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cpmA, a Gene Involved in an Output Pathway of the Cyanobacterial Circadian System

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We generated random mutations in *Synechococcus* sp. strain PCC 7942 to look for genes of output pathways in the cyanobacterial circadian system. A derivative of transposon Tn*5* was introduced into the chromosomes of reporter strains in which cyanobacterial promoters drive the *Vibrio harveyi luxAB* genes and produce an oscillation of bioluminescence as a function of circadian gene expression. Among low-amplitude mutants, one mutant, tnp6, had an insertion in a 780-bp open reading frame. The tnp6 mutation produced an altered circadian phasing phenotype in the expression rhythms of *psbAI*::*luxAB*, *psbAII*::*luxAB*, and *kaiA*::*luxAB* but had no or little effect on those of *psbAIII*::*luxAB*, *purF*::*luxAB*, *kaiB*::*luxAB*, *rpoD2*::*luxAB*, *ndhD*::*luxAB*, and *conII*::*luxAB*. This suggests that the interrupted gene in tnp6, named *cpmA* (circadian phase modifier), is part of a circadian output pathway that regulates the expression rhythms of *psbAI*, *psbAII*, and *kaiA*.

Circadian (daily) rhythms are ubiquitous regulatory functions, found in organisms from bacteria to mammals, that control various biological activities including behavior, metabolism, and gene expression (2, 12, 27, 32). The mechanism of the circadian clock has not been clarified. However, clock function can be modeled as having three common constituents: input pathways, a central oscillator, and output pathways (16). Light and temperature are the environmental stimuli recognized most commonly to act through input pathways to synchronize the phase of the circadian rhythm with environmental cycles. The central oscillator generates and sustains an oscillation that has an approximately 24-h period. The endogenous circadian oscillation is coupled to clock-controlled processes through output pathways.

Several genes that are involved in circadian clock function have been identified in the cyanobacterium *Synechococcus* sp. strain PCC 7942. Complementation of altered period and arrhythmic mutants and sequence analysis of the relevant loci revealed that all of the mutants carried a point (missense) mutation in one of three genes, designated *kaiA*, *kaiB*, and *kaiC*, which are clustered in the *Synechococcus* genome (15). Gene disruption and overexpression experiments suggest that the *kai* genes encode central oscillator components. Another gene, *pex*, was isolated by suppression of the short-period mutant SP22 (21). Disruption of *pex* shortens the circadian period and overexpression lengthens the period. The *pex* product is thought to modulate function of the central clock oscillator. A mutation in the *rpoD2* gene, which encodes a group 2 σ^{70}-like transcription factor, causes a low-amplitude phenotype in the *psbAI* gene's expression rhythm (34). A similar phenotype was observed for one other gene, *ndhD*, but the expression rhythms of several other genes were not affected by the mutation. The specificity of the mutation for a subset of genes indicates that the *rpoD2* gene is a component of an output pathway in the *Synechococcus* circadian clock.

Random insertion of promoterless *luxAB* genes, which encode luciferase from *Vibrio harveyi*, into the *Synechococcus* genome revealed robust gene expression rhythms as an output of the circadian clock. The approximately 800 insertion strains tested showed circadian oscillation of luciferase activity, measured as bioluminescence, and none was clearly arrhythmic (23). The genes were grouped into five categories ranging from class 1 to class 5 according to the waveforms of the bioluminescence rhythms: class 1 to class 3 rhythms have symmetrical waveforms but peak in different phases, whereas the others have asymmetric waveforms which are saw-toothed (class 4) or show bimodal peaks per cycle (class 5). These results suggest that there are several pathways of output from the circadian oscillator in this cyanobacterium, each of which generates a customized pattern of circadian expression for its target subset of genes.

Growth competition experiments have suggested that the different phase relationships of the expression of various genes may be important for optimal fitness (26). The relative phasing of circadian oscillations is described by the phase angle of each, or the time between the last synchronizing light-dark cycle and the peak or trough of the endogenous rhythm (27). One goal of our research is to map the output pathways responsible for the different phase angles of the expression rhythms in the *Synechococcus* genome.

We isolated a transposon-generated mutant of *Synechococcus* sp. strain PCC 7942, tnp6, which is affected in both amplitude and phasing of the *psbAI*::*luxAB* circadian expression rhythm. We examined the mutation's effect on circadian expression rhythms for several other genes and assigned a function of the tnp6 gene, renamed *cpmA* (circadian phase modifier), to an output pathway of the cyanobacterial circadian clock. Surprisingly, inactivation of *cpmA* dramatically changed the circadian phasing of promoter activity for one of the genes thought to encode a central clock component (*kaiA*::*luxAB*), but it had little or no effect on that which drives the other two clock genes (*kaiB*::*luxAB*). The net effect is a dramatic phase angle difference between these two gene expression oscillations. These data suggest that the coordinated expression of the *kai* genes is not essential for circadian timekeeping in *Synechococcus*.

