PsfR, a factor that stimulates \textit{psbAI} expression in the cyanobacterium \textit{Synechococcus elongatus} PCC 7942

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In this paper a gene (\textit{psfR}) is reported that regulates \textit{psbAI} activity in \textit{Synechococcus elongatus}, a unicellular photoautotrophic cyanobacterium that carries out oxygenic (plant-type) photosynthesis and exhibits global circadian regulation of gene expression. In \textit{S. elongatus}, a family of three \textit{psbA} genes encodes the D1 protein of the photosystem II reaction centre. Overexpression of \textit{psfR} results in increased expression of \textit{psbAI}, but does not affect the circadian timing of \textit{psbAI} expression. \textit{psfR} overexpression affected some, but not all of the genes routinely surveyed for circadian expression. PsfR acts (directly or indirectly) on the \textit{psbAI} basal promoter region. \textit{psfR} knockout mutants exhibit wild-type \textit{psbAI} expression, suggesting that other factors can regulate \textit{psbAI} expression in the absence of functional PsfR. PsfR contains two receiver-like domains (found in bacterial two-component signal transduction systems), one of which lacks the conserved aspartyl residue required for phosphoryl transfer. PsfR also contains a GGDEF domain. The presence of these domains and the absence of a detectable conserved DNA-binding domain suggest that PsfR may regulate \textit{psbAI} expression via protein–protein interactions or GGDEF activity (the production of cyclic dinucleotides) rather than direct interaction with the \textit{psbAI} promoter.

\textbf{INTRODUCTION}

Cyanobacteria are photosynthetic prokaryotes that carry out oxygenic (oxygen-evolving) plant-type photosynthesis. In the unicellular cyanobacterium \textit{Synechococcus elongatus} PCC 7942, gene expression is globally regulated by the circadian clock (Liu \textit{et al}., 1995). Expression of approximately 80\% of the genes in \textit{S. elongatus} peaks at subjective dusk (class 1 genes). The expression of a minority of genes, termed class 2 genes, peaks at subjective dawn. Class 1 genes include two of the three members of the \textit{psbA} gene family, which encode two forms of the D1 protein, a core component of the photosystem II reaction centre (Golden \textit{et al}., 1986).

The \textit{psbAI} promoter (\textit{PpsbAI}) is one of the strongest promoters in \textit{S. elongatus} (Andersson \textit{et al}., 2000; Liu \textit{et al}., 1995) and is often used to study circadian gene expression in this organism (Andersson \textit{et al}., 2000; Golden \textit{et al}., 1997). The promoter elements of \textit{psbAI} have been studied using \textit{psbAI}::\textit{lacZ} and \textit{psbAI}::\textit{luxAB} reporter gene fusions (Nair \textit{et al}., 2001). The functional elements of the \textit{psbAI} promoter include a positive element located between −115 and −54 and a basal promoter extending from −54 to +1. The \textit{psbAI} promoter is not expressed in \textit{Escherichia coli} (Schaefer & Golden, 1989). The \textit{psbAI} gene has a −35 region characteristic of \textit{E. coli} \textit{σ}70 promoters, but the −10 region contains the atypical sequence TCTCCT (Golden \textit{et al}., 1986).

Here we describe a gene that affects \textit{psbAI} expression levels, but does not affect the circadian timing of \textit{psbAI} expression. This gene was found during a search for random overexpression mutants with altered circadian expression of \textit{psbAI}. One isolate exhibited low amplitude, high bioluminescence expression of \textit{PpsbAI}::\textit{luxAB}. Further experiments confirmed that overexpression of the genomic DNA fragment in the sense orientation from \textit{PconII} caused the mutant phenotype. Experiments reported here showed that some, but not all genes that we routinely survey for circadian expression are affected and that the factor(s) that alters \textit{psbAI} expression, PsfR, acts upon the basal promoter region of \textit{psbAI}.

\textbf{METHODS}

\textbf{Strains and plasmids.} All cyanobacterial reporter strains were created in \textit{S. elongatus} PCC 7942. \textit{E. coli} strains DH10B, HB101 and

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These plasmids to create the overexpression library strain AM1454. In pAM1451. The Genomic DNA fragments ranging from 2 to 4 kb were isolated and genomic DNA was partially digested with BamHI. The P\textit{ksbAI}\textit{II} fragment from pAM1509 was recovered and amplified in an \textit{E. coli} host strain. Plasmids isolated from the \textit{E. coli} host strain were then used to retransform AMC149, the \textit{P\textit{ksbAI}\textit{II}}::\textit{luxAB} reporter strain.

