a detectable circadian phenotype in a deletion strain also has been observed for the circadian feedback regulator *lalA*, in which effects on rhythmic gene expression and cell growth are seen only as a consequence of overexpression (21).

The inability to complement the mutant phenotype with a tagged allele hindered our efforts to confirm overexpression in strains that carry inducible constructs and show no mutant phenotype. Notably, however, expression of native *crm* from the strong class I *psbAI* promoter in WT and *crm* deletion backgrounds resulted in substantial damping of *kaiBC* promoter rhythms over several days in free run (Fig. S3*A*), suggesting that Crm has a modulating effect on circadian rhythms.

In *kaiC* deletion strains, elevated RpaA phosphorylation correlates with high expression from the *kaiBC* promoter (Figs. 1 and 4), suggesting that P-RpaA is a prerequisite for activity of that promoter. In a previous study, the *crm1* allele was erroneously used as an *rpaA* deletion strain, under the assumption that the insertion blocked *rpaA* expression, consistent with arrhythmicity of reporter gene expression (8). The *crm1* allele, like a true *rpaA* or *sasA* null, suppresses the cell-division defect of *ΔcikA* mutant (8); in addition, we found that the combination of an *rpaA* null allele with either *crm1* or *crm* deletion alleles resulted in P_{kaiBC} reporter expression and light:dark sensitivity characteristic of an *rpaA* single mutant (Fig. S3*B*). These findings support the hypothesis that the *crm1* mutation disrupts RpaA activity. The observation that *crm1* suppresses high expression from the *kaiBC* promoter even in a *kaiC* deletion background (Fig. 3*B*) also supports this hypothesis. Moreover, the location of the *crm* gene just upstream of *rpaA* raises the possibility that transcription of *crm* itself is coupled to RpaA function. Few transcripts were identified through this region in deep-sequencing datasets, however (14).

Despite similar attenuation of KaiC levels, an *rpaA* null strain retains low-amplitude KaiC phosphorylation rhythms, whereas a *crm1* mutant does not. The *crm1* allele may influence *kaiBC* feedback regulation and synchrony and/or the mechanism of KaiC cycling. The lack of bioluminescence reporter rhythms in an *rpaA* null background suggests a decoupling or insensitivity to the KaiC oscillations. RpaA activity is critical for sustaining the transcription/translation feedback loop of KaiBC expression (20); however, a recent study suggested that the response regulator RpaB, not RpaA, binds directly to the promoters of rhythmically regulated target genes, including *kaiBC*, and represses them (22). The authors suggested that RpaA functions cooperatively with

RpaB in transcriptional regulation and postulated that phosphorylated RpaA dissociates RpaB from promoters. An interfering effect of a translation product from the *crm1* allele on RpaA's ability to associate with RpaB also would be consistent with this model. Alternatively, the arrhythmia observed in *crm1* mutant cells could be related to a defective mechanism of entrainment wherein cells cannot respond to LD cues and are asynchronous in the population.

We propose that the native form of Crm acts as an allosteric modulator of RpaA, although direct interaction with the oscillator cannot be ruled out. Crm likely forms a disordered coil, given that secondary structure prediction of the Crm amino acid sequence did not reveal recognized structural motifs. The genome of the multicellular nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 contains a short putative ORF, including 19 contiguous bases identical to those of *S. elongatus crm*, upstream of a two-component histidine kinase (all4261), but no *crm*-like sequence upstream of the *Anabaena* homolog of *rpaA*, suggesting that *crm*-like proteins exist in other cyanobacteria and perhaps function in other signaling pathways. A precedent for allosteric regulation of transcription factors by small proteins in cyanobacteria is provided by PipX, an 89-residue protein that interacts either with the carbon/nitrogen balance sensor PII or the transcription factor NtcA depending on nutrient conditions (23).

Similarly, Crm may interact transiently with RpaA, possibly in response to a state of the KaiABC oscillator, and also may interact with other clock components in the circadian regulatory network. However, the WT oscillations of gene expression in a *crm* null background indicate a more complex network in which redundancy is present; thus, the identity of *crm* is made possible by the negative effect of the neomorphic *crm1* allele. Other data support a wider network than has been described previously, with detection of RpaA phosphorylation in a *sasA* null background (Fig. 4*C*). Biochemical or proteomic approaches may reveal the mechanism of Crm activity and identify potential interaction partners.