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TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics or genotype
E. coli strains	
AM1452	HB101 containing helper plasmid pRL528[a] and transposon plasmid pAM1037
AM1460	HB101 containing plasmid pRK2013[b] to provide conjugal transfer functions
DH10B	Host for plasmids
Synechococcus strains[c]	
PCC 7942	Wild-type *Synechococcus*
AMC149	*psbAI*::*luxAB* reporter gene fusion inserted into NSI[d]
AMC393	*psbAI*::*luxAB* reporter gene fusion inserted into NSII[e] (Cm^r) *psbAI*::*luxCDE* fusion inserted into NSI (Sp^r)
AMC408	*purF*::*luxAB* reporter gene fusion inserted into NSII[e] (Cm^r) *psbAI*::*luxCDE* fusion inserted into NSI (Sp^r)
AMC412	Derivative of AMC149 with *psbAI*::*luxCDE* fusion inserted into NSII (from pAM1706)
AMC412(*cpmA*::Km)	Derivative of AMC412 in which *cpmA* has been inactivated by recombination with pAM2087 (Km^r)
AMC462	*kaiBC*::*luxAB* reporter gene fusion inserted into NSI
AMC509	*kaiA*::*luxAB* reporter gene fusion (translational) inserted into NSI
AMC520	*psbAII*::*luxAB* reporter gene fusion (translational) inserted into NSI
AMC537	*psbAIII*::*luxAB* reporter gene fusion inserted into NSI
AMC538	*rpoD2*::*luxAB* reporter gene fusion inserted into NSI
AMC539	*conII*::*luxAB* reporter gene fusion inserted into NSI
AMC540	Derivative of AMC149 with *psbAI*::*luxCDE* fusion and an ectopic copy of *cpmA* inserted into NSII (from pAM2089)
AMC540(*cpmA*::Km)	Derivative of AMC540 in which *cpmA* has been inactivated by recombination with pAM2087 (Km^r)
AMC543	*psbAI*::*luxAB* reporter gene fusion inserted at *Bst*EII site (Km^r selection) and *psbAI*::*luxCDE* inserted at *Bgl*II site (Cm^r selection) of NSII
AMC550	Derivative of AMC543 in which an IPTG-inducible ectopic copy of *cpmA* is inserted at NSI (from pAM1850)
LUX6	*ndhD*::*luxAB* reporter gene fusion; a 3.0-kb genomic DNA fragment including *ndhD* is integrated into the *ndhD* locus by single recombination
Plasmids	
pAM1037[e]	Tn*5* plasmid derivative pRL1058, further modified by insertion of a 0.7-kb fragment in the *Xba*I site to add outward-reading promoters from the *Anabaena* sp. strain PCC 7120 *glnA* and *rbcL* genes
pAM1706	Vector that transfers *psbAI*::*luxCDE* fusion to NSII (subsite NS2.1, *Bst*EII) of the *Synechococcus* chromosome
pAM1825	NSI vector that carries *lacI*^{q}, IPTG-inducible *trc* promoter, and ribosome-binding site upstream of unique *Nco*I and *Sma*I cloning sites
pAM1850	Vector that transfers *psbAI*::*luxCDE* fusion to NSII (subsite NS2.2, *Bgl*II) of the *Synechococcus* chromosome
pAM2086	pUC18 containing 0.9-kb *Eco*RI fragment including *cpmA* ORF in the same orientation as the *lacZ* promoter
pAM2087	46-bp *Nru*I fragment of pAM2086 was replaced by a Km^r cassette
pAM2088	46-bp *Nru*I fragment of pAM2086 was replaced by an Sp^r-Sm^r cassette
pAM2089	Derivative of pAM1706 that carries a P*lacZ*::*cpmA* fusion downstream of *psbAI*::*luxCDE*
pAM2107	Entire *cpmA* ORF inserted into pBR322 at the *Eco*RV site
pAM2108	0.9-kb *Eco*RI fragment from pAM2107 (with ends modified as described in Materials and Methods) inserted into pAM1825 for IPTG-inducible expression of *cpmA*

[a] Reference 8.
[b] Reference 7.
[c] All *Synechococcus* strains carry a *psbAI*::*luxCDE* fusion in NSII except PCC 7942, AMC149, AMC393, and AMC408; all except PCC 7942 and AMC149 are bioluminescent without exogenously added aldehyde substrate. All insertions into NSI were selected by Sp^r; all insertions into NSII were selected by Cm^r, except as otherwise noted for AMC543 and ACM550.
[d] Reference 19.
[e] Reference 1.

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All reporter strains were derived from *Synechococcus* sp. strain PCC 7942 by introducing *luxAB* fusions into the chromosome at neutral site I (NSI [6]) or, for *purF*::*luxAB* and in AMC393, at neutral site II (NSII [1]). *ndhD*::*luxAB* was introduced as a duplication at the native *ndhD* locus. All reporter genes are transcriptional fusions except two: *kaiA* and *psbAII* are translationally fused to *luxA* in AMC509 and AMC520, respectively. All *Synechococcus* strains were grown on modified BG-11 medium (BG-11M [5]), in liquid cultures or on agar plates (11), under continuous light (LL) at 30°C. Chloramphenicol (7.5 and 17 μg/ml) and/or spectinomycin (5 μg/ml and 20 μg/ml) was added to select for NSI and NSII targeting vectors in *Synechococcus* and in *Escherichia coli*, respectively. The tnp6 mutants were selected in the presence of either kanamycin (5 μg/ml for the *psbAI*::*luxAB*, *psbAII*::*luxAB*, *psbAIII*::*luxAB*, *kaiA*::*luxAB*, *kaiB*::*luxAB*, *rpoD2*::*luxAB*, and *conII*::*luxAB* strains) or spectinomycin (10 μg/ml for the *ndhD*::*luxAB* strain).

Transposon mutagenesis. pAM1037, which is a derivative of pRL1058 (36), was introduced into *Synechococcus* by conjugal transfer from *E. coli* (1). AMC149, a *psbAI*::*luxAB* reporter strain of *Synechococcus* (19), was grown to an optical density at 750 nm of 0.5. Cells from a 100-ml sample were harvested by centrifugation at 2,000 × *g* and resuspended in 10 ml of fresh BG-11M. Ten-milliliter overnight cultures of *E. coli* AM1452, which carries the helper plasmid pRL528 and the transposon plasmid pAM1037, and of *E. coli* AM1460, which carries the conjugal plasmid pRK2013, were each washed twice with 10 ml of fresh Luria-Bertani (LB) medium and resuspended in 10 ml of sterile water. Ten milliliters of *Synechococcus* cell suspension and 5 ml of each *E. coli* cell suspension were mixed, and 400-μl aliquots of the mixture were spread onto plates containing BG-11M plus 5% LB medium (vol/vol) (1). The plates were kept under dim light (10 to 30 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 30°C for 24 h. Kanamycin was underlaid beneath the agar (11) for a final concentration of 50 μg/ml, and the plates were incubated at 30°C under 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 4 weeks. Km-resistant (Km^r) transformants were streaked onto fresh BG-11M–kanamycin (50 μg/ml)

plates, incubated for an additional 2 weeks, and then inoculated into 250 μl of BG-11 liquid medium containing 5 μg of kanamycin/ml in 96-well microtiter plates.

Cloning and sequencing of the tnp6 region. Restriction enzymes and most modifying enzymes were purchased from Promega and used as directed by the manufacturer. Recovery of the transposon is described in the Results section. One cosmid clone, P2-C3, was isolated from a cosmid library of *Synechococcus* sp. strain PCC 7942 by DNA hybridization with a probe made from the flanking region of the recovered transposon (3). An *Eco*RI fragment of 0.9 kb which hybridized to the probe was cloned into the *Eco*RI site of pUC18 to produce pAM2086. We used this plasmid as a template for sequencing the tnp6 locus. Insert DNA was sequenced on both strands by using the cycle sequencing method (dye terminator cycle sequencing ready reaction, ABI PRISM; PE Applied Biosystems, Foster City, Calif.).

Construction of plasmids for disruption and complementation of *cpmA*. We digested pAM2086 with *Nru*I and inserted a 2.0-kb *Hin*cII fragment from pSKS101, including the Km^r gene, to produce pAM2087 (see Fig. 2B). We used pAM2087 to create the *cpmA* null mutation in *psbAI*::*luxAB*, *psbAII*::*luxAB*, *psbAIII*::*luxAB*, *kaiA*::*luxAB*, *kaiB*::*luxAB*, *purF*::*luxAB*, *rpoD2*::*luxAB*, and *conII*::*luxAB* reporter strains. We digested pAM2086 with *Nru*I and inserted a 2.1-kb *Sma*I fragment from pHP45Ω (28), which carries the spectinomycin-streptomycin resistance Ω fragment (Sp^r fragment), to produce pAM2088. We used this plasmid to introduce the *cpmA* mutation into the *ndhD*::*luxAB* reporter strain, which already encodes Km^r (34).

