

## Roles for Sigma Factors in Global Circadian Regulation of the Cyanobacterial Genome

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**The circadian clock of the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 imposes a global rhythm of transcription on promoters throughout the genome. Inactivation of any of the four known group 2 sigma factor genes (*rpoD2*, *rpoD3*, *rpoD4*, and *sigC*), singly or pairwise, altered circadian expression from the *psbAI* promoter, changing amplitude, phase angle, waveform, or period. However, only the *rpoD2* mutation and the *rpoD3 rpoD4* and *rpoD2 rpoD3* double mutations affected expression from the *kaiB* promoter. A striking differential effect was a 2-h lengthening of the circadian period of expression from the promoter of *psbAI*, but not of those of *kaiB* or *purF*, when *sigC* was inactivated. The data show that separate timing circuits with different periods can coexist in a cell. Overexpression of *rpoD2*, *rpoD3*, *rpoD4*, or *sigC* also changed the period or abolished the rhythmicity of *PpsbAI* expression, consistent with a model in which sigma factors work as a consortium to convey circadian information to downstream genes.**

Cyanobacteria are the simplest organisms known to exhibit circadian rhythms of biological activities, including gene expression (13). These are endogenously generated oscillations that have a period of ~24 h under constant conditions and are robustly maintained in the absence of external timing cues (26). In the cyanobacterium *Synechococcus elongatus* PCC 7942, some components of the circadian clock have been identified by genetic analysis, with luciferase gene fusions as phenotypic reporters of circadian gene expression (10, 12, 15, 19, 29, 32). Genetic complementation of mutants with circadian periods between 14 and 60 h and of arrhythmic mutants with distorted waveforms (17) identified three novel genes, *kaiA*, *kaiB*, and *kaiC* (10). Disruption of any individual *kai* gene abolishes circadian rhythms, suggesting that these genes are central to the timing mechanism (10). Although putative *kai* orthologs exist in other groups of prokaryotes, the deduced amino acid sequences of Kai proteins have little similarity with proteins of known function from other organisms (10).

The specific functions of the Kai proteins are still cryptic, but some of their biochemical properties are known. KaiB and KaiC protein levels cycle robustly under constant conditions, whereas KaiA abundance shows little oscillation (33). The three Kai proteins form homo- and heteromultimeric complexes, and the physical interactions among them seem to be important for generation of circadian rhythms (11, 34). KaiC shows autophosphorylation and ATP-binding activities, which are also likely to be significant for circadian function (25).

In *S. elongatus*, the circadian clock globally regulates expression of the entire genome, although many promoters are expressed with a specific waveform or relative phasing that indicates specialization of circadian expression (20). Even an

artificial *Escherichia coli* consensus promoter, PconII (6), drives rhythmic expression of the luciferase (*luxAB*) genes in the cyanobacterium (32). This is contrary to observations from well-characterized eukaryotic circadian model organisms in which only a small percentage of genes are known to be under circadian control and specific *cis* elements confer rhythmicity (2, 8, 35). How temporal information is transduced from the central Kai oscillator globally to the ~3,000 genes in the *S. elongatus* genome is not known. Circadian control is so extensive in *S. elongatus* that it is almost inconceivable that each set of genes has its own dedicated transcription factor(s) responsible for its circadian expression. Instead, we propose that regulatory mechanisms that are more global are responsible for transducing temporal information to all downstream genes.

Previous work implicated the basic transcription machinery in circadian control. Inactivation of *rpoD2*, an *S. elongatus* group 2 sigma factor gene, results in circadian rhythms with low amplitude, albeit high expression levels, from a subset of reporter genes including *psbAI* (32). This observation placed *rpoD2* as a component of an output pathway from the circadian oscillator that affects transmission of temporal information to some genes (32). Group 2 comprises sigma factors that are sigma 70-like in sequence but not essential for growth (23). Cyanobacteria are unusual in having several closely related group 2 sigma factors in addition to the essential, principal sigma, RpoD1 (30). Genetic and biochemical evidence suggests that the holoenzyme comprising different group 2 sigma factors can recognize *S. elongatus* promoters (7, 24).

Two models were proposed to explain the limited effect of *rpoD2* inactivation on the amplitude of oscillation from some genes. In one, RpoD2 is necessary for transcribing a gene that encodes a factor responsible for generating the circadian trough (e.g., a time-dependent repressor of *psbAI* expression). In another, the loss of RpoD2 allows access of other sigma factors to the *psbAI* promoter by relieving competition.

In order to distinguish between these possibilities, we disrupted other known group 2 sigma factor genes, *rpoD3*, *rpoD4*, and *sigC*, to see whether their loss would cause a circadian

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defect as predicted by a sigma competition model. We found that removing any one of the four group 2 sigma factors of *S. elongatus* affects circadian properties of a subset of reporter genes, suggesting that the combinatorial action of sigma factors contributes to wild-type circadian rhythmicity. All pairwise inactivations of these sigma factor genes also affected the circadian properties of the *psbAI* reporter, but only two combinations had a notable effect on the expression of *PkaiB* and *PpurF*. Taken together, the data indicate that these sigma factors, although structurally similar and partially overlapping in function, are not entirely redundant. Circadian alterations accompanied overexpression of each of four group 2 sigma factors, consistent with a model in which rhythmicity of gene expression derives from temporal modulation of the holoenzyme pool. Disruption of one sigma factor gene, *sigC*, showed a lengthening of the period of expression from the *psbAI* promoter by about 2 h but did not affect that from the *kaiB* and *purF* promoters to the same extent. These data indicate that it is possible for two or more oscillations of different periodicities to coexist in *S. elongatus*. Immunoblot analysis revealed oscillation of RpoD4 in protein levels during the circadian cycle.

#### MATERIALS AND METHODS

**Inactivation of sigma factor genes.** Inactivation of each of the four sigma factor genes has been described previously for a *PpsbAI* reporter strain (24). Single or pairwise inactivations were made in the reporter strains AMC393 (−185 to +224 of *PpsbAI* fused to the *luxAB* reporter integrated in neutral site II [NS2] and *psbAI::luxCDE* in neutral site I [NS1]; Gm<sup>r</sup> cassette in *sigC*), AMC669 (−185 to +224 of *PpsbAI::luxAB* and *PpsbAI::luxCDE* integrated in NS2; Km<sup>r</sup> cassette in *rpoD2*, *rpoD3*, or *rpoD4*), AMC777 (−54 to +43 of *PpsbAI* fused to *luxAB* integrated in NS2 and *PpsbAI::luxCDE* in NS1; used for pairwise sigma factor inactivation effect on *PpsbAI*), AMC462 (*PkaiB::luxAB* in NS2 and *PpsbAI::luxCDE* in NS1), and AMC408 (*PpurF::luxAB* in NS2 and *PpsbAI::luxCDE* in NS1). Transformants were selected on BG-11 M agar with kanamycin (20 μg/ml) and/or gentamicin (2 μg/ml), as appropriate.