DH5\textsubscript{a} were used as plasmid hosts during cloning (Sambrook & Russell, 2001). The reporter strains and plasmids are summarized in Table 1.

### Table 1. Reporter strains and plasmids

<table>
<thead>
<tr>
<th>Reporter strain* or plasmid</th>
<th>Description†</th>
<th>Antibiotic resistance‡</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMC149</td>
<td>\textit{P\textit{ksbAI}}::\textit{luxAB} in neutral site 1</td>
<td>Sp</td>
<td>Kondo \textit{et al.} (1993)</td>
</tr>
<tr>
<td>AMC408</td>
<td>\textit{P\textit{purF}}::\textit{luxAB} in neutral site 2</td>
<td>Cm, Sp</td>
<td>Katayama \textit{et al.} (1999)</td>
</tr>
<tr>
<td>AMC412</td>
<td>\textit{P\textit{ksbAI}}::\textit{luxAB} in neutral site 1</td>
<td>Cm, Sp</td>
<td>Lab collection</td>
</tr>
<tr>
<td>AMC462</td>
<td>\textit{P\textit{kaiB}}::\textit{luxAB} in neutral site 1</td>
<td>Cm, Sp</td>
<td>Lab collection</td>
</tr>
<tr>
<td>AMC537</td>
<td>\textit{P\textit{ksbAI\textit{II}}}::\textit{luxAB} in neutral site 1</td>
<td>Cm, Sp</td>
<td>Katayama \textit{et al.} (1999)</td>
</tr>
<tr>
<td>AMC589</td>
<td>\textit{P\textit{cikA}}::\textit{luxAB} in neutral site 1</td>
<td>Cm, Sp</td>
<td>Lab collection</td>
</tr>
<tr>
<td>AMC669</td>
<td>\textit{P\textit{ksbAI}}::\textit{luxAB} in neutral site 2.1</td>
<td>Cm</td>
<td>Nair \textit{et al.} (2002)</td>
</tr>
<tr>
<td>AMC776</td>
<td>\textit{P\textit{ksbAI}} (−115 to +43)::\textit{luxAB} in neutral site 2</td>
<td>Cm, Sp</td>
<td>Nair \textit{et al.} (2001)</td>
</tr>
<tr>
<td>AMC777</td>
<td>\textit{P\textit{ksbAI}} (−54 to +43)::\textit{luxAB} in neutral site 2</td>
<td>Cm, Sp</td>
<td>Nair \textit{et al.} (2001)</td>
</tr>
<tr>
<td>AMC781</td>
<td>\textit{P\textit{ksbAI}} (−115 to +1)::\textit{luxAB} in neutral site 2</td>
<td>Cm, Sp</td>
<td>Nair \textit{et al.} (2001)</td>
</tr>
<tr>
<td>AMC1042</td>
<td>\textit{P\textit{sigC}}::\textit{luxAB} in neutral site 2</td>
<td>Cm, Sp</td>
<td>Lab collection</td>
</tr>
<tr>
<td>AMC1264</td>
<td>\textit{P\textit{ksbAI\textit{II}}}::\textit{luxAB} in neutral site 1</td>
<td>Cm, Sp</td>
<td>Lab collection</td>
</tr>
</tbody>
</table>

*pAll reporter strains (except for AMC149) also contain \textit{P\textit{ksbAI}}::\textit{luxCDE}. AMC669 contains both \textit{P\textit{ksbAI}}::\textit{luxAB} and \textit{P\textit{ksbAI}}::\textit{luxCDE} in neutral site 2.1. The other reporter strains contain \textit{P\textit{ksbAI}}::\textit{luxCDE} in the neutral site (1 or 2) not occupied by the \textit{luxAB} reporter.†See Methods for full descriptions of plasmids.‡Ap, Ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin.