For complementation of the *cpmA* mutation, a 1.4-kb *Pvu*II fragment in which the *cpmA* gene is preceded by the *lacZ* promoter, in the same orientation, was excised from pAM2086, ligated with the *Kpn*I linker 5′-GGGTACCC-3′, and cloned into the *Kpn*I site of pAM1706 to produce pAM2089. In this plasmid the *cpmA* gene was inserted downstream of the *luxCDE* genes in the same orientation (see Fig. 2C). We transformed wild-type AMC149 with pAM2089 to create AMC540 and then transformed this strain with pAM2087. Transformants that carried the *cpmA*::Km^r mutation in the original *cpmA* locus, and an ectopic copy of *cpmA* in NSI, were identified by genomic Southern hybridization (3) and assayed for their circadian phenotypes. One such clone was designated AMC540(*cpmA*::Km^r).

Overexpression of *cpmA*. A DNA fragment which includes the initiation codon and N-terminal coding portion of the *cpmA* gene was amplified by PCR with the primer set 5′-GTGCTCATGATTGATTCACAG-3′ (AMO282) and 5′-CCTGAAGTCGGGGAATGCG-3′ (AMO283). This 288-bp fragment was made blunt ended by T4 DNA polymerase and cloned into the *Eco*RV site of pBR322; a 0.3-kb *Ban*II fragment of this plasmid was then replaced by the 1.1-kb *Ban*II fragment of pAM2086 to complete the gene and produce pAM2107. A 0.9-kb *Eco*RI fragment was removed from pAM2107, blunted with T4 DNA polymerase, digested with *Bsp*HI, and cloned into *Nco*I-*Sma*I sites of pAM1825 to produce pAM2108. pAM1825 is an NSI targeting vector that includes the $lacI^q$ gene, the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *trc* promoter, and a Shine-Dalgarno ribosome-binding sequence (30). pAM2108 was introduced into the *psbAI*::*luxAB* reporter strain AMC543 to produce AMC550. For induction of *cpmA* expression, samples were inoculated onto BG-11M agar pads containing 1 mM IPTG (Research Products International Corp., Mt. Prospect, Ill.), incubated in LL for 24 h, and entrained by a 12-h dark pulse before bioluminescence measurement began.

Assay of bioluminescence rhythms. The original tnp6 mutant was identified by continuous bioluminescence monitoring of Tn*5*-generated mutants by using a turntable apparatus described previously (20). The genetic background, AMC149, carried *psbAI*::*luxAB*; decanal was added exogenously in the vapor phase as a substrate for luciferase (20). When the mutation was recreated in AMC149 and other reporter strains, long-chain aldehyde production was engineered in vivo by insertion of the *luxCDE* genes of *Xenorhabdus luminescens*, driven by the *psbAI* promoter, at NSII (1). Cultures grown on BG-11M agar in LL were inoculated onto BG-11M agar in 96-well microtiter plates. An antibiotic (kanamycin or spectinomycin, as appropriate) was included in the agar for monitoring of *cpmA*-inactivated strains. These sample plates were incubated for 24 h under standard LL conditions and then subjected to a 12-h dark interval to synchronize the clocks of all cells in the population. Bioluminescence rhythms were measured by using a Packard TopCount luminometer (1). Measurements of bioluminescence from firefly luciferase fusions were performed similarly, except that the reporter strains did not carry *luxCDE* genes for aldehyde production; luciferin (Biosynth International, Naperville, Ill.) was added to the top of the agar in each well for a final concentration of 190 nM.

Nucleotide sequence accession number. The *cpmA* gene sequence was entered into the GenBank database (accession no. AF117208).

RESULTS

Isolation of the mutant tnp6. We generated random transposon mutations in a bioluminescent reporter strain of *Synechococcus* sp. strain PCC 7942 (AMC149) to isolate mutants affected in circadian clock function. The transposon was delivered by conjugal transfer from *E. coli* of pAM1037, which carries a Tn*5* derivative to which the tandem *rbcL* and *glnA* promoters of *Anabaena* sp. strain PCC 7120 have been added at one end of the transposon (1). In addition to simple gene disruption, the modified Tn*5* can, theoretically, cause the over- or underexpression of adjacent genes by the activities of these strong outward-reading promoters. The modified Tn*5* also bears a p15A replication origin to facilitate recovery in *E. coli* after genomic insertion (36). AMC149 carries a reporter gene in which the promoter region of *psbAI*, which encodes form I of the photosystem II D1 protein, is fused to promoterless *luxAB* genes of *V. harveyi* (19). AMC149 shows a circadian bioluminescence rhythm that has peaks at subjective dusk and troughs at subjective dawn (Fig. 1A). Among approximately 3,000 independent Km^r exconjugants, we identified a mutant, tnp6, which showed both a low-amplitude oscillation and an early-phase-angle phenotype (Fig. 1B).

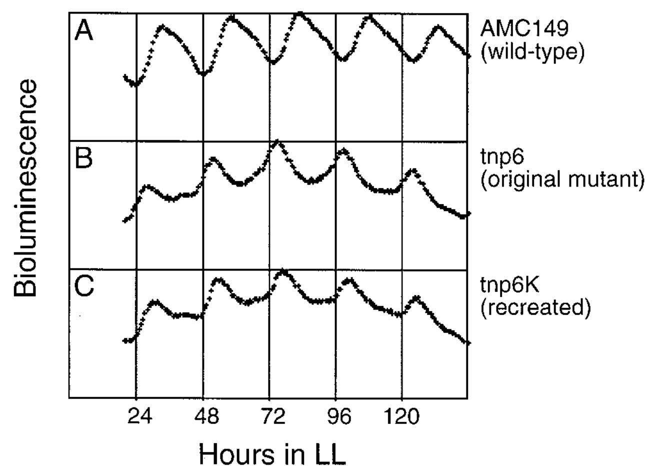

FIG. 1. Bioluminescence traces from the tnp6 mutant of *Synechococcus* AMC149. Bioluminescence was measured from streaks on agar plates of wild-type AMC149 (A); original tnp6 mutant (B); and recreated tnp6 mutant, tnp6K (C). Time shown on the *x* axis refers to hours in LL after a synchronizing 12-h dark incubation. The *y* axis indicates bioluminescence (counts/3-min bin) from baseline of 0 to maxima of 101,000 (panel A), 57,300 (panel B), and 64,100 (panel C) as detected by a cooled charge-coupled device camera (20).