**Bioluminescence assays.** All strains were derived from *S. elongatus* PCC 7942 and carry, in addition to the *luxAB* gene set, a *PpsbAI::luxCDE* construct that directs synthesis of the long-chain aldehyde substrate for luciferase in vivo, making the reporter strains autonomously bioluminescent. Cultures grown on BG-11 M agar (3) were inoculated onto BG-11 M agar in 96-well microtiter plates. Appropriate antibiotics were included in the agar. The microtiter plates with samples were incubated in constant light (LL) conditions for 6 h and then subjected to a 12-h entraining dark incubation to synchronize the clocks of all cells in the population (15). Bioluminescence rhythms were assayed from luciferase reporter strains in at least two independent experiments on a Packard TopCount luminometer (1, 15). Because light intensity has a minor effect on circadian period (less than 1 h) and because absolute expression levels (but not circadian parameters) vary with cell number, all mutant and control analyses reported here were calculated from samples carefully paired on the monitoring device for equivalent illumination from samples grown in parallel under identical conditions. Note that some cycle-to-cycle variations in amplitude and expression levels are normal. Data were imported into Microsoft Excel 2000 for plotting. Phase, amplitude, and period were calculated by using the Fourier analysis software, FFT-NLLS, version 98.5, from the Import & Analysis software package (M. Straume, National Science Foundation [NSF] Center for Biological Timing, University of Virginia).

**Overexpression of sigma factor genes.** Each sigma factor gene was amplified from *S. elongatus* PCC 7942 chromosomal DNA by PCR with Pwo polymerase (Roche Molecular Biochemicals, Indianapolis, Ind.) and primers that incorporated restriction enzyme sites for cloning into the cyanobacterial overexpression vector p322*Prc* (19). Each sigma factor coding region was cloned directly in frame with the ATG of the vector at the *NcoI* site. Primer pairs and resulting restriction sites for each gene were as follows: *rpoD2*, 5'-CTCTCTCATGAGT ACCTCTCCGC-3' (*BspHI*; compatible with *NcoI* site in the vector) and 5'-TCTCGGATCCAACCTAGCTAGCTGCG-3' (*BamHI*); *rpoD3*, 5'-TCATGCCA TGGCCAAAACCTGAAACCCC-3' (*NcoI*) and 5'-TCTCGGATCCACACTAG CTAGCCAAG-3' (*BamHI*); *rpoD4*, 5'-CTCTCTCACTCTCTCATGAACGTA

CAGGAACGC-3' (*BspHI*) and 5'-CGATCGAGCTCAGCCTAGCAAGCCTC TGG-3' (*SacI*); *sigC*, 5'-CTCTCTCATGACGCTGCC-3' (*BspHI*) and 5'-CG ATCGAGTCCGACTAGTGCACCAACCC-3' (*SacI*).

Fusions of *Prc* to *rpoD3*, *rpoD4*, and *sigC* were then cloned as *BglII* fragments into the NS1 vector pAM1303 to incorporate the overexpression constructs into the cyanobacterial genome (18). A 4-kb *BstZ171-NgoMIV* fragment bearing the *rpoD2* coding region fused to the *Prc* promoter was cloned into the NS1 vector pAM2314 cut with *XmaI* and *StuI*. The nucleotide sequence of each construct was confirmed by using the cycle sequencing method (dye terminator cycle sequencing ready reaction, ABI PRISM; PE Applied Biosystems, Foster City, Calif.). AMC669 was transformed with each overexpression construct, and clones were selected on BG-11 M plates with spectinomycin (20 μg/ml). Bioluminescence from overexpression strains was monitored after inducing expression of the sigma factors by adding 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and subjecting the cells to a 12-h dark pulse to synchronize the clock.

**Complementation of the *sigC*, *rpoD3*, and *rpoD4* mutations.** *sigC*, *rpoD3*, and *rpoD4* were inactivated in AMC669, a *PpsbAI::luxAB* reporter strain. The resultant strains bearing *rpoD3* and *rpoD4* mutations were transformed with the *rpoD3* and *rpoD4* overexpression constructs, respectively, with the ectopic copies of the sigma factor genes in NS1 (4). Bioluminescence rhythms were measured in the absence of IPTG; the *Prc* promoter allows low-level constitutive expression of genes in *S. elongatus*. A 3.1-kb *BamHI* fragment from pAM2330, bearing the *sigC* gene, was cloned into pAM1303 to create pAM2337. This construct was used to transform a *PpsbAI::luxAB* strain in which *sigC* was disrupted.

**Preparation of protein extracts and immunoblots to detect overexpression of RpoD3 and RpoD4.** Cultures (100 ml) of wild-type and overexpression strains of RpoD3 and RpoD4 were grown to an optical density of ~0.7 at 750 nm in shaking flasks in CO<sub>2</sub>-enriched air (1%) at a light intensity of 150 microeinsteins m<sup>-2</sup> s<sup>-1</sup>. The cultures were divided, and 2 mM IPTG was added to one half of each culture to induce the expression of the respective sigma factor. The flasks were incubated for an additional 12 h, then cells were collected by centrifugation, washed with 10 mM sodium phosphate buffer, pH 7.0, and resuspended in the same buffer to give a final volume of 2 ml. The cell suspension was passed twice through a prechilled French pressure cell at 137.9 MPa (20,000 lb/in<sup>2</sup>). Fifteen micrograms of the protein extract from the uninduced sample and 7.5 and 15 μg of protein from the induced sample were suspended in a β-mercaptoethanol-containing denaturing buffer system, boiled for 5 min, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (BA85S; Schleicher and Schuell) as previously described (22). Proteins were immunostained with anti-RpoD3 and anti-RpoD4 antisera at a dilution of 1:15,000 and detected by the ECL kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Antisera raised against RpoD2, RpoD3, and RpoD4, expressed in *E. coli*, were provided by Kan Tanaka, University of Tokyo. To determine the degree to which each cross-reacts with other sigma factors, we compared immunostains of extracts prepared from wild-type and specific *rpoD*-inactivated and *rpoD*-overexpressing *S. elongatus* strains. The results indicated that the anti-RpoD4 serum recognizes itself and overexpressed RpoD3; however, the amount of RpoD3 in wild-type cells was too low to be detected by anti-RpoD3 antiserum. We concluded that anti-RpoD4 primarily recognizes RpoD4 in wild-type cells.

