Illumination stimulates cAMP receptor protein-dependent transcriptional activation from regulatory regions containing class I and class II promoter elements in *Synechocystis* sp. PCC 6803

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The cAMP receptor protein (Crp) is a global transcriptional regulator that binds sequence-specific promoter elements when associated with cAMP. In the motile cyanobacterium *Synechocystis* sp. strain PCC 6803, intracellular cAMP increases when dark-adapted cells are illuminated. Previous work has established that Crp binds proposed Crp target sites upstream of *slr1351* (*murF*), *slr1874* (*chlA*), *slr1708* (*narL*), *slr0442* and *slr1268* in *in vitro*; and that *slr0442* is downregulated in a *crp* mutant during photoautotrophic growth. To identify additional Crp target genes in *Synechocystis*, 11 different Crp binding sites proposed during a previous computational survey were tested for *in vitro* sequence-specific binding and crp-dependent transcription. The results indicate that *murF*, *chlA*, and *slr0442* can be added as ‘target genes of Sycrp1’ in *Synechocystis*. Promoter mapping of the targets revealed the same close association of RNA polymerase and Crp as that found in *Escherichia coli* class I and class II Crp-regulated promoters, thereby strongly suggesting similar mechanisms of transcriptional activation.

INTRODUCTION

The cAMP receptor protein (Crp, or ‘Sycrp1’ in *Synechocystis* where required for clarity) can act as a transcriptional regulator when bound to the cAMP ligand (Botsford & Harman, 1992). Intracellular cAMP levels change dynamically to control gene regulation (Cann, 2004; Hammer et al., 2006; Kolb et al., 1993; Ohmori & Okamoto, 2004; Sakamoto et al., 1991). Various environmental conditions signal *Synechocystis* to maintain low, moderate and high intracellular cAMP levels that can be defined accordingly. Following dark adaptation, intracellular cAMP levels are low, at 0.02–0.04 pmol cAMP [μg chlorophyll (Chl) a]−1 (Terauchi & Ohmori, 2004). During regular photoautotrophic growth, intracellular cAMP levels are moderate [0.14–0.20 pmol cAMP (μg Chl a)−1] (Ochoa de Alda et al., 2000; Terauchi & Ohmori, 1999). Following illumination with either blue or white light, dark-adapted cells increase intracellular cAMP to high levels [0.60–0.80 pmol cAMP (μg Chl a)−1] (Masuda & Ono, 2004; Terauchi & Ohmori, 1998, 2004). These spectrum-specific photoreponses support the current view that the cAMP–Crp complex is ecologically beneficial for optimal positioning of motile cells relative to incident light (Bhaya et al., 2006; Masuda & Ono, 2004). Indeed, it has been shown that intracellular cAMP is necessary and sufficient to restore phototactic motility used by cells to escape from the confines of a colony during suboptimal illumination (Bhaya et al., 2006; Terauchi & Ohmori, 1999). Both Crp and cAMP are required for transcriptional activation of genes encoding type IV pilin biosynthesis proteins involved in motility, thereby strongly suggesting a role for regulation of motility by Crp (Yoshimura et al., 2002a, b).

To predict additional candidate genes for Crp regulation, a computational survey has previously proposed 11 different Crp binding target sequences based on the observation that Sycrp1 binds the *Escherichia coli* consensus ICAP Crp binding site (Ochoa de Alda & Houmard, 2000). Recently, a biochemical study by Omagari et al. (2008) demonstrated *in vitro* that systematic substitution of bases in ICAP could be used to fairly accurately predict the observed free energy change (ΔΔG_total) of Crp binding to any given DNA sequence (Omagari et al., 2008). The limit for detection of
Crp binding \textit{in vitro} was $\Delta \Delta C_{\text{total}}^{A} < 3.1$, and all intergenic sequences in the \textit{Synechocystis} genome containing calculated $\Delta \Delta G_{\text{total}}^{A} < 3.1$ were bound by Crp. The study by Omagari et al. (2008) demonstrated Crp binding to three of the 11 target sequences (\textit{slr1351}, \textit{slr1874} and \textit{slr1708}) predicted by Ochoa de Alda & Houmard (2000). Most recently, an interspecies bioinformatic comparison of cyanobacterial genomes (Xu & Su, 2009) has been performed, based in part on Crp binding sites in the \textit{Synechocystis} Crp transcriptome as identified by Yoshimura et al. (2002). Of the 53 target sequences that Xu & Su (2009) predicted for \textit{Synechocystis}, seven (\textit{slr1732}, \textit{slr1667}, \textit{slr1351}, \textit{slr1708}, \textit{slr1874}, \textit{slr0442} and \textit{slr1268}) were bound by Crp in the Omagari et al. (2008) study, and three (\textit{slr1351}, \textit{slr1708} and \textit{slr1874}) were also predicted by Ochoa de Alda & Houmard (2000). These predictive and \textit{in vitro} binding studies have not provided \textit{in vivo} evidence, nor elucidated possible mechanisms of transcriptional activation by Crp (i.e. whether Sycrp1-dependent promoters demonstrate the same well-characterized promoter organization as in \textit{Escherichia coli}).