Genomic DNA was extracted from tnp6 and digested by *Stu*I, which does not cut within the transposon. Ligated genomic DNA was used to transform *E. coli*. The transposon, flanked by approximately 24 kb of *Synechococcus* genomic DNA, was recovered as a Km^r plasmid. We transformed wild-type AMC149 with the recovered Km^r plasmid, which had been linearized by digestion with *Stu*I, to recreate the tnp6 mutant. The recreated mutant, tnp6K, showed the same phenotype as the original tnp6 mutant (Fig. 1C), confirming that the phase change resulted from the transposon insertion rather than from an unrelated secondary mutation. In addition to the circadian phenotype, tnp6 and tnp6K had a paler green color and slower growth on BG-11M agar medium than the wild type under light at 100 to 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. Under stronger light conditions (250 to 300 $\mu E \cdot m^{-2} \cdot s^{-1}$) or in liquid media, revertants that showed wild-type color and growth rate and normal phasing and amplitude of circadian bioluminescence oscillation were readily obtained. We routinely maintained tnp6 mutant strains by streaking on BG-11M agar medium with selective antibiotics under light at 100 to 150 $\mu E \cdot m^{-2} \cdot s^{-1}$, and we did not observe reversion of any of the mutant phenotypes under these conditions.

Sequencing the tnp6 locus and determining the insertion site of the transposon. We determined the insertion site of Tn*5*

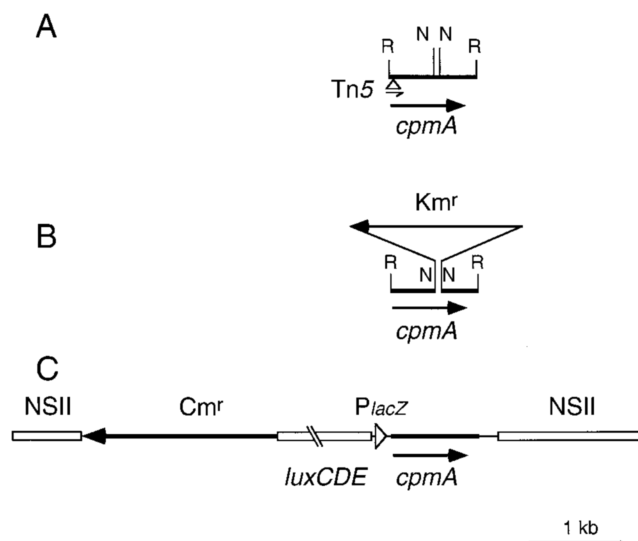

FIG. 2. Chromosomal maps of specific loci from recombinant cyanobacterial strains. Shown are physical maps of original tnp6 mutant of *Synechococcus* (A), the inactivated *cpmA* locus in which an *Nru*I fragment was replaced by a Km^r gene cartridge (B), and the NSII loci in AMC540 and AMC540(*cpmA*::Km^r) (C).

from the recovered transposon-based plasmid using a primer that is complementary to one end of the transposon and reads outward into the flanking *Synechococcus* DNA. The wild-type locus was isolated from a cosmid library by using the recovered tnp6 plasmid as a probe. Southern hybridization against DNA from the cosmid clone indicated that the transposon inserted into the chromosome in a region, of which we subsequently determined the nucleotide sequence, bounded by *Eco*RI sites 0.9 kb apart (Fig. 2A). The *Eco*RI fragment included a 780-bp open reading frame (ORF). Comparison with the sequence we obtained from the recovered transposon showed that the insertion occurred at nucleotide 34 of this ORF. The direction of transcription of the tandem *glnA* and *rbcL* promoters on the transposon was the same as that of the 780-bp ORF. We designated this ORF the *cpmA* gene (circadian phase modifier).

Database searches showed that the deduced CpmA protein has extensive sequence similarity to predicted proteins of unknown function that have been identified through total genome sequence determinations (Fig. 3). CpmA has the following degrees of identity to hypothetical proteins: 53.8% identity to sll1489 of *Synechocystis* sp. strain PCC 6803 (17), 53.2% identity to MJ0165 of *Methanococcus jannaschii* (4), 42.0% identity to the phosphoribosylaminoimidazole carboxylase-related protein of *Methanobacterium thermoautotrophicum* (31), and 40.6% identity to AF1275 of *Archaeoglobus fulgidus* (18). The degrees of identity among these amino acid sequences, along their entire lengths, and striking blocks of identical residues, suggest that they are CpmA homologs. The putative CpmA protein also shows some sequence similarity to the *purE* product from diverse prokaryotes (Fig. 3). *purE* encodes the catalytic subunit of the de novo purine biosynthesis enzyme 5′-phosphoribosyl-5-amino-4-imidazole (AIR) carboxylase, which catalyzes the carboxylation of AIR to 5′-phosphoribosyl-5-aminoimidazole-4-carboxylic acid (33, 35). The hydrophobicity profile of CpmA indicates that there are two hydrophobic regions (amino acids 185 to 205 and 214 to 234 in the sequence of *Synechococcus* CpmA) (Fig. 3) in the part of the protein that is similar to PurE. These hydrophobic regions exist in the putative cyanobacterial and archaeal CpmA homologs as well.

Regeneration and complementation of the tnp6 mutation. The transposon inserted close to the initiation codon (position 12 in the amino acid sequence) of *cpmA* and the direction of transcription from the strong *glnA-rbcL* promoter on the transposon were the same as those of *cpmA* (Fig. 2A). Therefore, it was considered possible that the phenotype of tnp6 was caused either by overexpression of a portion of *cpmA* or by interference with the transcription of a downstream gene. To exclude these possibilities, we generated a *cpmA* null mutation by replacement of the internal *Nru*I fragment with a Km^r cartridge in the *psbAI*::*luxAB* reporter strain; the orientation of the Km^r gene was reversed relative to that of *cpmA* (Fig. 2B). This mutant (Fig. 4B) showed the same circadian phenotype as the original tnp6 mutant (Fig. 1B). This result confirmed that disruption of expression, rather than overexpression, can cause the phenotype.

We provided the *cpmA* gene in *trans* to determine whether loss of function of this gene alone was responsible for the mutant phenotype. Sequence data showed that *cpmA* is located 21 bp downstream of an ORF that is similar to the oligopeptide transport permease gene of *Synechocystis* sp. strain PCC 6803 (sll1699; data not shown) and transcribed in the same direction. In addition, a 1.0-kb *Eco*RI fragment immediately upstream of *cpmA* did not drive a *luxAB* reporter gene in *Synechococcus* above the basal level of bioluminescence (data not shown). Therefore, we considered it likely that *cpmA* is part of an operon together with this upstream ORF and does not have its own promoter. We removed *cpmA* from pAM2086 in such a way as to provide the *lacZ* promoter from the pUC18 vector and cloned it into an NSII vector (pAM1706) downstream of the *luxCDE* genes (Fig. 2C), which direct the synthesis of the luciferase long-chain aldehyde substrate in vivo. We transformed AMC149 with this plasmid (pAM2089) to create AMC540 (which carries both native and ectopic copies of *cpmA*) and in parallel with pAM1706 (to create the autonomously bioluminescent wild-type reporter strain AMC412). The *cpmA* null mutation was then introduced into both genetic backgrounds to create strains AMC540(*cpmA*::Km) and AMC412(*cpmA*::Km), respectively. Southern blot analysis was performed to distinguish whether the disrupted *cpmA* gene replaced the native or ectopic *cpmA* copy in the AMC540 background. The mutation was created after addition of the ectopic copy because the reversion frequency of the *cpmA* mutant in liquid culture precluded transformation of that strain.

