PsfR, a factor that stimulates psbAI expression in the cyanobacterium Synechococcus elongatus PCC 7942

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In this paper a gene (psfR) is reported that regulates psbAI activity in Synechococcus elongatus, a unicellular photoautotrophic cyanobacterium that carries out oxygenic (plant-type) photosynthesis and exhibits global circadian regulation of gene expression. In S. elongatus, a family of three psbA genes encodes the D1 protein of the photosystem II reaction centre. Overexpression of psfR results in increased expression of psbAI, but does not affect the circadian timing of psbAI expression. psfR overexpression affected some, but not all of the genes routinely surveyed for circadian expression. PsfR acts (directly or indirectly) on the psbAI basal promoter region. psfR knockout mutants exhibit wild-type psbAI expression, suggesting that other factors can regulate 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 psbAI expression via protein–protein interactions or GGDEF activity (the production of cyclic dinucleotides) rather than direct interaction with the psbAI promoter.

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes that carry out oxygenic (oxygen-evolving) plant-type photosynthesis. In the unicellular cyanobacterium Synechococcus elongatus PCC 7942, gene expression is globally regulated by the circadian clock (Liu et al., 1995). Expression of approximately 80% of the genes in 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 psbA gene family, which encode two forms of the D1 protein, a core component of the photosystem II reaction centre (Golden et al., 1986).

The psbAI promoter (PpsbAI) is one of the strongest promoters in S. elongatus (Andersson et al., 2000; Liu et al., 1995) and is often used to study circadian gene expression in this organism (Andersson et al., 2000; Golden et al., 1997). The promoter elements of psbAI have been studied using psbAI:: lacZ and psbAI:: luxAB reporter gene fusions (Nair et al., 2001). The functional elements of the psbAI promoter include a positive element located between −115 and −54 and a basal promoter extending from −54 to +1. The psbAI promoter is not expressed in Escherichia coli (Schafer & Golden, 1989). The psbAI gene has a −35 region characteristic of E. coli σ70 promoters, but the −10 region contains the atypical sequence TCTCCT (Golden et al., 1986).

Here we describe a gene that affects psbAI expression levels, but does not affect the circadian timing of psbAI expression. This gene was found during a search for random overexpression mutants with altered circadian expression of psbAI. One isolate exhibited low amplitude, high bioluminescence expression of PpsbAI:: luxAB. Further experiments confirmed that overexpression of the genomic DNA fragment in the sense orientation from 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 psbAI expression, PsfR, acts upon the basal promoter region of psbAI.

METHODS

Strains and plasmids. All cyanobacterial reporter strains were created in S. elongatus PCC 7942. E. coli strains DH10B, HB101 and
DH5α were used as plasmid hosts during cloning (Sambrook & Russell, 2001). The reporter strains and plasmids are summarized in Table 1.

**Construction of the PconII overexpression library.** The cloning vector pAM1153 was created by inserting a kanamycin resistance cassette into the PstI site of pBR322 (Sambrook & Russell, 2001). The ORF was inserted into the unique BamHI site of the vector pAM1153 to create the overexpression library vector pAM1451. The S. elongatus genomic DNA was partially digested with Sau3A1. The PconII fragment was cloned into pAM1153 to create the overexpression library strain AM1454.

**Isolation and sequencing of the psfR genomic DNA fragment.** The overexpression library was transferred to the PpsbAI::luxAB reporter strain AM149 by conjugation (Andersson et al., 2000). The overexpression library plasmids integrate into the S. elongatus genome by homologous recombination, as shown in Fig. 1. Exconjugants were screened for altered circadian expression of ppsbA. One exconjugant, AM371, exhibited low amplitude, high bioluminescence expression of ppsbA.

To demonstrate linkage of the mutant phenotype to the plasmid, AM371 was cured of the plasmid by growth on non-selective medium. When a plasmid integrates into a homologous site on the 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 et al., 2000). After growing AM371 in the absence of the antibiotic selection marker, excised plasmids (pAM1509) were recovered and amplified in an E. coli host strain. Plasmids isolated from the E. coli host strain were then used to retransform AM149, the PpsbAI::luxAB reporter strain.