In an attempt to elucidate possible mechanisms of transcriptional activation by Crp, sequence-specific Crp/DNA binding, transcriptional start sites, and Crp-dependent regulation of the \textit{slr1667}--1668 operon have been demonstrated (Yoshimura et al., 2002a). Even though the results have not established a plausible mechanism (see discussion), this operon has subsequently been discussed in the context of Crp regulation (Dienst et al., 2008; Singh et al., 2008; Summerfield & Sherman, 2008). Furthermore, the Kazusa Cyanobase describes these genes as 'target genes of Sycrp1', based on data that demonstrated both (1) \textit{in vitro} sequence-specific binding and (2) Crp-dependent gene expression. These two criteria will be referenced as such throughout this text. No other genes have been so annotated in the \textit{Synechocystis} genome to date.

To identify additional 'target genes of Sycrp1', all Crp targets proposed by Ochoa de Alda & Houmard (2000) and a target (\textit{slr0442}) proposed by Omagari et al. (2008) were studied using a motile \textit{Synechocystis} strain capable of displaying large increases in intracellular CAMP following illumination. These proposed targets were tested \textit{in vitro} for sequence-specific Crp/DNA binding, and expression was monitored in wild-type and \textit{crp} cells to assess Crp-dependent regulation during a dark to light environmental change that causes a low to high intracellular CAMP change. The results indicate that \textit{slr1351} (\textit{murF}), \textit{slr1874} (\textit{chlaB}) and \textit{slr0442} can be classified as 'target genes of Sycrp1' in \textit{Synechocystis}. Plausible Crp activation mechanisms of these cyanobacterial Crp targets are discussed based on transcriptional start sites mapped in \textit{Synechocystis} and similar expression of promoter--reporter constructs derived from these targets and expressed in \textit{E. coli}.

**METHODS**

**Strains and growth conditions.** The wild-type motile glucose-sensitive \textit{Synechocystis} PCC sp. strain 6803 was obtained from the Pasteur Culture Collection of Cyanobacteria. All \textit{Synechocystis} cells were pre-grown for 11 days in BG-11 medium (Stanier et al., 1971) containing 75.0 mM TES, pH 7.75, 10.0 mM bicarbonate, and were supplemented with 5.0 mM bicarbonate every 12 h in a manner that maintained exponential growth at pH 7.75 in an inorganic carbon-replete condition. \textit{Synechocystis} cells were grown at 30 °C and illuminated with 30.0 μmol photons m$^{-2}$ s$^{-1}$ from cool white fluorescent lamps. Cultures in mid-exponential phase (OD$_{730}$ 0.6) were washed in fresh media and transferred to the dark 16 h prior to sampling. Samples for RNA extraction were rapidly chilled on ice water, pelleted in a prechilled rotor for 10 min at 4000 g, and flash-frozen immediately following sampling in the dark, 30 and 60 min following illumination. \textit{E. coli} K-12 M182 Δlac wild-type (Casadanb et al., 1980; Casadaban & Cohen, 1980) and \textit{crp} mutant (Busby et al., 1983) stock cultures were kindly provided by Stephen Busby (University of Birmingham), and were maintained in Luria–Bertani (LB) medium supplemented with 30.0 μg streptomycin ml$^{-1}$ and 50.0 μg ampicillin ml$^{-1}$, respectively. \textit{E. coli} clones containing reporter plasmids were grown in LB with or without 3% glucose in a roller drum at 37 °C for 48 h and assayed for green fluorescent protein (Gfp) signal as described below. All antibiotics were omitted from experimental cultures. \textit{E. coli} DH5x MCR was used for plasmid amplification.