Figure 4 shows bioluminescence traces of the wild-type AMC412 (Fig. 4A), the *cpmA* mutant AMC412(*cpmA*::Km) (Fig. 4B), and the complemented strain AMC540(*cpmA*::Km) (Fig. 4C). The phase and amplitude of the bioluminescence rhythm in the complemented strain were almost identical to those of the wild-type strain (Fig. 4A). The complemented strain also had wild-type pigmentation and growth rate. These data showed that the loss of function of the *cpmA* gene was responsible for the phenotypes of the tnp6 mutant.

Three mechanisms could account for the high frequency of reversion observed for the *cpmA* phenotypes when cells were exposed to higher light intensities. (i) The transposon (or antibiotic resistance cassette) might be lost somehow. (ii) Wild-type chromosomes may persist in the cells in addition to *cpmA*-inactivated genomes. In either case, degradation of the selecting antibiotics during incubation might allow wild-type (or reverted) segregants to overgrow the population. Alternatively, (iii) mutation at a second locus in the presence of a

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Synco-CpmA              MIDSQALQTLLEGVATGAIAPQQALEQLRYLDTETISDFARID  43
Syncy-CpmA   MVSGPVFQFSAPMTPDSLQQLLTAIASGHISSQEGFEQLKHLSFQAIDDFAKVD  54
Metco-CpmA                MGKNLRDLLLAFKNGDISLDEIEKQIKLNYYEEIEERLKLD  41
Metba-CpmA                 MMPVRSILEELLRGRISLEEAERAIESAQLSLGDR-VRFD  39
Arhog-CpmA                                     MRRADELKNLAN------LD  14

Synco-CpmA   HHRQLRTGFPEVIWGQDKTTEQILTLFRAYAARNQAAIATRIEPDR-----IPR  92
Syncy-CpmA   HQRQLRTGFPEVVWGPGKTPEQIEQIIQVLAVHNPVVLVTRVEPEV-----ADL 103
Metco-CpmA   INRQFRTGVPEVVYGKGKDIDEIIKATLKLVEKNGIALATKIEDIEKLSDEIRK  95
Metba-CpmA   IFREKRTGFPEAVFAPGKSDEDIVNIVKAVG----DVLVTRLPPDRAE-GILKE  88
Arcog-CpmA   FERFERAGKPEAVFAEGKSVEDLVKIVKRFIEEGKSTLVTRLSRE------QIE  62

Synco-CpmA   LQAQLPELTYDSIARIAALQPQPPQPQP-GRLAVVCAGTSDLPVAEEAAVVAEL 145
Syncy-CpmA   LGDRIPLLQYYPQARICALVQTPLQIKYSGTIGVLSAGTADLPVAEEAAITASL 157
Metco-CpmA   WNLKNYDIKINKKAKTLIIKNKNYEVKKIGKVGILTAGTSDIPVAEEAKDTLEI 149
Metba-CpmA   LGDIDRRIEYHETAGVLAARSG--DPQFIGRIGLLSAGTSDIPVAEEARVVAEE 133
Arcog-CpmA   ALKSFEEVKINERGRIAIVGKP--VSAKAGKVAILTAGTSDIPVAEEAAVTAEF 114
Syncy-PurE                           MTSPSPLVGIIMGSDSDLPTMAAAIAVCEE  30
Metco-PurE                                 MICIIMGSESDLKIAEKAVNILKE  24
Mycba-PurE                            MTRQPRVGVIMGSDSDWSVMQDAAHALAE  29
Esche-PurE                          MSSRNNPARVAIVMGSKSDWATMQFAAEIFEI  32

Synco-CpmA   WGFRVDRIRDVGVAGLHRLLAE--RSRLDQAD----VLIVVAGMEGA--LPSVI 191
Syncy-CpmA   CGFKVEKLWDVGVAGIHRLLSH--RELIQAMD----VLIVVAGMEGA--LPSVV 203
Metco-CpmA   MGVEAITAYDVGIAGIHRLFPALKRMIEEDVC----CIIVVAGMEGA--LPSVI 197
Metba-CpmA    SGCEVINAYDVGVAGLHRLLDPLRRMHESGVK----ALIVVAGMEGA--LPSVV 188
Arcog-CpmA   LGLEVLRFYDVGVAGLHRIVEPVKRIREENVD----SAIVVAGMEGA--LPSVI 162
Syncy-PurE   FAVPTE----VAIISAHRTPER--MVEYAQTAHQRGLRIIIAGAGGAAHLPGMV  78
Metco-PurE   FGVEFE----VRVASAHRTPE-----LVEEIVKNSKADVFIAIAGLAAHLPGVV  69
Mycba-PurE   FDIPIE----VRVVSAHRTPAE--MFDYARNAVDRSIAVIIAGAGGAAHLPGMV  77
Esche-PurE   LNVPHH----VEVVSAHRTPDK--LFSFAESAEENGYQVIIAGAGGAAHLPGMI  80

Synco-CpmA   AGLVACPIIAVPTSVGYGASFQGLAALLSMLNSCAPGMGVVNIDNG----FGAA 241
Syncy-CpmA   AGLVDCPVIGVPTSVGYGTSFGGVAPLLTMLNSCAVGVGVVNIDNG----FGAA 253
Metco-CpmA   ASMVDIPVIGVPTSTSYGIK---ITPLLTMLHSCSPGIAVVNIDNG----FGAG 244
Metba-CpmA   AGLVDVPVIGVPTSVGYGVGEGGSVALKSMLQSCSPGIGVVNIDNG----FGAA 238
Arcog-CpmA   AGLVDVPVLAVPTSVGYGVNLGGITTLFAMLQSCSSGVAVVNIDNG----FGAA 212
Syncy-PurE   AALTPLPVIGVPVQT---KTLQGVDSLYSIVQMPG-GIPVATVAIG--NAKNAG 126
Metco-PurE   ASLTTKPVIAVPVDA---K-LDGLDALLSSVQMPP-GIPVATVGID--RGENAA 116
Mycba-PurE   ASATPLPVIGVPVPL---ARLDGLDSLLSIVQMPA-GVPVATVSIG--GARNAG 125
Esche-PurE   AAKTLVPVLGVPVQS---AALSGVDSLYSIVQMPR-GIPVGTLAIGKAGAANAA 130