**Immunoblots to detect cycling of RpoD4.** Cells were grown in culture tubes suspended in a 30°C water-filled aquarium under aeration (1% CO<sub>2</sub>) in LL conditions of 200 microeinsteins m<sup>-2</sup> s<sup>-1</sup> (28). Cultures were exposed to two light-dark pulses of 12 h each to synchronize the circadian clock and then returned to LL conditions. Samples corresponding to 2 ml at an optical density at 750 nm of 0.55 were collected at 4-h intervals and frozen at −20°C. Samples were resuspended in a β-mercaptoethanol-containing denaturing buffer and boiled for 5 min in a water bath. Ten microliters of the protein sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis as described above. The anti-RpoD4 antiserum was used at a dilution of 1:15,000. Posttransfer gels were stained with Coomassie blue to verify even loading of total protein.

**Monitoring *rpoD2*, *rpoD3*, and *sigC* gene expression as bioluminescence from a luciferase reporter.** A 1-kb *SmaI*-to-*PstI* fragment carrying the upstream region of *rpoD3* recovered from pAM1309 was blunt ended with T4 DNA polymerase and cloned into pAM1580 digested with *StuI*. The upstream region of *sigC* (1.2-kb *EcoRV*-*SalI* fragment) from pAM2330 was cloned into pAM1580 cut with *SmaI* and *SalI*. Strain AMC395, carrying *psbAI*-driven *luxCDE* genes to provide an aldehyde substrate for luciferase and a spectinomycin-streptomycin resistance marker in NS1, was transformed with the pAM1580 derivatives. The *PpoD2::luxAB* fusion was reported previously (15). Bioluminescence from these strains was monitored as described above. No stable promoter fragment clone was obtained from the upstream region of *rpoD4* (data not shown).

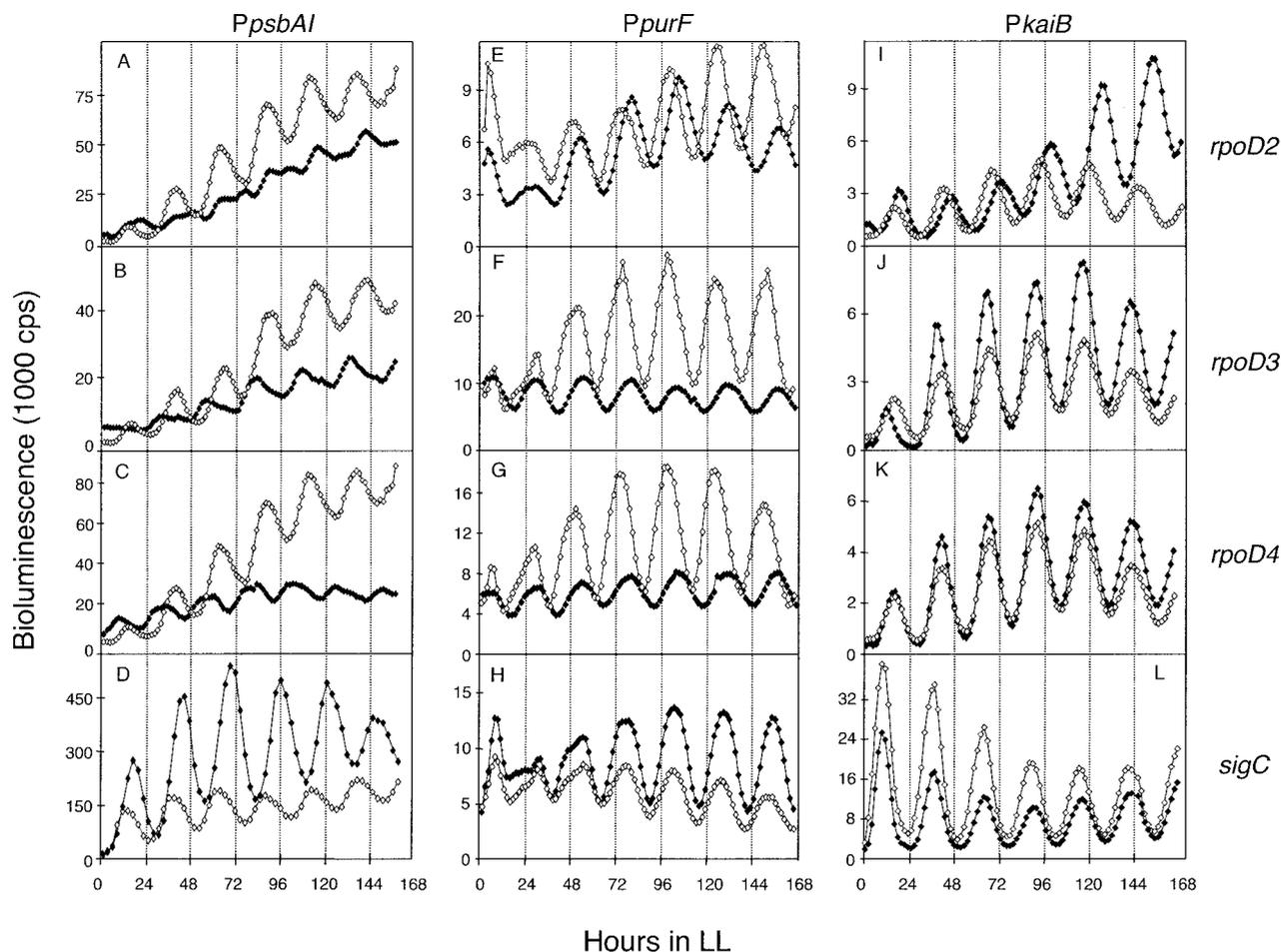


FIG. 1. Circadian expression rhythms of *PpsbAI::luxAB*, *PpurF::luxAB*, and *PkaiB::luxAB* reporter strains in which the *rpoD2*, *rpoD3*, *rpoD4*, or *sigC* gene has been inactivated. Bioluminescence traces are shown from wild-type (open diamonds) and sigma-inactivated mutant (closed diamonds) strains that carry the reporter gene shown above each column of panels. The gene inactivated in each reporter strain is indicated at the end of each row. The x axis shows time in hours after the cells were released into LL. The y axis indicates bioluminescence (in units of 1,000 cps). Sigma factor gene inactivations are: *rpoD2* (A, E, and I), *rpoD3* (B, F, and J), *rpoD4* (C, G, and K), and *sigC* (D, H, and L). Traces are representative of bioluminescence measured from at least three replicates of four independent transformants for each sigma factor mutant. Note that the wild-type *psbAI::luxAB* reporter strain used for inactivation of *sigC* in panel D is of a different genetic background than that used for inactivation of the other sigma factor genes in panels A, B, and C (see Materials and Methods). The overall expression level is higher for the AMC393 reporter than for AMC669, but the circadian properties of the strains are equivalent.