To demonstrate linkage of the mutant phenotype to the plasmid, AMC371 was cured of the plasmid by growth on non-selective medium. When a plasmid integrates into a homologous site on the \textit{S. elongatus} chromosome by a single crossover event (as shown in Fig. 1), plasmid excision can occur spontaneously in the absence of antibiotic selection (Andersson \textit{et al.}, 2000). After growing AMC371 in the absence of the antibiotic selection marker, excised plasmids (pAM1509) were recovered and amplified in an \textit{E. coli} host strain. Plasmids isolated from the \textit{E. coli} host strain were then used to retransform AMC149, the \textit{P\textit{ksbAI}}::\textit{luxAB} reporter strain.

The pAM1509 genomic DNA fragment was sequenced by the cycle sequencing method. The NCBI GenBank BLAST e-mail server (Altschul \textit{et al.}, 1997) was used for DNA and protein database searches.

**Construction of the \textit{PconII} overexpression library.** The cloning vector pAM1153 was created by inserting a kanamycin resistance cassette into the \textit{P\textit{stl}} site of pBR322 (Sambrook & Russell, 2001). pNN396 (Elledge & Davis, 1989) was cut with \textit{NorI}, blunted with Klenow DNA polymerase, then cut with \textit{BamHI} to release \textit{PconII}. pAM1153 was cut with \textit{EcoRI}, blunted with Klenow DNA polymerase, then cut with \textit{BamHI}. The \textit{PconII} fragment was cloned into pAM1153 to create the overexpression library vector pAM1451. \textit{S. elongatus} genomic DNA was partially digested with \textit{Sau3AI}. Genomic DNA fragments ranging from 2 to 4 kb were isolated and cloned into the unique \textit{BamHI} site downstream of the \textit{conII} promoter in pAM1451. The \textit{E. coli} HB101 host strain was transformed with these plasmids to create the overexpression library strain AM1454.

**Isolation and sequencing of the \textit{psfR} genomic DNA fragment.** The overexpression library was transferred to the \textit{P\textit{ksbAI}}::\textit{luxAB} reporter strain AMC149 by conjugation (Andersson \textit{et al.}, 2000). The overexpression library plasmids integrate into the \textit{S. elongatus} genome by homologous recombination, as shown in Fig. 1. Exconjugants were screened for altered circadian expression of \textit{psbAI}. One exconjugant, AMC371, exhibited low amplitude, high bioluminescence expression of \textit{psbAI}.

To demonstrate linkage of the mutant phenotype to the plasmid, AMC371 was cured of the plasmid by growth on non-selective medium. When a plasmid integrates into a homologous site on the \textit{S. elongatus} chromosome by a single crossover event (as shown in Fig. 1), plasmid excision can occur spontaneously in the absence of antibiotic selection (Andersson \textit{et al.}, 2000). After growing AMC371 in the absence of the antibiotic selection marker, excised plasmids (pAM1509) were recovered and amplified in an \textit{E. coli} host strain. Plasmids isolated from the \textit{E. coli} host strain were then used to retransform AMC149, the \textit{P\textit{ksbAI}}::\textit{luxAB} reporter strain.

The pAM1509 genomic DNA fragment was sequenced by the cycle sequencing method. The NCBI GenBank BLAST e-mail server (Altschul \textit{et al.}, 1997) was used for DNA and protein database searches.

**Construction of \textit{psfR} overexpression and knockout plasmids.** Diagrams of the \textit{psfR} overexpression and knockout plasmids are shown in Fig. 2. A 1-6 kb \textit{BamHI} fragment from pAM1509 was cloned into the \textit{BamHI} site of the overexpression vector pAM1451 to create pAM1767 and pAM1768.
pAM1509 was cut with Xhol, pAM1451 was cut with BamHI, and both were blunted with Klenow DNA polymerase. The 2-4 kb Xhol fragment was cloned into pAM1451 to create pAM1769 and pAM1770.

A 1-6 kb BamHI fragment from pAM1509 was cloned into the BamHI site of pUC18 (Vieira & Messing, 1982) to create pAM1761. pAM1761 was cut with HindII and BamHI, pAM1451 was cut with BamHI, and both were blunted with Klenow DNA polymerase. The 1-4 kb HindII-BamHI fragment from pAM1761 was cloned into pAM1451 to create pAM17781 and pAM17782.