Synco-CpmA   QLAGRILRRLHRLGRSDDQ                                  260
Syncy-CpmA   MLAGQIL                                              279
Metco-CpmA   VFAGLIAKIMHK                                         256
Metba-CpmA   VLAVKILRACSL                                         250
Arcog-CpmA   VFAALISRVKRKAAGED                                    229
Synco-PurE   LLAVQILASHNPVLLEKVQQYRQSLETMVLDKQAELERLGYRAYLDQQNQ   176
Metco-PurE   ILALEILALKDENIAKKLIEYREKMKKKVYASDEKVKEMFK            157
Mycba-PurE   LLAVRILGSSDLQLRAQLVAFQDRLADTVRAKDADLQRFRGKLIGD       171
Esche-PurE   LLAAQILATHDKELHQRLNDWRKAQTDEVLENPDPRGAA              169
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FIG. 3. Comparison of amino acid sequences including that deduced from the *cpmA* gene of *Synechococcus* sp. strain PCC 7942 (Synco-CpmA), putative *cpmA* homologs from *Synechocystis* sp. strain PCC 6803 (Syncy-CpmA), *M. jannaschii* (Metco-CpmA), *M. thermoautotrophicum* (Metba-CpmA), and *A. fulgidus* (Arcog-CpmA), and the sequences of PurE proteins of *Synechocystis* sp. strain PCC 6803 (Syncy-PurE), *M. jannaschii* (Metco-PurE), *Mycobacterium leprae* (Mycba-PurE), and *E. coli* (Esche-PurE). Residues identical among all CpmA (but not all PurE) sequences are boxed; those common to all aligned sequences are shown in boldface.

fully-segregated inactive *cpmA* gene (pseudoreversion) might be responsible for loss of the mutant phenotypes.

We confirmed the complete segregation of the *cpmA* mutation in AMC412(*cpmA*::Km) by genomic Southern hybridization with the 0.9-kb *Eco*RI fragment (*cpmA*) used as a probe (data not shown). We extracted genomic DNA after several subcultures on BG-11M–kanamycin plates and digested it with *Eco*RV. We detected a 4.5-kb band in the wild-type strain and a 6.5-kb band, larger than the wild-type band by the size of the Km^r gene cartridge, in the *cpmA* mutant. No wild-type band was detectable in the mutant (data not shown).

We also removed selective pressure to determine whether wild-type genomes, which could segregate in the absence of antibiotics, persisted, at a level not detectable by Southern analysis. We streaked AMC412(*cpmA*::Km) onto BG-11M plates that lacked antibiotics to obtain single colonies and then transferred 100 colonies to a plate containing BG-11M and kanamycin to assess antibiotic resistance. All colonies grew in the presence of kanamycin, indicating that the *cpmA*-disrupting insertion was intact and supporting the Southern analysis data that showed complete segregation of the mutant allele. However, all colonies had normal pigmentation and growth

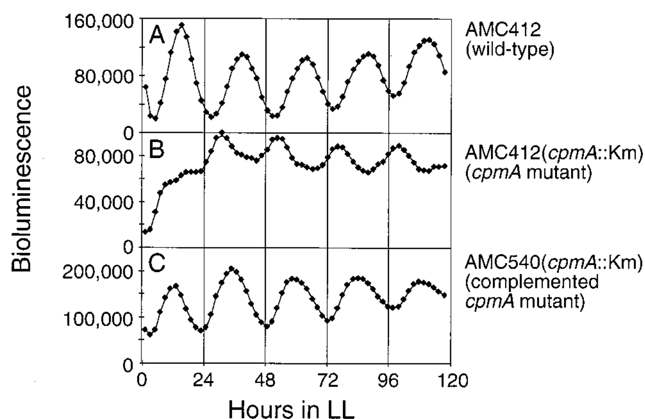

FIG. 4. Loss of *cpmA* function causes low amplitude and early phase angle in the *psbAI*::*luxAB* bioluminescence rhythm. Bioluminescence traces were obtained from AMC412, the wild-type strain (A), AMC412(*cpmA*::Km^r), the mutant in which the *cpmA* gene was inactivated by the replacement of an internal *Nru*I fragment with a Km^r gene cartridge (B), and AMC540(*cpmA*::Km), the *cpmA* mutant that carries an ectopic wild-type allele of *cpmA* in *trans* (C).

rate, and the circadian phenotypes of the six clones tested were wild type. Southern analysis of these clones again confirmed the absence of a wild-type *cpmA* locus (data not shown). Thus, the Km^r clones that show wild-type phenotypes are pseudorevertants rather than true revertants of the original mutation. We concluded that the gene is not essential for viability of *Synechococcus*, that *cpmA* disruption instead causes a severe growth defect, and that there is a strong selection for a second mutation that reverts the phenotype under all conditions that expose cells to high light intensity. Plating for single colonies, like suspension in liquid culture or incubation under strong light, seems to increase the amount of irradiation received by individual cells. The nature of the selective pressure is not yet obvious.

Effect of *cpmA* inactivation on the circadian expression rhythms of other genes. Most, if not all, genes in *Synechococcus* show a circadian rhythm of expression, and the majority of these rhythms are in the same phase, having a peak at subjective dusk and a trough at subjective dawn (class 1 rhythm) (23). Exceptionally, the expression rhythm of *purF*, which encodes a regulatory enzyme in the de novo purine synthetic pathway, glutamine phosphoribosyl pyrophosphate amidotransferase, shows a rhythm with an opposite phase (class 2) (22). We were interested in whether mutation of *cpmA* affects the expression rhythms of different genes in *Synechococcus* in the same way. We introduced the *cpmA* disruption into several reporter strains in which *luxAB* is driven by the following promoters: *psbAII* and *psbAIII*, which encode form II of the photosystem II reaction center D1 protein (10, 29); *purF*; *kaiA* and *kaiB*, which encode circadian clock component proteins (15); *ndhD*, which encodes subunit D of NADH dehydrogenase (34); *rpoD2*, which encodes a group 2 σ^{70}-like transcription factor that has been shown to influence the expression rhythm of *psbAI* (34); and the artificial promoter, *conII*, whose sequence matches the *E. coli* -35 and -10 elements (9).

Figure 5 shows traces of the bioluminescence rhythms from each reporter strain. The *cpmA* mutation changed the phase angles in *psbAII* and *kaiA* expression rhythms by about 10 h, as was seen for the *psbAI* expression rhythm (Fig. 4A and 5). The mutation had little or no effect on the phases of the expression rhythms of *psbAIII*, *purF*, *kaiB*, *ndhD*, *rpoD2*, and *conII* (Fig. 5). Compared with the effect on phase, the effect on amplitude was less clear. The *cpmA* mutation reproducibly lowered the expression rhythm amplitude for *psbAI*, *kaiA*, and *kaiB* and increased the amplitude for *purF* (Fig. 5). The effect of *cpmA* disruption on amplitude was not consistent for the expression rhythms of the other promoters.