## RESULTS

**Differential effects of inactivating group 2 sigma factor genes on circadian expression of genes in different output pathways.** We inactivated each of the four group 2 sigma factor genes, *rpoD2*, *rpoD3*, *rpoD4*, and *sigC*, singly in a *PpsbAI::luxAB* reporter strain and monitored bioluminescence levels in LL after an entraining 12-h dark pulse. As reported earlier by screening with a cooled charge-coupled device camera and uniform sample illumination, the disruption of *rpoD2* decreased the peak-to-trough amplitude of the circadian expression rhythm from *PpsbAI::luxAB* (32).

However, in our present screening device, the low-amplitude phenotype could not be assigned to loss of trough and was conditional, depending on light intensity reaching the sample, i.e., evident at 230 microeinsteins  $\text{m}^{-2} \text{s}^{-1}$  (Fig. 1A) but not at 50 microeinsteins  $\text{m}^{-2} \text{s}^{-1}$  (data not shown). An approximately 50% decrease in amplitude also resulted from the loss of *rpoD3*

( $5.3 \times 10^3 \pm 0.8 \times 10^3$  versus  $8.2 \times 10^3 \pm 0.5 \times 10^3$ ,  $n = 12$ ) or *rpoD4* ( $4.5 \times 10^3 \pm 1.9 \times 10^2$  versus  $8.2 \times 10^3 \pm 0.5 \times 10^3$ ,  $n = 12$ ). The phasing of the peaks was advanced by  $\sim 4$  h in both the *rpoD3* and *rpoD4* mutant backgrounds relative to that of the wild-type strain (Fig. 1B and C, respectively). Inactivation of *sigC* caused a lengthening of the *PpsbAI::luxAB* expression rhythm by about 2 h ( $27.2 \pm 0.4$  h versus  $25.1 \pm 0.3$  h,  $n = 20$ ) and consistently increased overall bioluminescence and amplitude of the oscillation (Fig. 1D). Genetic complementation confirmed that the observed phenotypes resulted from the loss of the given sigma factor gene rather than from polar effects on adjacent loci (32) (also data not shown).

Thus, disruption of any of the group 2 sigma factors affected the circadian expression of the *psbAI* promoter. This is more consistent with a model in which RpoD2 depletion allows access to the *psbAI* promoter by an RNA polymerase associated with other sigma factors than with one based on RpoD2-

dependent expression of a specific circadian regulatory factor. The sigma factor inactivations did not identically affect the *psbAI* promoter, indicating that the activities of the sigma factors are not entirely redundant.

We chose the *PpurF::luxAB* and *PkaiB::luxAB* reporter strains as representative of classes of promoters different from *PpsbAI* (15) to determine whether the loss of each of the group 2 sigma factors also affected their rhythmic expression. The circadian expression pattern of *PpurF* is nearly opposite that of *PpsbAI*, i.e., its expression peaks at subjective dawn (circadian times [CT] of 24 and 48 h, etc., where 1 CT h = 1/24 of one circadian cycle) (20). The *kaiB* promoter drives very high amplitude expression of *kaiB* and *kaiC*, which are central clock genes in *S. elongatus* (10). Mutations in other genes have been shown to affect the circadian expression of *PpsbAI* but not of *PpurF* or *PkaiB*, suggesting that these genes are in different output pathways (15, 32).

As shown in Fig. 1E, and as reported previously, disruption of *rpoD2* had no effect on the amplitude of *PpurF::luxAB* expression (32). However, we observed an increase in period length by about 1 h ( $26.6 \pm 0.5$  h versus  $24.8 \pm 0.3$  h,  $n = 20$ ), which was not detected previously (32). The circadian oscillation of *PkaiB::luxAB* expression was also lengthened by about 1 h ( $26.4 \pm 0.5$  h versus  $25.1 \pm 0.3$  h,  $n = 20$ ), with no change in amplitude (Fig. 1I). The period lengthening is, by eye, also possible to detect for *PpsbAI*, but the low amplitude of that oscillation impaired calculation of the period value (Fig. 1A). We assume that this reflects a small fundamental change in the period of the central oscillator in the *rpoD2* mutant.

Inactivation of other sigma factor genes affected additional circadian properties of *PpurF*. The disruption of *rpoD3* or *rpoD4* changed the phase angle (relative timing of the peaks), amplitude, and expression level of a *PpurF::luxAB* reporter strain (Fig. 1F and G). Either mutation consistently delayed the peaks of *PpurF::luxAB* bioluminescence by about 3 h and decreased the amplitude of expression to one half and the expression level itself to one third of those in the wild type. The same mutations had no significant effect on the bioluminescence rhythms of a *PkaiB::luxAB* reporter strain (Fig. 1J and K).

The *sigC* null mutant, which consistently lengthened the period of *psbAI* expression by 2 h (Fig. 1D), had little or no effect on the period of expression from the *PpurF::luxAB* ( $25.2 \pm 0.2$  h versus  $24.8 \pm 0.3$  h,  $n = 12$ ) and *PkaiB::luxAB* ( $24.9 \pm 0.3$  h versus  $25.1 \pm 0.1$  h,  $n = 12$ ) reporter strains (Fig. 1H and L, respectively). In some experiments, *sigC* inactivation increased *PpurF::luxAB* and *PkaiB::luxAB* periods  $\sim 0.8$  h over the periods of the respective wild-type reporter strains but never to the extent seen in the *PpsbAI* reporter. The loss of *sigC* extended the period of expression from *PkaiA*, *PpsbAIII*, and *PconII* similarly to that from *PpsbAI* (data not shown).

The individual inactivation of the four group 2 sigma factors dissimilarly affected the circadian expression patterns of different reporter genes, supporting the assignment of these genes to different output pathways from the circadian clock. However, none of the sigma factors was genuinely essential for expression of any of the tested genes or for circadian rhythmicity.