pAM2992, used to overexpress psfR in the neutral site 1 (NS1) of *S. elongatus* (Andersson et al., 2000), was constructed as follows. A 4-1 kb Asp718–Sphl fragment from pAM1509 was cloned into the Asp718–Sphl site of pUC20H (Marsh et al., 1984) to create pAM1801. pAM1801 was digested with Smal and HindII to remove orfXY, but this also removes 1-4 kb of psfR as a HindII fragment. The psfR HindII fragment was isolated and ligated into the Smal–HindII site of pAM1801. Fragment orientation was checked by restriction mapping and a plasmid with the psfR fragment cloned in the proper orientation was isolated (pAM2886). pNN396 (Elledge & Davis, 1989) was cut with NotI, blunted with T4 DNA polymerase, then cut with Asp718 to release PconII. The PconII fragment was cloned into the EcoRV–Asp718 site of pAM2886 to create pAM2985, which contains psfR (minus orfXY) downstream of PconII in the sense orientation. The PconII–psfR fragment was released from pAM2985 by digestion with HindIII and cloned into the HindIII site of the NS1 vector pAM2314.

A 2-4 kb Xhol fragment from pAM1509 was cloned into the Xhol site of pUC1819RI to create pAM1762. The pUC1819RI cloning vector is similar to the pUC1819H3 vector described previously (Golden & Wiest, 1988). pUC1819RI contains the small Scal–EcoRI fragment from pUC18 ligated to the large Scal–EcoRI fragment of pUC19.

pAM165 was digested with BamHI to release the spectinomycin resistance cassette. The spectinomycin resistance cassette was cloned into the BamHI site of pAM1762 to create pAM1788. Three more psfR knockout constructs were made by inserting the spectinomycin resistance cassette into the Clal (pAM1787), Scal (pAM1786) or Xhol (pAM1790) sites within the psfR ORF.

**Construction of psfR overexpression strains.** psfR overexpression and knockout plasmids were transferred to reporter strains by conjugation (Andersson et al., 2000) or transformation, followed by selection on BG-11 M agar (Bustos & Golden, 1991) containing the appropriate antibiotics. For each mutant strain created, a minimum of four independently isolated transformants was assayed for bioluminescence. Ectopic psfR fragments were inserted at ‘neutral sites’ in the *S. elongatus* chromosome: loci at which insertions of ectopic DNA and the selection marker are inserted into the chromosome: loci at which insertions of ectopic DNA and the selection marker are inserted into the chromosomal location occurs by homologous recombination at the neutral site. The ectopic DNA and the selection marker are inserted into the chromosome at the neutral site, while the other vector sequences are lost (Golden et al., 1987). All other psfR overexpression strains were created by insertion of overexpression plasmids at the psfR locus by single-crossover homologous recombination, as shown in Fig. 1.

**Bioluminescence assays.** All reporter strains used in this study (except AMC149) are autonomously bioluminescent. In addition to the luxA:B reporter, they contain *PsbaI*: luxCDE, which directs the synthesis of the long-chain aldehyde substrate for luciferase in *vivo* (Andersson et al., 2000). *S. elongatus* strains were grown on BG-11 M agar (Bustos & Golden, 1991); for screening by a Packard TopCount luminometer (Andersson et al., 2000), samples were inoculated onto BG-11 M agar in 96-well microtitre plates. BG-11 M
agar was always supplemented with the appropriate antibiotics for selection purposes. Inoculated Petri or microtitre plates were incubated in constant light for 6–18 h, then incubated in the dark for 12 h to synchronize the clocks of all of the cells on the plates (Katayama et al., 1999). The initial screen used a turntable device and CCD camera as described previously (Kondo et al., 1994). In subsequent analyses (all data shown in this paper) strains were assayed for bioluminescence on a Packard TopCount luminometer. The psfR knockout strain used for the experiment shown in Fig. 8 was monitored for bioluminescence on two different microtitre plates in a single TopCount assay. All other strains were assayed in at least two independent assays.

Because absolute expression levels vary with cell number, many comparisons of reporter strain bioluminescence to mutant strain bioluminescence were graphed for each TopCount experiment. To control for the effect of light intensity on reporter gene expression levels, comparisons were made only between strains from wells exposed to similar light intensities (Katayama et al., 2003). TopCount data were imported into Microsoft Excel 2000 using the Import & Analysis software package (M. Straume, National Science Foundation Center for Biological Timing, University of Virginia).