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

**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>
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<tr>
<td>AMC149</td>
<td>PpsbAI::luxAB in neutral site 1</td>
<td>Sp</td>
<td>Kondo et al. (1993)</td>
</tr>
<tr>
<td>AMC408</td>
<td>Ppurf::luxAB in neutral site 2</td>
<td>Cm, Sp</td>
<td>Katayama et al. (1999)</td>
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<td>PpsbAI::luxAB in neutral site 1</td>
<td>Cm, Sp</td>
<td>Lab collection</td>
</tr>
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<td>PkaiB::luxAB in neutral site 1</td>
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<td>AMC537</td>
<td>PpsbAII::luxAB in neutral site 1</td>
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</tr>
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<td>PcellA::luxAB in neutral site 1</td>
<td>Cm, Sp</td>
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<tr>
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<td>AMC1264</td>
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<table>
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<td>Km</td>
<td>This work</td>
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<td>This work</td>
</tr>
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<td>pAM1769</td>
<td>2/3 of psfR+35 bp upstream DNA (sense)</td>
<td>Km</td>
<td>This work</td>
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<td>This work</td>
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<td>pAM2992</td>
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*All reporter strains (except for AMC149) also contain PpsbAI::luxCDE. AMC669 contains both PpsbAI::luxAB and PpsbAI::luxCDE in neutral site 2.1. The other reporter strains contain PpsbAI::luxCDE in the neutral site (1 or 2) not occupied by the luxAB reporter.
†See Methods for full descriptions of plasmids.
‡Ap, Ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin.
Fig. 1. Integration of an \textit{S. elongatus} genomic DNA fragment into the chromosome by single recombination. This figure shows integration of the genomic DNA fragment originally isolated during a screen for circadian clock mutants. This fragment contains \textit{PconII} upstream of two small ORFs (\textit{orfXY}) and the N-terminal two-thirds of a larger ORF (\textit{psfR}). Homologous recombination results in the partial duplication of the genomic DNA fragment. After recombination, \textit{PconII} drives expression from \textit{orfXY} and the complete \textit{psfR} ORF.

pAM1509 was cut with XhoI, pAM1451 was cut with BamHI, and both were blunted with Klenow DNA polymerase. The 2-4 kb XhoI fragment was cloned into pAM1451 to create pAM1769 and pAM1770. A 1-6 kb \textit{BamHI} fragment from pAM1509 was cloned into the \textit{BamHI} site of pUC18 (Vieira & Messing, 1982) to create pAM1761. pAM1761 was cut with \textit{HincII} and \textit{BamHI}, pAM1451 was cut with \textit{BamHI}, and both were blunted with Klenow DNA polymerase. The 1-4 kb \textit{HincII–BamHI} fragment from pAM1761 was cloned into pAM1451 to create pAM1781 and pAM1782.

pAM2992, used to overexpress \textit{psfR} in the neutral site 1 (NS1) of \textit{S. elongatus} (Andersson \textit{et al}., 2000), was constructed as follows. A 4-1 kb \textit{Asp718–SpeI} fragment from pAM1509 was cloned into the \textit{Asp718–SpeI} site of pIC20H (Marsh \textit{et al}., 1984) to create pAM1801. pAM1801 was digested with \textit{SmaI} and \textit{HincII} to remove \textit{orfXY}, but this also removes 1-4 kb of \textit{psfR} as a \textit{HincII} fragment. The \textit{psfR} \textit{HincII} fragment was isolated and ligated into the \textit{SmaI–HincII} site of pAM1801. Orientation was checked by restriction mapping and a plasmid with the \textit{psfR} fragment cloned in the proper orientation was isolated (pAM2886). pNN396 (Elledge \& Davis, 1989) was cut with \textit{NotI}, blunted with T4 DNA polymerase, then cut with \textit{Asp718} to release \textit{PconII}. The \textit{PconII} fragment was cloned into the \textit{EcoRV–Asp718} site of pAM2886 to create pAM2985, which contains \textit{psfR} (minus \textit{orfXY}) downstream of \textit{PconII} in the sense orientation. The \textit{PconII–psfR} fragment was released from pAM2985 by digestion with \textit{HindIII} and cloned into the NS1 vector pAM2314.

A 2-4 kb \textit{XhoI} fragment from pAM1509 was cloned into the \textit{XhoI} 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 \textit{SacI–EcoRI} fragment from pUC18 ligated to the large \textit{SacI–EcoRI} fragment of pUC19.

pAM165 was digested with \textit{BamHI} to release the spectinomycin resistance cassette. The spectinomycin resistance cassette was cloned into the \textit{BamHI} site of pAM1762 to create pAM1788. Three more \textit{psfR} knockout constructs were made by inserting the spectinomycin resistance cassette into the \textit{ClaI} (pAM1787), \textit{SacI} (pAM1786) or \textit{XhoI} (pAM1790) sites within the \textit{psfR} ORF.

\textbf{Construction of \textit{psfR} overexpression strains.} \textit{psfR} overexpression and knockout plasmids were transferred to reporter strains by conjugation (Andersson \textit{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 \textit{psfR} fragments were inserted at ‘neutral sites’ in the \textit{S. elongatus} chromosome: loci at which insertions of ectopic DNA fragments can be made without any apparent phenotype (Andersson \textit{et al}., 2000). Neutral site vectors contain cloning sites and a selection marker flanked by neutral site sequence. These vectors can replicate in \textit{E. coli}, but not in \textit{S. elongatus}. Transformation 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 \textit{et al}., 1987). All other \textit{psfR} overexpression strains were created by insertion of overexpression plasmids at the \textit{psfR} locus by single crossover homologous recombination, as shown in Fig. 1.