**Pairwise inactivation of sigma factor genes affects a broader spectrum of gene expression rhythms.** We assessed the effects of pairwise inactivation of group 2 sigma factors on the *PpsbAI*, *PpurF*, and *PkaiB* expression patterns in order to determine whether a larger subset of genes would be affected by their loss. All six combinations of sigma factor mutations affected the phase, amplitude, or expression from *PpsbAI* (Fig. 2D and data not shown). However, only two combinations had an effect on either *PpurF* or *PkaiB*. The *rpoD2 rpoD3* double mutation lengthened, and the *rpoD3 rpoD4* double mutation shortened, the circadian periods from all three reporter strains by  $\sim 1.5$  h (Fig. 2). The *rpoD2 sigC* double mutation led to a  $\sim 1.5$ -h-long period of *PpsbAI::luxAB* expression but had no significant effect on the periods of expression of the *PpurF::luxAB* and *PkaiB::luxAB* reporters, confirming the ability of mutations to differentially affect the periods of different reporters (Fig. 2D).

The absence of effect on *PkaiB* when either *rpoD3* or *rpoD4* is missing (Fig. 1J and K), but mutant phenotype when both are lost (Fig. 2C and D), suggests that these sigma factors redundantly recognize *PkaiB*. We propose that this short period effect on *PkaiB*, which drives expression of the essential clock components KaiB and KaiC, cascades to the other promoters *PpsbAI* and *PpurF* via a change in the central oscillator period.

**Effects of sigma factor overexpression on *psbAI* expression rhythms.** If sigma factors compete for association with the core RNA polymerase, and if different forms of holoenzyme can recognize *S. elongatus* promoters (7), we would predict that disrupting the normal pool by overabundance of any one sigma factor would result in abnormal rhythmicity of gene expression. We placed the coding region of each of the four group 2 sigma factor genes under transcriptional control of the IPTG-inducible *trc* promoter and assayed the effects of overexpression on *PpsbAI::luxAB* expression. As shown in Fig. 3A, B, and D, the period of *PpsbAI* expression rhythm increased by approximately 2 h with overexpression of SigC ( $26.8 \pm 0.3$  h versus  $24.8 \pm 0.3$  h,  $n = 6$ ), RpoD2 ( $26.7 \pm 0.3$  h versus  $24.4 \pm 0.3$  h,  $n = 6$ ), or RpoD4 ( $26.9 \pm 0.4$  h versus  $24.8 \pm 0.3$  h,  $n = 6$ ). Overexpression of RpoD3 resulted in arrhythmic expression of *PpsbAI::luxAB* (Fig. 3C). Immunoblot analysis confirmed that RpoD3 and RpoD4 (for which antisera were available) were overexpressed after administration of IPTG, reaching eight- and fourfold, respectively, over their corresponding uninduced levels (data not shown).

We wanted to determine whether arrhythmic expression of *PpsbAI* when RpoD3 is overexpressed represented a complete cessation of Kai clock function itself. Many mutations, including the sigma factor inactivations reported here, dramatically alter *PpsbAI* oscillation while leaving *PkaiB* expression unaffected. Therefore, we assayed a *PkaiB::luxAB* strain in which RpoD3 was overexpressed. IPTG induction reduced the magnitude of expression by a factor of 10 and decreased the amplitude of oscillation, but the expression was still notably rhythmic (Fig. 3E). Thus, overexpression of RpoD3 does not stop the clock altogether.

**Expression patterns of *rpoD2*, *rpoD3*, and *sigC*.** We constructed transcriptional fusions between promoterless *luxAB* and upstream regions of *rpoD2*, *rpoD3*, and *sigC* to determine the relative phasing and expression patterns of the sigma factor genes themselves (15). Attempts to produce a similar reporter

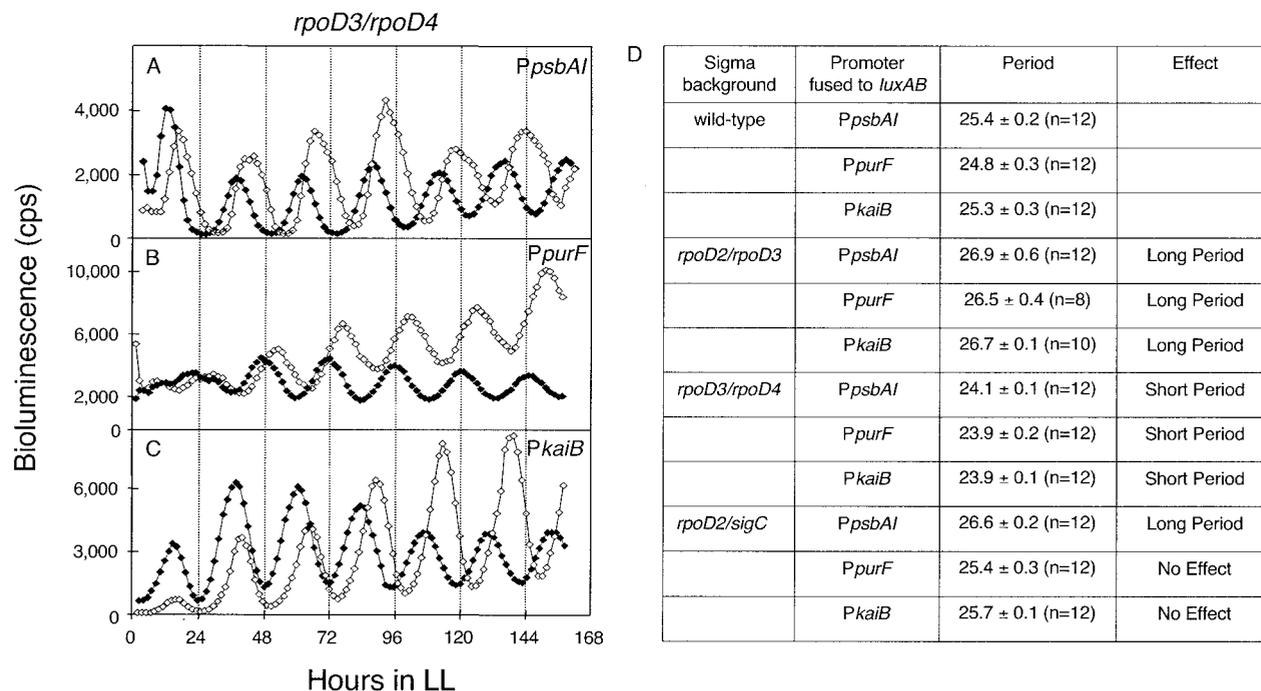


FIG. 2. Effects of pairwise inactivations of sigma factors on period lengths of *PpsbAI*, *PpurF*, and *PkaiB* expression rhythms. Effect of the *rpoD3 rpoD4* double null mutation on *PpsbAI::luxAB* (A), *PpurF::luxAB* (B), and *PkaiB::luxAB* (C) bioluminescence rhythms. Open diamonds and closed diamonds represent wild-type and mutant traces, respectively. The *x* and *y* axes are labeled as described for Fig. 1 except that the units for bioluminescence are counts per second. Traces are representative of bioluminescence measured from at least three replicates of four independent transformants. (D) Effects of pairwise inactivations of *rpoD2-rpoD3*, *rpoD3-rpoD4*, and *rpoD2-sigC* on the periods of *PpsbAI::luxAB*, *PpurF::luxAB*, and *PkaiB::luxAB* expression rhythms.

for *rpoD4* produced only unstable clones, so expression of that gene could not be assayed.