**RESULTS**

**Isolation of a gene that affects psbAI expression levels**

A locus that affects psbAI expression levels was isolated during a search for components of the *S. elongatus* circadian clock. Plasmids that contain genomic DNA fragments cloned downstream of a constitutively expressed promoter (PconII) were transferred to a psbAI reporter strain (AMC149) by conjugation (Andersson et al., 2000). Successful conjugation results in the insertion of the entire plasmid into the *S. elongatus* chromosome by single crossover homologous recombination at a single site (Fig. 1). Exconjugants were screened by Carl H. Johnson (Vanderbilt University) for altered circadian expression of PpsbAI::luxAB using a custom built turntable and CCD camera like that described previously (Kondo et al., 1994).

One of the exconjugants exhibited low amplitude, high bioluminescence expression of the psbAI reporter gene (data not shown, but similar to that shown for derivative strains screened using a Packard TopCount luminometer in Fig. 3a and c). To confirm that this phenotype was caused by overexpression of the genomic DNA fragment driven by PconII, the plasmid was recovered by spontaneous loopout in the absence of antibiotic selection and reintroduced into a wild-type psbAI reporter strain as described elsewhere (Andersson et al., 2000). Strains cured of the plasmid had wild-type expression of the psbAI reporter gene, whereas strains transformed with the recovered plasmid (pAM1509) had the low amplitude, high bioluminescence phenotype (data not shown).

Sequence analysis of the genomic DNA fragment in pAM1509 revealed two small ORFs of unknown function (orfX and orfY) and a larger ORF (Fig. 2). We named the larger ORF psfR (*psbAI*-stimulating factor) for reasons described below. Conserved domain searches revealed that the derived Psfr amino acid sequence predicts two receiver-like domains (found in bacterial two-component regulatory systems) and a GGDEF domain (Fig. 4). The C-terminal

**Fig. 3.** Overexpression of the orfXY–psfR fragment in a psbAI reporter strain. Bioluminescence traces (counts per second) are shown for the psbAI reporter strain AMC412 (closed circles) and clones overexpressing psfR (open circles). (a, b) Overexpression of psfR in the sense (a) and antisense (b) orientations. (c) The data shown in graph (a) were plotted on two axes to clearly show low-amplitude expression of psbAI. LL, Continuous light.

<table>
<thead>
<tr>
<th><strong>Psfr</strong> (796 aa, 88 kDa)</th>
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<tbody>
<tr>
<td>Pseudo-receiver domain (5–123)</td>
</tr>
<tr>
<td>Psfr (796 aa, 88 kDa)</td>
</tr>
<tr>
<td>Receiver domain (496–610)</td>
</tr>
<tr>
<td>GGDEF domain (601–782)</td>
</tr>
</tbody>
</table>

**Fig. 4.** Conserved domains of Psfr. See text for details.
receiver domain contains the conserved aspartyl residue required for phosphoryl-transfer activity (Stock et al., 2000). The N-terminal receiver domain appears to be a pseudo-receiver, as it lacks the conserved aspartyl residue.

To delimit the region of the genomic DNA fragment required for the mutant psbAI phenotype, smaller fragments of S. elongatus genomic DNA from pAM1509 were cloned into the expression library vector and reintroduced into a psbAI reporter strain (Fig. 2). When the psbAI reporter strain AMC412 was transformed with pAM1767, the mutant phenotype was observed (Fig. 3a and c). pAM1767 carries orfXY and 2/3 of the N-terminal coding region of psfR downstream of PconII in the sense orientation. Wild-type expression of psbAI was observed when AMC412 was transformed with pAM1768, which carries the pAM1767 genomic DNA fragment in the antisense orientation (Fig. 3b). As shown in Fig. 1, integration of these plasmids into the S. elongatus chromosome results in the partial duplication of the genomic DNA fragment. After recombination, PconII drives expression of the complete psfR ORF.

**Overexpression of psfR affects a subset of genes in S. elongatus**

To determine whether psfR overexpression affects genes other than psbAI, we overexpressed psfR in several different S. elongatus reporter strains. The reporter strains were transformed with pAM1767 or pAM1768 to drive the sense or the antisense orientation, respectively, of orfXY and psfR. Transformants were tested for rhythmic expression of bioluminescence using a Packard TopCount luminometer.

Like psbAI, kaiB is a class 1 gene whose expression peaks at subjective dusk (Liu et al., 1995). KaiB is a component of the central circadian oscillator in S. elongatus (Ishiura et al., 1998). As shown in Fig. 5(a and b), overexpression of psfR in either orientation had no effect on kaiB expression.