**Molecular biology techniques.** Plasmid purifications, isolation, PCR, ligation reactions, Southern blotting and transformations were performed according to standard protocols (Ausubel et al., 2000) using commercial kits for DNA purification. \textit{Synechocystis} sp. PCC 6803 genomic DNA was harvested as previously described (Summers et al., 1995).

**sycrp1 mutant construction.** The \textit{sycrp1} gene (\textit{slr1371}) was amplified from genomic DNA using primers ATTCAGAG-TTTACTGAGGCGT and CCTGAGTTGGCACCAGCTGA and cloned into pCR2.1 (Invitrogen). The cloned gene was then inactivated by insertion of a \textit{PsvI} fragment of \textit{pZeo} (Stevens et al., 1996), containing the \textit{ble} zeocin-resistance of \textit{pZeo}, into the \textit{Smal} site within \textit{slr1371} to produce pK2L. This insertion was verified by sequencing at the California State University sequencing facility. The wild-type strain was subject to natural transformation with pK2L and selection in zeocin to yield CAMP receptor protein \textit{sycrp1::ble mutants} (\textit{crp}). Zeocin-resistant \textit{crp} mutant stock cultures were maintained in BG-11 supplemented with 6.0 μg zeocin ml$^{-1}$.

**Gfp reporter construction and quantification.** A novel \textit{SphI} site was introduced into the multiple cloning site of the pIGA transcriptional reporter plasmid (Kunert et al., 2000) via a custom adaptor created by annealing GAGGGTACCGCATGCGGTACCTCA and TGAGGTACCAGCATGGGTACCTCA. \textit{KpnI} digestion and ligation of the adaptor (\textit{KpnI-SphI-KpnI}) located downstream of a strong T7 transcription terminator sequence, but upstream of \textit{gfp}, into the \textit{KpnI} site of \textit{pIGA} created \textit{pIGS}. The promoter region and 5′ N terminus of the indicated \textit{Synechocystis} genes were amplified by PCR using gene-specific primer sets (Supplementary Table S1) that added restriction sites for \textit{KpnI} or \textit{SphI}. The PCR product was digested, and ligated into \textit{pIGA} or \textit{pIGS}. Primers flanking the multiple cloning site (Argueta et al., 2004) were used to sequence each insert, thereby confirming the proper orientation and absence of mutation. The \textit{slr0442} reporter construct was created by ligation of a partial \textit{Hsp92II} \textit{Synechocystis} genomic digest into \textit{pIGS} and screening of \textit{E. coli} DH5x clones with and without glucose. The identified clone contained the almost complete \textit{slr0442} intergenic region from chromosomal positions 2080 200 to 2080 940 (Kaneko et al., 1996). These resultant reporter plasmids were used to transform \textit{E. coli} K-12 M182 Δlac wild-type and \textit{crp} mutant strains via electroporation followed by selection in LB supplemented with 50.0 μg kanamycin ml$^{-1}$. 
E. coli M182 cells containing reporter constructs were washed twice in PBS and normalized to OD\textsubscript{595} 0.25 immediately prior to measuring Gfp fluorescence, as previously described (Argueta & Summers, 2005).

**RNA isolation and reverse transcriptase-mediated quantitative PCR (RT-QPCR).** RNA was isolated as previously described (Kim et al., 2006) and visualized for integrity in a formaldehyde gel. Genomic DNA was removed by two rounds of RQ1 DNase (Promega) digestion and Zymoclean (Zymo Research) column purification according to the manufacturers’ instructions. The absence of genomic DNA in the resultant RNA samples was confirmed by the absence of product following PCR using rnpB primers and a genomic control. RNA samples were normalized to 1.0 g l\textsuperscript{-1} immediately prior to measuring OD\textsubscript{595} 0.25 immediately prior to measuring Gfp fluorescence, as previously described (Argueta & Summers, 2005).

**Sequences of coding strand (complement not shown) of the double-stranded blunt-ended oligonucleotide DNA used in EMSA and calculated ΔΔG\textsubscript{total} values for the consensus**