All three sigma factor promoters were strongly expressed with peaks of reporter activity at subjective dusk (class 1 phase) like most genes in *S. elongatus* (Fig. 4A). The amplitude of the *PsigC::luxAB* reporter is one order of magnitude higher than that of *PrpoD2::luxAB* or *PrpoD3::luxAB*. No difference in relative phasing of the rhythms was observed. However, the reporter cannot identify differences in transcription, mRNA stability, protein accumulation, or properties of proteins such as activity or ability to bind to the RNA polymerase core, any of which may vary over CT.

To assess the effect of the loss of each sigma factor on its own gene's oscillation, we inactivated *rpoD2*, *rpoD3*, and *sigC* genes in their respective reporter fusion backgrounds. The inactivation of *rpoD2* lead to an ~4-fold-elevated expression from an *rpoD2::luxAB* fusion (Fig. 4C). The period of *PrpoD2::luxAB* expression was not affected by the loss of *rpoD2*, whereas *PpurF::luxAB* and *PkaiB::luxAB* rhythms were extended by ~1 h (Fig. 1E and I). We concluded that *rpoD2* negatively regulates its own expression, either directly or indirectly. The inactivation of *rpoD3* (data not shown) and *sigC* (Fig. 4B) had no effect on their own oscillations. We propose that while *rpoD3* and *sigC* are involved in the recognition of promoters like *PpsbAI*, their own promoters may be recognized solely by RNA polymerase complexed with the principal sigma factor RpoD1.

**Cycling of RpoD4 protein under constant conditions.** Because the transcriptional luciferase reporter fusions do not report posttranscriptional events for the genes under study, we wanted to determine whether sigma factors themselves accumulate in a rhythmic manner. Although the promoter activities of all genes tested in *S. elongatus* are rhythmic when fused with the luciferase reporter, and mRNA abundances for *psbAI*, *psbAII*, *purF*, *kaiA*, and *kaiBC* oscillate in a circadian fashion, only the clock proteins KaiB and KaiC have been demonstrated to oscillate at the protein level under LL conditions (10, 12, 16, 33). An antiserum raised against RpoD4 (a gift from K. Tanaka, University of Tokyo) was sufficiently specific to track the abundance of that protein over a CT scale.

Immunoblot analysis showed that the RpoD4 protein level oscillates as a function of time, with peaks near 28 and 52 h in LL conditions (Fig. 5). The timing of these peaks is almost opposite to those of the bioluminescence patterns from *rpoD2::luxAB*, *rpoD3::luxAB*, and *rpoD2::luxAB*. However, we do not know whether this reflects a difference in the timing of expression of the *rpoD4* and the other sigma factor genes or a difference between peaks of transcription and protein accumulation.

## DISCUSSION

The mechanism by which temporal information is transduced to downstream genes in *S. elongatus* poses a dilemma

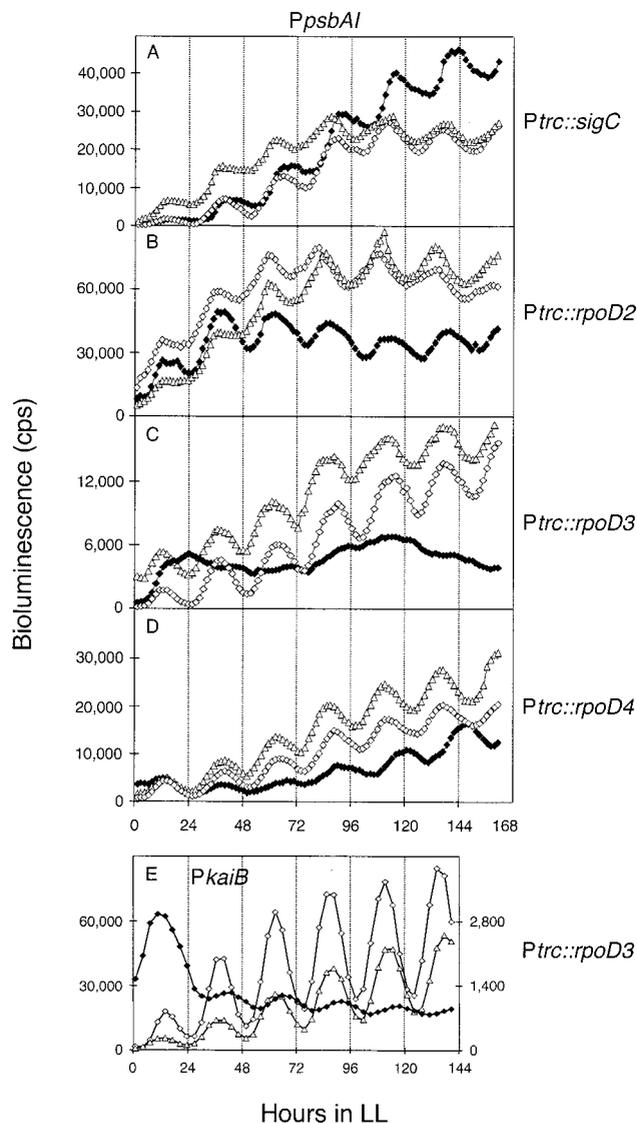


FIG. 3. Differential effects of sigma factor overexpression. The overexpression of *sigC* (A), *rpoD2* (B), *rpoD3* (C), or *rpoD4* (D) on rhythms from a *PpsbAI::luxAB* and *rpoD3* (E) on a *PkaiB::luxAB* reporter strain. Overexpression was induced in bioluminescent reporter strains by the addition of 2 mM IPTG immediately before the entraining 12-h dark pulse. Open triangles, bioluminescence rhythms from wild-type reporter strains; open and closed diamonds, rhythms from reporter strains bearing sigma factor overexpression constructs in the absence and presence, respectively, of the inducer. The gene overexpressed from the *trc* promoter is indicated at the end of each row. The *x* and *y* axes are the same as in Fig. 2. Traces are representative of bioluminescence measured from at least six independent samples for each sigma factor overexpression.

because circadian control is not limited to a small subset of genes; rather, it pervades expression of the entire genome (20). Although the transcriptional level is not the only level at which gene expression may be regulated by the clock, it is the control point most readily assayed via luciferase reporter genes in which the cyanobacterial promoter is the only variable among tested loci. A large body of reporter data showing rhythmic bioluminescence from defined promoters and from randomly