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**Fig. 5.** Overexpression of psfR affects expression from a subset of reporter strains. Bioluminescence traces are shown from reporter strains (closed circles) and reporter strains overexpressing psfR (open circles). Overexpression of psfR in the kaiB reporter strain AMC462 (a, b), purF reporter strain AMC408 (c, d), cikA reporter strain AMC589 (e, f), sigC reporter strain AMC1042 (g, h), psbAll reporter strain AMC1264 (i, j) and psbAIII reporter strain AMC537 (k, l) in the sense and antisense orientations, respectively. Graph (e) shows two representative overexpression traces from the same clone. Graph (g) shows two representative overexpression traces from two independently isolated clones.
To test the effect of psfR overexpression on a class 2 gene (whose expression peaks at subjective dawn), we overexpressed psfR in a purF reporter strain (AMC408). The purF gene encodes a protein involved in purine biosynthesis (Liu et al., 1996). Fig. 5(c and d) show that overexpression of psfR in either orientation had no effect on purF expression.

We also tested the effect of psfR overexpression on two other well characterized class 1 genes: cikA and sigC. The cikA gene encodes a protein that is a member of the extended bacteriophytochrome family and appears to be part of the input pathway to the circadian clock (Schmitz et al., 2000). Overexpression of psfR in a cikA reporter strain (AMC589) reduced the amplitude of oscillation from the cikA promoter, and often reduced cikA expression levels as well (Fig. 5e), whereas overexpression of the fragment in the antisense orientation had no effect (Fig. 5f).

The sigC gene encodes a group 2 sigma factor involved in psbAI expression (Nair et al., 2002). If the sigC gene is inactivated, psbAI expression levels increase, the amplitude of the oscillation increases and the period of psbAI expression increases by 2 h. This long-period phenotype is also seen when sigC is overexpressed. This suggests that other sigma factors can recognize psbAI in the absence of sigC, but wild-type sigC expression is required for normal circadian expression of psbAI. Overexpression of psfR in a sigC reporter strain (AMC1042) resulted in reduced expression and a reduction in the amplitude of oscillation from the sigC promoter approximately 85% of the time. (Fig. 5g). In approximately 15% of samples, overexpression of psfR in AMC1042 did not significantly reduce expression from the sigC promoter. This phenotype was seen in two independently isolated exconjugants, each of which usually displayed reduced expression from the sigC promoter when psfR was overexpressed. Therefore, it is unlikely that this phenotype is the result of suppressor mutations. Overexpression of psfR in the antisense orientation did not affect expression from the sigC promoter (Fig. 5h).

The psbAI gene is part of a family of three genes that encode the D1 protein in S. elongatus (Golden et al., 1986). Overexpression of psfR in bioluminescent reporter strains for psbAII (AMC1264) and psbAIII (AMC537) resulted in elevated expression from those promoters as was seen for psbAI (Fig. 5i, k), although no effect was seen when the antisense construct was overexpressed (Fig. 5j, l). Thus, the psbA family as a whole is responsive to PsfR.

**Overexpression of the psfR ORF alone is sufficient for altered psbAI gene expression**

To determine whether the small ORFs orfX and orfY are required for elevated psbAI expression, we transformed the psbAI reporter strain AMC412 with plasmid vectors that contain PconII–psfR but lack orfXY (pAM1781, pAM1769). pAM1781 contains 85 bp upstream of psfR, including 30 bp of orfY. pAM1769 contains 35 bp upstream of psfR (Fig. 2). When S. elongatus is transformed with either plasmid, the plasmid is inserted into the S. elongatus chromosome at the psfR locus by homologous recombination and the entire psfR ORF is driven by PconII (Fig. 1). As shown in Fig. 6(a and c), overexpression of psfR from either plasmid was sufficient to elevate psbAI expression and overexpression of these psfR fragments in the antisense orientation had no effect (Fig. 6b and d). PconII–psfR was also sufficient to elevate expression from psbAII and psbAIII reporter genes (data not shown).