Taken together, our data are largely consistent with current models in which phosphorylated RpaA represents an active state corresponding to promotion of circadian gene expression from most genes. However, we found that in the absence of KaiC, a substantial ratio of phospho-RpaA is continuously present in the cell, suggesting a more critical role for the oscillator in regulating RpaA dephosphorylation. Although a recent study did not detect RpaA phosphorylation in the absence of SasA, those experiments were conducted using a strain induced to restore native levels of KaiC (11). Our observation that phosphorylated RpaA is present in the absence of SasA in vivo suggests that an alternative interaction partner of RpaA may help maintain residual gene expression rhythms from the *kaiBC* promoter in an *sasA* null (20), a hypothesis that is consistent with a network organization model for two-component systems in *S. elongatus* (24).

In addition, the detectable sensitivity of RpaA phosphorylation level to an LD transition may imply a connection between the redox-sensing input system and the output acting in parallel with the central oscillator. In this model, association of RpaA with a previously described dynamic multiprotein circadian complex, termed the periodosome, consisting of input proteins, output factors, and the Kai oscillator (25, 26), best explains the interplay between traditionally defined output proteins and the core of the clock, such as the negative regulation of RpaA by CikA (10, 11). Such an arrangement would facilitate the integration of environmental and time signals and ensure robust circadian cellular responses.

Materials and Methods

Bacterial Strains, Media, and Culture. *S. elongatus* PCC 7942 and transformed derivatives (Table S2) were maintained in BG-11 liquid or solid medium with antibiotics as needed for selection at standard concentrations (27).

Bioluminescence Monitoring. Bioluminescence of P_{kaiBC}–*luc* firefly luciferase fusion reporter strains was monitored at 30 °C under LL conditions as described previously (27). Data were analyzed with the Biological Rhythms Analysis Software System (http://millar.bio.ed.ac.uk/pebrown/brass/brasspage.htm) import and analysis program using Microsoft Excel. Results shown are representative of at least three independent experiments.

RT-PCR. Cyanobacterial strains in liquid medium were grown for 3 d under a 12:12 LD regimen, and 10–15 mL of culture was collected at 4 h after lights on. cDNA was prepared from total RNA using the SuperScript III Kit (Life Technologies). qRT-PCR was performed with the Applied Biosystems StepOne Plus system (Life Technologies) and SybrGold Master Mix (Life Technologies) with three biological replicates per strain. Abundance was calculated via the $\Delta\Delta C_T$ method and normalized to *rpoA* as a control.

Construction of Plasmids. Plasmids (Table S3) were constructed using the GeneArt Seamless Cloning and Assembly Kit (Life Technologies) or GeneArt *Synechococcus* pSyn1_D/TOPO Vector Kit (Life Technologies) without the addition of nickel.

Site-Directed Mutagenesis. Site-directed mutagenesis designed to substitute two stop codons for the start codon of the *crm* ORF was performed on plasmid pAM4666 (Table S3) following the Quickchange method (Stratagene) using complementary primers 5′-CCTGATTACTTGAGTAGTAGATCAAGGCTCAG-3′ and 5′-CTGAGCCTTGATCTACTACTCAAGTAATCAGG-3′.

Immunoblotting. SDS/PAGE was performed according to standard methods with the following exceptions. Phosphorylation of RpaA and KaiC was detected using 10% SDS-polyacrylamide gels supplemented with Phos-tag ligand (Wako Chemicals USA) at a final concentration of 25 μM and manganese chloride at a final concentration of 50 μM. Gels were incubated once for 10 min in transfer buffer supplemented with 100 mM EDTA, followed by a 10-min incubation in transfer buffer without EDTA before standard wet transfer. For phospho-RpaA detection, protein extracts and electrophoretic apparatus were kept chilled to minimize hydrolysis of heat-labile phospho-Asp. Protein extracts for use in Phos-tag gels were prepared in Tris-buffered saline, and extracts for standard SDS/PAGE were prepared in PBS. RpaA antiserum (a gift of E. O'Shea, Harvard University, Cambridge, MA) was used at a dilution of 1:1,000. KaiC immunoblotting was performed as described previously (28) with modifications described elsewhere (8). Densitometric analyses were performed using National Institutes of Health ImageJ software.

Live-Cell Fluorescence Microscopy. Strains were patched on BG-11 solid medium supplemented with appropriate antibiotics and then inoculated into liquid BG-11 medium. Immediately before imaging, 1 μL of culture was placed onto 1% agarose dissolved in BG-11 medium, coverslipped, and imaged on a DeltaVision RT inverted epifluorescence microscope (Applied Precision) with an Olympus Plan Apochromat 100× objective at 24 °C using appropriate filters. Images were captured using a CoolSnap HQ CCD camera (Photometrics) and deconvolved using the SoftWorx imaging program (Applied Precision). Micrographs were saved as 16-bit gray-scale images and then converted to 8-bit images, and channels were combined in ImageJ.

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