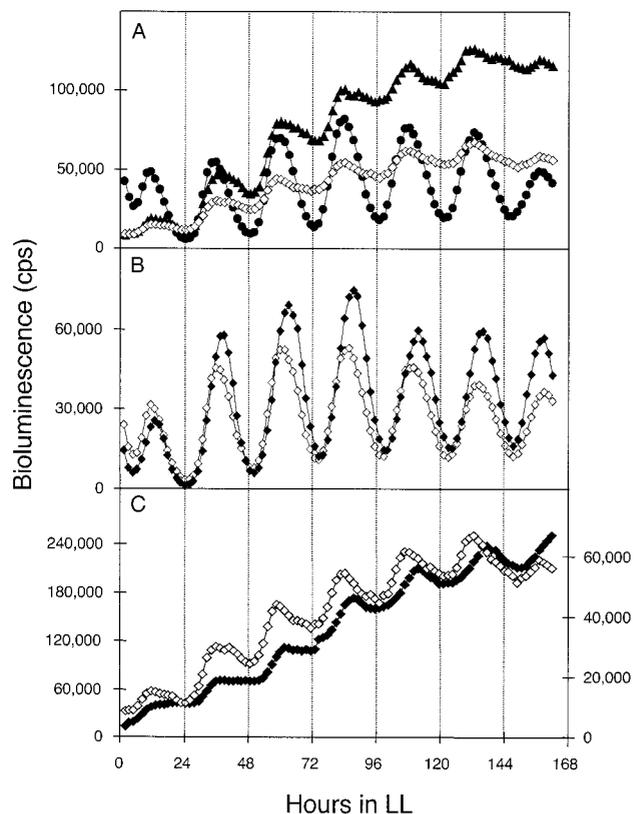


FIG. 4. Expression patterns of *rpoD2*, *rpoD3*, and *sigC* and the effect of the loss of *sigC* and *rpoD2* on their respective oscillations. (A) The upstream regions of *rpoD2*, *rpoD3*, and *sigC* were fused to *luxAB*, and the expression patterns of these sigma factor genes were monitored. Closed triangles, *PrpoD3::luxAB*; closed circles, *PsigC::luxAB*; open diamonds, *PrpoD2::luxAB*. (B) Inactivation of *sigC* in a *PsigC::luxAB* reporter strain. Open diamonds, wild-type *PsigC::luxAB* reporter strain; closed diamonds, *sigC* mutant *PsigC::luxAB* reporter strain. (C) Inactivation of *rpoD2* in a *PrpoD2::luxAB* reporter strain. Open diamonds, wild-type *PrpoD2::luxAB* reporter strain, with *y* axis scale at right; closed diamonds, *PrpoD2::luxAB rpoD2* mutant trace, with *y* axis scale at left. The *x* and *y* axes are the same as for Fig. 2. Traces are representative of bioluminescence measured from at least three replicates of four independent transformants.

sampled genomic segments suggests that the fundamental transcription machinery (RNA polymerase [RNAP] core plus sigma factors) is important in maintaining wild-type circadian rhythmicity.

Our working model for the global regulation of gene expression by the circadian clock is that the transcription apparatus oscillates in a circadian manner, in phase with the class 1 (peak at dusk) genes (20). There may be accessory (transcription) factors that modulate specific circadian properties, e.g., the opposite-phase expression of *PpurF::luxAB* or the high-amplitude expression of *PkaiB::luxAB*. We propose that changes in the pool of sigma species during the circadian cycle are at least partially responsible for global circadian expression.

We found that removal of any single group 2 sigma factor modifies expression from the *psbAI* gene of *S. elongatus* and that different aspects of the circadian rhythm can be affected: the circadian period, the amplitude of the oscillation, and the

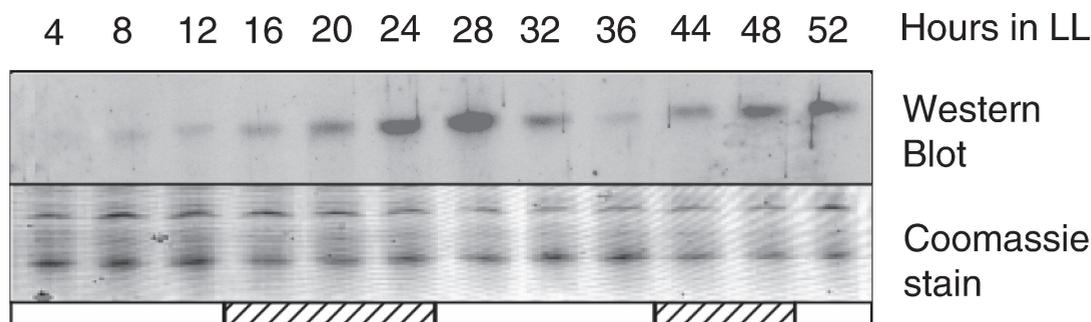


FIG. 5. Rhythmic abundance of RpoD4 during the circadian cycle. An entrained wild-type strain was sampled at 4-h intervals between hours 4 and 52 in LL ( $200 \text{ microeinsteins m}^{-2} \text{ s}^{-1}$ ) and examined by immunoblot analysis with an RpoD4 antiserum (one sample was lost at 40 h). The posttransfer gel was stained with Coomassie blue to verify equal loading of protein samples. White and hatched bars indicate time points corresponding to subjective day and night, respectively.

relative phasing of the peaks. These data suggest an important role for sigma factors in the circadian regulation of gene expression. Because all individual and pairwise sigma factor gene inactivations affected *PpsbAI* expression, we expect that the *psbAI* promoter is recognized by all four group 2 sigma factors. This is consistent with previously reported genetic data for overall expression in sigma mutant backgrounds (24) and with biochemical evidence that the holoenzymes reconstituted with RpoD1, RpoD3, or RpoD4 all recognized a number of *S. elongatus* and *E. coli* promoters, albeit with different preferences (7).

Our data are most consistent with a model in which different sigma factors compete for association with the core RNAP, such that the composition of the holoenzyme pool varies over a CT scale. Temporal changes in the relative abundance of active sigmas, and differences among them in affinity for specific promoters, could account, at least in part, for individualized patterns of gene expression in an overall rhythmic background. In *Arabidopsis*, the circadian waves of members of a putative signal transduction protein family have been proposed as conveyors of temporal information (21). The expression of five members of the APRR1/TOC1 family of pseudo response regulators is coordinated in a circadian manner with the abundance of each message peaking at a distinct time. The authors propose a bar code model to explain how CT might be read out based on the relative abundance of these factors (21). Expression patterns of *PrpoD2*, *PrpoD3*, and *PsigC* fusions to the *luxAB* gene set showed no evidence for a progressive change from one sigma factor to another at different times during the circadian cycle; all showed peaks of expression in the class 1 phase (Fig. 4A). However, even though transcriptional peaks of these sigma factors are not differently phased, their activities, such as ability to associate with the RNAP core, may vary on a CT scale.