Overexpression from a neutral site on the S. elongatus chromosome of a fragment that contains orfXY and the N-terminal half of the psfR ORF (pAM1799) results in wild-type psbAI expression (data not shown). Unlike the integration event shown in Fig. 1, neutral site recombination does not reconstruct a full-length psfR gene. This further suggests that elevated expression of psfR, rather than orfX or orfY, is responsible for the increase in psbAI expression noted in the
previous mutants. To confirm that the psfR ORF alone is sufficient for elevated psbAl expression, we overexpressed the psfR ORF from a neutral site on the S. elongatus chromosome (pAM2992). In this situation, the native psfR locus is intact and PconII drives an ectopic psfR. As shown in Fig. 7, overexpression of psfR from an S. elongatus neutral site results in elevated psbAl expression.

In contrast, inactivation of the psfR gene had no effect on psbAl expression. The knockout mutants shown in Fig. 8(a and b) (using plasmid pAM1788) contain a spectinomycin resistance cassette insertion in the BamHI site of psfR. Three additional knockout constructs were made (pAM1786, pAM1787, pAM1790) within the psfR ORF (Fig. 2); none of these knockout mutants displayed altered psbAl expression (data not shown). The transcription terminators in the spectinomycin resistance gene (Ω cassette) work as terminators in S. elongatus (Golden & Stearns, 1988). Therefore, insertion of the spectinomycin resistance gene prevents transcription of any psfR sequence downstream of it.

**psfR acts directly or indirectly at the basal promoter region of psbAl**

The psbAl reporter strain AMC412 has end points extending from approximately −75 to +180 relative to the psbAl transcription start site. To determine which elements in the psbAl promoter region are acted upon by psfR (directly or indirectly), we overexpressed psfR in psbAl reporter strains that contain different segments of the psbAl promoter region driving luxAB. AMC776, AMC781 and AMC777 contain sequences extending from −115 to +43, −115 to +1 and −54 to +43, respectively. All three reporter fusions contain the psbAl basal promoter region, which extends from −54 to +1 (Nair et al., 2001). AMC777 lacks the positive regulatory element that extends from −115 to −54 (Nair et al., 2001), and expression of PpsbAl::luxAB is much lower in this strain than in AMC776 and AMC781. Note that the AMC777 data presented in Fig. 9 are graphed on a lower scale than the data from AMC776 and AMC781.

These reporter strains were transformed with plasmid vectors that carry the orfXY–psfR fragment downstream of the conII promoter in the sense or antisense orientation (pAM1767, pAM1768); recombination results in the insertion of the entire plasmid into the S. elongatus genome at the psfR locus (Fig. 1). As shown in Fig. 9(a, c and e), overexpression of psfR in any of these reporter strains resulted in increased expression from the psbAl promoter, whereas overexpression of psfR in the antisense orientation had no effect (Fig. 9b, d and f). The only psbAl sequence shared by all three reporter strains is the basal promoter region, which extends from −54 to +1. Therefore, we conclude that PsfR acts upon the psbAl basal promoter region, either by directly binding to this region or by affecting the activity of an unknown psbAl-binding factor.

**DISCUSSION**

The psfR ORF was isolated during a screen for overexpression mutants with altered circadian expression of
psbAI. However, while elevated psfR expression causes increased psbAI expression, it does not genuinely alter its circadian expression pattern. Further experiments showed that overexpression of psfR does not affect kaiB gene expression, indicating that psfR does not regulate the circadian clock itself. Experiments with a class 2 reporter gene (purF) showed that overexpression of psfR does not globally affect gene expression. Dramatic increases in psbAI expression levels were consistently observed when psfR was overexpressed in psbAI reporter strains, suggesting that the PsfR protein plays a role in psbAI gene regulation. For this reason, we named the locus psf for psbAI-stimulatory factor. Experiments with different psbAI reporter strains indicate that PsfR acts (either directly or indirectly) at the basal promoter region of psbAI.

PsfR overexpression resulted in decreased expression of sigC in many assays. Loss of sigC is known to increase expression from psbAI (Nair et al., 2002). However, the phenotypes are different, as sigC inactivation increases the amplitude of psbAI expression, whereas PsfR overexpression decreases it. Thus, the effect of PsfR overexpression cannot be explained entirely by a loss of sigC. In addition, the psbAI elevation phenotype showed complete penetration, whereas sigC suppression did not.