The product of the *luxAB* genes, bacterial luciferase, requires reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde as substrates for bioluminescence (13). $FMNH_2$ is produced in the cyanobacterium by photosynthesis, and synthesis of the aldehyde is directed by the *luxCDE* genes which we introduced into the reporter strain at a neutral site independent of the *luxAB* reporter fusion (1). Therefore, it was considered possible that the phenotype observed in *cpmA* mutants was caused by an effect on the synthesis of the luciferase substrates. We tested whether the *cpmA* disruption caused the same phenotype when the *psbAI* expression rhythm was measured by a different reporter. Firefly luciferase is another real-time reporter that has a half-life sufficiently short for visualization of circadian troughs (25). Firefly luciferase, like bacterial luciferase, is an oxidase, but its other substrate requirements are different, being ATP and a molecule known as a luciferin (added exogenously). We introduced the *cpmA* disruption into strains in which the *psbAI* or *purF* promoter is fused to a promoterless firefly luciferase gene (*psbAI*::*luc* and *purF*::*luc*, respectively). The results for the *luc* reporter strains were consistent with those for *luxAB* reporter strains: early phase angle of the expression rhythm of *psbAI* and little or no effect on the phase angle of the rhythm of *purF* (data not shown). We concluded that the effect of *cpmA* inactivation is a genuine change in the phasing of *psbAI* expression rather than a modification of bioluminescence through alteration of substrate levels.

Overexpression of *cpmA*. An IPTG-inducible promoter was used to drive expression of an ectopic copy of *cpmA* in an otherwise wild-type *psbAI*::*luxAB* reporter strain. No alteration of the wild-type circadian bioluminescence pattern was observed in either the presence or absence of IPTG (data not shown).

DISCUSSION

We concluded that *cpmA* is involved in an output pathway of the circadian clock, rather than an input pathway or the central oscillator itself, from the following observations. Mutation of *cpmA* altered the phasing of the circadian rhythm for a subset of genes; it changed the phase angles of the *psbAI*, *psbAII*, and *kaiA* expression rhythms dramatically, but it had little effect on those of *psbAIII*, *purF*, *rpoD2*, *ndhD*, and *conII*. Theoretically, a change in the central oscillator should be reflected in all downstream behaviors. Also, *cpmA* inactivation did not affect the phase of the expression rhythm of the *kaiB* promoter, which directs a dicistronic message that encodes KaiB and KaiC. It has been suggested that KaiC functions as a central clock component in this organism because disruption of *kaiC* eliminates the circadian rhythm, missense mutations in *kaiC* change the periods of all gene expression rhythms in the cell, and pulsed overexpression of *kaiC* can shift the phase of the circadian rhythm (15).

It is surprising that the *cpmA* mutation dramatically changed the phase of the *kaiA* expression rhythm (phase angle difference of approximately 10 h) but had a very minor effect on the phase of the *kaiB* expression rhythm. Disruption of *kaiA* eliminates the circadian rhythm of *kaiBC* expression, and overexpression of *kaiA* leads to high-level and low-amplitude expression of *kaiBC*, suggesting that *kaiA* functions as an activator of the *kaiBC* promoter (15). Furthermore, some period-altering

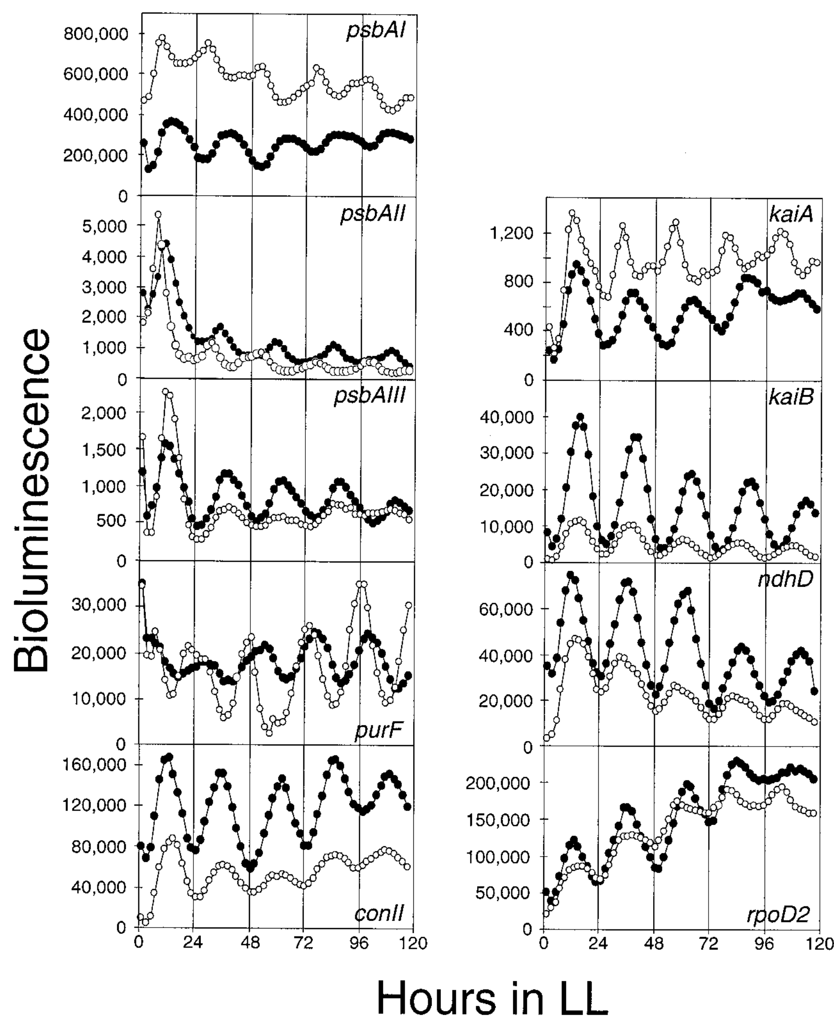

FIG. 5. Effect of *cpmA* inactivation on the expression rhythms of various reporter genes. Closed circles, wild-type traces; open circles, *cpmA* mutant traces. The *x* axis is labeled as for Fig. 1. The *y* axis values are counts per second. A representative trace is shown for each genotype, and the identity of the *Synechococcus* gene fused to *luxAB* is indicated for each panel. The fraction of independent experiments and the fraction of total independent colonies for which marked phase-change phenotypes (several hours' phase angle difference) were observed, respectively, follow in parentheses for each reporter strain: *psbAI*::*luxAB* (9/10; 37/48), *psbAII*::*luxAB* (6/10; 22/46), *psbAIII*::*luxAB* (1/6; 4/31), *purF*::*luxAB* (0/9; 0/41), *kaiA*::*luxAB* (8/9; 38/42), *kaiB*::*luxAB* (0/10; 0/47), *rpoD2*::*luxAB* (3/9; 12/31), *ndhD*::*luxAB* (1/9; 1/37), and *conII*::*luxAB* (2/7; 10/35) reporter fusions.

mutations map to *kaiA*, which is one criterion for its role as a central clock component; if it is such a component, the relative concentrations of KaiA and KaiC at different times during the circadian cycle should be important for sustaining the rhythm. In the *cpmA* mutant background, the *kaiA* and *kaiB* promoters are out of phase by 8 h or more. Our results indicate that synchronization of the phase of the circadian expression rhythms of *kaiA* and *kaiBC* may not be necessary for normal clock function.