From immunoblot analysis, the protein level of RpoD4 oscillates with peaks of abundance near CT 24 and 48 h (Fig. 5), although the bioluminescence peaks from the majority of gene fusions are at CT 12 and 36 h, etc. Despite the robust cycling of RpoD4 evident in two experiments, oscillation was not obvious in another. Thus, changes in sigma factor protein abundance may not be necessary for circadian rhythmicity. Temporal changes in activity may occur that are not evident by immunoblot analysis. The inability to distinguish among sigma

factors with existing antisera currently precludes determining whether the protein abundance oscillation phasing is different among the factors. Aside from absolute abundance changes, sigma factors may experience temporal regulation of activity. For example, sigmas may be sequestered and rendered inactive by anti-sigma factors, as occurs in many developmental systems (9). A search of the genome of another cyanobacterium, *Synechocystis* sp. strain PCC 6803, revealed two predicted anti-sigma factors, slr1856 (homolog of an anti-sigma B factor antagonist) and slr1912 (homolog of an anti-sigma F factor antagonist) (14) ([www.kazusa.or.jp/cyano](http://www.kazusa.or.jp/cyano)). Complete genome analysis of *S. elongatus* PCC 7942 is under way and will determine whether the identification of this class of regulatory factors is present in *S. elongatus* (C. Holtman, P. Youderian, and S. S. Golden, unpublished data). Another regulatory mechanism for altering sigma activity has been proposed in plants in which phosphorylation states of sigma-like factors affects DNA binding characteristics in different plastid types (31).

None of the single or pairwise sigma factor gene disruptions severely reduced expression of *PpsbAI*, *PpurF*, or *PkaiB* reporter strains, indicating that the remaining sigma factors in the pool are capable of transcribing these promoters even if the usual mechanism involves progressive interchange of subunits during the circadian cycle.

Overexpression of RpoD3 resulted in arrhythmicity of a *PpsbAI* reporter strain (Fig. 3C). However, overexpression in the *PkaiB::luxAB* reporter strain decreased the magnitude and amplitude of the bioluminescence rhythm without abolishing it, indicating that intrinsic timing is still intact in this situation (Fig. 3E). The decreased amplitude of expression from *PkaiB*, which encodes a central component of the clock mechanism, may be sufficient to explain why the oscillation from *PpsbAI*, which is controlled by the clock but not part of the clock, becomes arrhythmic. Alternatively, the difference between a lower amplitude rhythm from *PkaiB::luxAB* and arrhythmicity from *PpsbAI::luxAB* may merely reflect the lower amplitude of the wild-type *psbAI* oscillation.

The relative insensitivity of *PkaiB* expression to the loss of sigma factor genes supports the idea that this promoter is relatively insulated, or buffered, from other gene expression pathways (Fig. 1). However, the promoter for *kaiA*, which also encodes a clock component and is immediately upstream of *kaiBC* in the genome, shows the same sensitivity to output

pathway mutations as does *psbAI* (15; this study and A. Sivan, N. Tsinoremas, and S. S. Golden, unpublished data [for mutations in the *tmp5* gene]). These data indicate that *PkaiA* and *PkaiB* are on different regulatory circuits and that *kaiA* expression can be dramatically altered without changing the fundamental timing mechanism, even though KaiA is necessary for normal levels of *PkaiB* expression (10). The marked effect of *rpoD3* and *rpoD4* inactivation on the amplitude of oscillation and magnitude of expression for *purF* is the first evidence for a gene involved in the circadian expression of a class 2 (peaks at dawn) gene, although the sigma factors do not seem to regulate circadian phasing.

Inactivation of *rpoD2* led to an approximately fourfold increase in expression from *PrpoD2::luxAB* (Fig. 4C). At this time we do not know whether the interaction of RpoD2 with its own promoter is biochemical or solely genetic. RpoN of *Rhizobium leguminosarum* bv. viciae strain VF39SM negatively autoregulates expression from *rpoN* by binding to its upstream region (5), but the RpoD2 data could be explained as easily by an indirect mechanism.

The *sigC* null mutation caused the period of *PpsbAI* expression to be longer than those of *PkaiB* and *PpurF* (Fig. 1D, H, and L). These results reveal that it is possible for multiple timing circuits to coexist in *S. elongatus*. The first report that clearly demonstrates the presence of two oscillations with different circadian periods in one cell is from the unicellular alga *Gonyaulax polyedra* (27). In this organism, rhythms of bioluminescence and aggregation run with different periods, and the authors proposed that each rhythm is controlled by its own separate oscillator (27).

This phenomenon is not limited to *sigC* disruption. The inactivation of *rpoD3* dissimilarly affects even different segments of the *psbAI* regulatory region: it shortened the period of the bioluminescence rhythm by  $\sim 1$  h ( $23.9 \pm 0.2$  h versus  $25.0 \pm 0.3$  h,  $n = 10$ ) in a reporter strain that bears the  $-54$  to  $+43$  element of *PpsbAI* fused to *luxAB* (data not shown); however, it advanced the relative phase of expression by about 4 h without affecting the period in a reporter strain that carries a longer upstream region ( $-185$  to  $+224$  element of *PpsbA*) (Fig. 2B). Moreover, the *rpoD2 sigC* double mutation led to an  $\sim 1.5$ -h-long period of *PpsbAI::luxAB* expression but had no significant effect on the period of expression of the *PpurF::luxAB* and *PkaiB::luxAB* strains, confirming the ability of mutations to differentially affect the circadian periods of different reporters (Fig. 2D). These results reveal that it is possible for multiple timing circuits to coexist in the cell and that there is flexibility in circadian timing in *S. elongatus*.

A comprehensive model for the roles of sigma factors in circadian control of gene expression must account for the dissimilar effects on different promoters, the ability to control genes with timing circuits of different periods, and increased expression from some genes when a sigma factor is missing. The data describe a complex network in which both direct and indirect effects of changes in gene expression are likely to contribute to the observed phenotypes. Direct assay of the RNAP holoenzyme and its sigma factor constituents over time will contribute greatly to assembling the puzzle and determining whether the basic tenets of the model are correct.

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