As shown in Fig. 4, sequence analysis of the putative PsfR protein predicted an N-terminal pseudo-receiver domain that lacks the conserved aspartyl residue that would be needed for phosphoryl transfer from a histidine protein kinase in a bona fide receiver, a C-terminal typical receiver domain and a C-terminal GGDEF domain. Conserved domain searches did not detect a putative DNA-binding domain. While this does not rule out the possibility that PsfR is a DNA-binding protein, the presence of a pseudo-receiver domain suggests that PsfR may regulate psbAI expression via protein–protein interactions rather than by direct interaction with the psbAI promoter DNA. Although the genomes of other cyanobacteria encode proteins with these motifs, there is no clear homologue of psfR in the available sequences.

A pseudo-receiver domain of known function is present in the AmiR regulatory protein of Pseudomonas aeruginosa (O’Hara et al., 1999). Free AmiR activates expression from the aliphatic amidase operon via a transcription anti-termination mechanism. When aliphatic amides are not present, the AmiC protein binds to the pseudo-receiver domain of AmiR, sequestering AmiR and allowing transcription termination to occur, so that the aliphatic amidase operon is not expressed. In a similar fashion, PsfR could indirectly regulate psbAI expression by interacting with a protein that binds to the basal promoter region of psbAI, affecting activity of that protein. Pseudo-receiver domains have been found in the S. elongatus proteins CikA (Schmitz et al., 2000) and KaiA (Williams et al., 2002). Members of the Arabidopsis thaliana family of pseudo-response regulators (APRR family), which includes the putative plant clock protein (TOC1), contain pseudo-receiver domains as well (Imamura et al., 1999; Makino et al., 2000; Strayer et al., 2000). All lack the aspartyl residue that would be necessary for two-component system receiver function.

The two receiver-like domains of PsfR may act together to regulate the activity of the protein. Phosphorylation of the C-terminal receiver could make it easier for the N-terminal pseudo-receiver to bind to its protein target, or binding of the pseudo-receiver to its target could regulate

**Fig. 9.** Overexpression of psfR in psbAI reporter strains that contain different psbAI promoter fragments. Bioluminescence traces are shown from the reporter strains (closed circles) and from clones that overexpress psfR (open circles). (a, b) Overexpression of psfR in sense (a) and antisense (b) orientations in the −115/+43 psbAI reporter strain AMC776. (c, d) Overexpression of psfR in sense (c) and antisense (d) orientations in the −115/+1 psbAI reporter strain AMC781. Overexpression of psfR in sense (e) and antisense (f) orientations in the −54/+43 psbAI reporter strain AMC777.
phosphorylation of the receiver. In either case, a conformational change in the PsfR protein would result in a change in PsfR activity.

GGDEF domains are found in many multidomain signal transduction proteins; in most, the role of this domain has not been determined. GGDEF domains have sequence similarity to the eukaryotic adenyl cyclase catalytic domain, suggesting that GGDEF domains could be regulatory enzymes involved in nucleotide cyclization (Pei & Grishin, 2001). Studies of cellulose production in Rhizobium leguminosarum bv. trifolii and Agrobacterium tumefaciens suggest that GGDEF domains are involved in the synthesis of bis-(2',5')-cyclic diguanylic acid (cyclic di-GMP) and that cyclic di-GMP is an activator of cellulose production in these bacteria (Ausmees et al., 2001). Therefore, the role of GGDEF domains in some proteins is the production of cyclic di-GMP, a signalling molecule in some regulatory pathways. If the GGDEF domain of PsfR produces cyclic di-GMP, perhaps its activity is controlled by conformational changes in PsfR, through phosphorylation of the receiver or protein–protein interactions with the pseudo-receiver.

Because a phenotype was detected in an overexpression mutant, but not an inactivation mutant, PsfR may be part of a family that serves similar roles in the cell, such that its loss is compensated. It is also possible that the phenotype results from a ‘crosstalk’ phenotype that does not reflect the true function of PsfR. However, we now know that the conII promoter is low to moderate in strength in Synechococcus elongatus, such that overexpression of the protein is likely to be modest (Katayama et al., 1999).

In conclusion, we identified a regulatory gene that affects the expression of a subset of genes in Synechococcus elongatus, but does not regulate the Synechococcus elongatus circadian clock. Our work shows that overexpression of the psfR ORF is sufficient for elevated psbAI expression and that PsfR acts (either directly or indirectly) at the basal promoter region of psbAI.

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