It is not clear that the early-phase-angle and low-amplitude phenotypes are closely related. Changes in amplitude resulting from *cpmA* inactivation were less reproducible than the early-phase-angle phenotype. Both phenotypes were always observed in the *psbAI* reporter strain. However, the low-amplitude phenotype was not reproducibly observed in the *psbAII* and *kaiA* rhythms, even though their phasing was affected by the mutation. Furthermore, sometimes the low-amplitude phenotype was observed in the expression rhythms of *psbAIII*, *kaiB*, and *conII*, whose phases were not markedly affected by the mutation. For the *purF* rhythm, this mutation reliably increased the amplitude of the oscillation.

In this organism the expression of the *conII* promoter fragment, an entirely heterologous sequence that includes the consensus elements recognized by the *E. coli* σ^{70} subunit, shows circadian oscillation with class 1 phase and waveform. This result suggests that the class 1 rhythm is generated through the basic transcription machinery as an original mode of the circadian expression rhythm and that other components in specific output pathways modify this rhythm to generate the different waveforms represented by the different classes (23). Mutation of *cpmA* dramatically affected the phase relationships of a subset of class 1 gene expression rhythms, those of *psbAI*, *psbAII* and *kaiA*, but had only a modest effect on the phase angles of the *conII* expression rhythm or other class 1 rhythms, such as those of *psbAIII*, *kaiB*, *ndhD*, and *rpoD2*. This indicates that the category of class 1, identified as constituting up to 85% of the expression patterns of the genome, actually encompasses two or more subclasses that are controlled by

different output pathways. Mutation of *cpmA* did not affect the phase of the class 2 *purF* expression rhythm, which is opposite in phase to class 1. This confirms the separation of class 2 from class 1 as being controlled by independent output pathways. In all, our results indicate that *cpmA* functions in an output pathway that modulates the phasing (and amplitude) of a subset of class 1 rhythms.

Another *Synechococcus* gene, *rpoD2*, was previously identified as a component of an output pathway of the circadian clock (34). Disruption of *rpoD2* decreases the amplitude of the *psbAI* expression rhythm. The *rpoD2* sequence predicts a product that is a group 2 σ factor of RNA polymerase. Generally, cyanobacteria have, in addition to a group 1 σ factor which is essential for exponential growth, multiple group 2 sigmas that are dispensable but have extensive sequence similarity to the group 1 σ factor. Tsinoremas et al. suggested a model in which temporal incorporation of the RpoD2 sigma factor into RNA polymerase decreases the expression of *psbAI* during subjective night and generates the high-amplitude circadian oscillation of *psbAI* expression (34). Inactivation of *rpoD2* decreases the amplitude of the expression rhythm of *ndhD* as well as that of *psbAI* but does not affect the *purF* expression rhythm (34). The effect of the *cpmA* mutation was different in that it affected phase as well as amplitude of the *psbAI* rhythm and separated *ndhD* and *psbAI* into separate subclasses.

Constitutive high expression of *cpmA* did not affect the phase or amplitude of the *psbAI* expression rhythm. This result suggests that temporal oscillations in the amount of the *cpmA* product are not necessary to determine the correct amplitude and phase. This contrasts with the case of components that are involved in circadian output pathways in other organisms. The expression of the *Atgrp7* gene, encoding a glycine-rich RNA-binding protein of *Arabidopsis thaliana*, shows circadian oscillation in its mRNA and protein levels; overexpression of *At*-GRP7 greatly suppresses and eliminates the circadian oscillation of *Atgrp7* expression (14). In another example, the *lark* gene of *Drosophila melanogaster* encodes an RNA-binding protein that is thought to function as a negative repressor of the circadian eclosion rhythm (24). *lark*/+ heterozygotes, which have a decreased amount of Lark protein, have an early-eclosion phenotype, and an increase in gene dosage of *lark* causes a late-eclosion phenotype. In both cases the overexpression of the output component disrupts the normal circadian output pathway.

Database searches revealed that a cyanobacterium, *Synechocystis* sp. strain PCC 6803, and three species of *Archaea*, *M. janaschii*, *M. thermoautotrophicum*, and *A. fulgidus*, have a gene that is very similar to *cpmA*. It is interesting that all of these organisms have a putative *kaiC* homolog. Because KaiC may function as a central clock component in the circadian system of *Synechococcus*, it is possible that the *kaiC* genes in other prokaryotes also encode a clock component and that *cpmA* is a circadian output factor in those organisms as well. Because all of these organisms also carry *purE* homologs, we expect that CpmA will be found to function distinctly from PurE; however, it is likely to have a similar biochemical activity. The nature of this activity may be more apparent when the locus or loci that allow pseudoreversion of the *cpmA* phenotypes are identified.

Mutation of *cpmA* caused a severe growth defect in the cyanobacterium. One possible explanation is that its product is involved in basic cell metabolism. CpmA may have a carboxylase activity similar to that of PurE, such that its loss causes disruption of an unidentified metabolic pathway. Another possibility is that the detrimental effect on growth is a more direct result of a circadian role for CpmA because loss of this function causes a conflict among gene expression rhythms of different phases in this organism. In unicellular cyanobacteria some processes that are incompatible are known to be separated temporally. For example, in unicellular diazotrophic cyanobacteria, the oxygen-sensitive enzyme nitrogenase, which is irreversibly inactivated by exposure to oxygen, is expressed in the night, in phase opposite to those of expression rhythms of (oxygen-evolving) photosynthesis-related genes (12). In *Synechococcus* the expression rhythm of *purF*, which encodes the oxygen-sensitive enzyme glutamine phosphoribosyl pyrophosphate amidotransferase, is expressed in phase (class 2 phase) opposite to that of *psbAI*, which encodes the photosystem II reaction center D1 protein. Furthermore, bioluminescence patterns following random insertion of promoterless *luxAB* genes suggest that this organism has several genes expressed with a class 2 phase (23). It is possible that the activities of the enzymes which are specifically expressed in the night, like *purF*, are inhibited by the approach of the peak of a subset of class 1 rhythms, including the rhythms of the photosynthesis genes *psbAI* and *psbAII*, into their phase.

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