\textbf{Bioluminescence assays.} All reporter strains used in this study (except AMC149) are autonomously bioluminescent. In addition to the \textit{luxAB} reporter, they contain \textit{PpsbAl::luxCDE}, which directs the synthesis of the long-chain aldehyde substrate for luciferase in vivo (Andersson \textit{et al}., 2000). \textit{S. elongatus} strains were grown on BG-11 M agar (Bustos & Golden, 1991); for screening by a Packard TopCount luminometer (Andersson \textit{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 PsbAI::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 loop-out 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
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 *psbAII* phenotype, smaller fragments of *S. elongatus* genomic DNA from pAM1509 were cloned into the expression library vector and reintroduced into a *psbAII* reporter strain (Fig. 2). When the *psbAII* 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 *psbAII* 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 *psbAII*, 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 *psbAII*, *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), *psbAII* 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 $psbA$ expression (Nair et al., 2002). If the $sigC$ gene is inactivated, $psbA$ expression increases by 2 h. This long-period phenotype is also seen when $sigC$ is overexpressed. This suggests that other sigma factors can recognize $psbA$ in the absence of $sigC$, but wild-type $sigC$ expression is required for normal circadian expression of $psbA$. Overexpression of $psfR$ in a $sigC$ reporter strain (AMC1042) resulted in reduced expression from the $sigC$ promoter (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 $psbA$ 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 $psbA$ gene expression**

To determine whether the small ORFs $orfX$ and $orfY$ are required for elevated $psbA$ expression, we transformed the $psbA$ reporter strain AMC412 with plasmid vectors that contain P$conII$–$psfR$ 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 P$conII$ (Fig. 1). As shown in Fig. 6(a and c), overexpression of $psfR$ from either plasmid was sufficient to elevate $psbA$ expression and overexpression of these $psfR$ fragments in the antisense orientation had no effect (Fig. 6b and d). P$conII$–$psfR$ 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 $psbA$ 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 of $orfX$ or $orfY$, is responsible for the increase in $psbA$ expression noted in the

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**Fig. 6.** Overexpression of $psfR$ fragments that lack $orfXY$ in the $psbA$ reporter strain AMC412. Bioluminescence traces are shown from the reporter strain (closed circles) and clones that overexpress $psfR$ (open circles). (a, b) $psfR$ overexpressed from pAM1781 (sense orientation) and pAM1782 (antisense orientation). (c, d) $psfR$ overexpressed from pAM1769 (sense orientation) and pAM1770 (antisense orientation).
previous mutants. To confirm that the \textit{psfR} ORF alone is sufficient for elevated \textit{psbAI} expression, we overexpressed the \textit{psfR} ORF from a neutral site on the \textit{S. elongatus} chromosome (pAM2992). In this situation, the native \textit{psfR} locus is intact and \textit{PconII} drives an ectopic \textit{psfR}. As shown in Fig. 7, overexpression of \textit{psfR} from an \textit{S. elongatus} neutral site results in elevated \textit{psbAI} expression.

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

\textbf{\textit{psfR} acts directly or indirectly at the basal promoter region of \textit{psbAI}}

The \textit{psbAI} reporter strain AMC412 has end points extending from approximately −75 to +180 relative to the \textit{psbAI} transcription start site. To determine which elements in the \textit{psbAI} promoter region are acted upon by \textit{psfR} (directly or indirectly), we overexpressed \textit{psfR} in \textit{psbAI} reporter strains that contain different segments of the \textit{psbAI} promoter region driving \textit{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 \textit{psbAI} basal promoter region, which extends from −54 to +1 (Nair \textit{et al}., 2001). AMC777 lacks the positive regulatory element that extends from −115 to −54 (Nair \textit{et al}., 2001), and expression of \textit{PpsbAI::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 \textit{orfXY−psfR} fragment downstream of the \textit{conII} promoter in the sense or antisense orientation (pAM1767, pAM1768); recombination results in the insertion of the entire plasmid into the \textit{S. elongatus} genome at the \textit{psfR} locus (Fig. 1). As shown in Fig. 9(a, c and e), overexpression of \textit{psfR} in any of these reporter strains resulted in increased expression from the \textit{psbAI} promoter, whereas overexpression of \textit{psfR} in the antisense orientation had no effect (Fig. 9b, d and f). The only \textit{psbAI} sequence shared by all three reporter strains is the basal promoter region, either by directly binding to this region or by affecting the activity of an unknown \textit{psbAI}-binding factor.

\textbf{DISCUSSION}

The \textit{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 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 loss is compensated. It is also possible that the phenotype of a family that serves similar roles in the cell, such that its mutant, but not an inactivation mutant, PsfR may be part of protein–protein interactions with the pseudo-receiver.

In conclusion, we identified a regulatory gene that affects the expression of a subset of genes in S. elongatus, but does not regulate the S. 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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REFERENCES