The putative Sycrp1 core binding consensus is indicated in bold type. ORF identifiers of genes demonstrating both sequence-specific binding and sycrp1-dependent transcriptional regulation are in bold underlined type. ΔΔG\textsubscript{total} values <3.1 are in bold type. The value of ΔΔG\textsubscript{total} was calculated by strict summation of position values given by the position-specific scoring matrix in Omagari et al. (2008), except that G and C substitution ΔG values at positions 9 and 14 were switched to accurately reflect the authors’ intent.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>ΔΔG\textsubscript{total}</th>
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</thead>
<tbody>
<tr>
<td>slr1667</td>
<td>ATACACAACAGGTGTTGGTGCTGGTCACAACCAGTTGAGTGA</td>
<td>0.34*†</td>
</tr>
<tr>
<td>Rndm.</td>
<td>AAGCCGTTAGACCTAATGTGAAAGTGTCCTCAAGACTTCAC</td>
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<td>str1991</td>
<td>AGGCTCCCTGAATGGGACACGGTCAGGACCTTACTATTA</td>
<td>5.85§</td>
</tr>
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<td>TTCCTAATCTATGTGAGGAGTTTTTGCGAAATGCAAGCTTT</td>
<td>8.04§</td>
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<tr>
<td>str0194</td>
<td>AAACCGAAGCTGTCGATAAGTTTGCGACCTTGAATTT</td>
<td>9.01§</td>
</tr>
<tr>
<td>slr1351</td>
<td>GCACACCATGGGAAGTTGCTTAGTACAGATACAGATAAAATAGGC</td>
<td>0.67*†††§§</td>
</tr>
<tr>
<td>str1375</td>
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<td>7.35§</td>
</tr>
<tr>
<td>slr1708</td>
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<td>3.07*†††§§</td>
</tr>
<tr>
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<td>7.85§</td>
</tr>
<tr>
<td>slr0041</td>
<td>GAATTCACTTCTTCGGAAGAGCTACATCTTCAC</td>
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</tr>
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<td>str1200</td>
<td>CAAATGGCAACATGTGATATGTTCTCAGCTTGCCCCCACCTGCCCAGAC</td>
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<td>0.00*†§§</td>
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<td>slr1268</td>
<td>TCGCCAATAGTTGTTATCATGACATATGACAGGGCCACGCG</td>
<td>0.00*†§§</td>
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*Oligonucleotide sequences demonstrating sequence-specific His–Sycrp1 binding in this work.
†Sycrp1 binding sequences proposed by Xu & Su (2009).
§Sycrp1 binding sequences proposed by Ochoa de Alda & Houmard (2000).
§§Sycrp1 binding sequences proposed by Omagari et al. (2008) to demonstrate His–Sycrp1 binding.
visualization substrate from Novagen, were used according to the manufacturers’ recommendations.

5′-Phosphate-free oligonucleotides were synthesized by Integrated DNA Technologies without HPLC purification, mixed 1:1 with their complement, annealed by boiling followed by slow cooling over 3 h, and gel-purified by UV-shadowing following native PAGE of DNA alone. This process allowed visualization of bands for excision and gel-purified by UV-shadowing following native PAGE of DNA.

For native PAGE gel-shift experiments, the binding reaction buffer contained 20 μM cAMP, 1.0 nM labelled dsDNA, His–SyCrp1 as indicated, 50 mM Tris/HCl, pH 7.5, 60 mM NaCl, 1.0 mM EDTA, 8.3% (v/v) glycerol, and 0.1 mg acetylated BSA ml⁻¹. Reactions were incubated at 22 °C for 25 min, then on ice for 15 min. The reactions were quickly and directly loaded onto the gel without loading buffer or dye. A voltage of 90 V was immediately applied for 10 min, and then increased to 200 V for an additional 35–45 min. The gel apparatus and buffers were pre-chilled and maintained at 4 °C. A voltage of 90 V was applied for at least 30 min prior to loading to remove mobile charged molecules. All running buffers were titrated to pH 8.0 to match the reaction buffer at 4 °C. Components that varied in the reaction buffer are indicated in the legend to Fig. 1.

RESULTS

Crp sequence-specific binding was demonstrated

To demonstrate Crp binding in vitro, a positive binding control, slr1667, a randomly generated sequence, and all target sites proposed by Ochoa de Alda & Houmard (2000) were screened for Crp binding (Fig. 1a, Table 1). The chlA, narL, mcrp, and mcrp target sites were bound by His–SyCrp1. These proposed target sites are the only loci common to all Omagari et al. (2008), Ochoa de Alda & Houmard (2000) and Xu & Su (2009) predictions. These results were in accord with the inability of Omagari et al. (2008) to detect binding to any proposed site that has a calculated ΔΔGₜₐₜₒ₉₉ >3.1. In Fig. 1(a), binding to narL was detected, ΔΔGₜₐₜₒ₉₉ = 3.1; consequently, sensitivity similar to that achieved by Omagari et al. (2008) was demonstrated in this assay. The Kₐ of His–SyCrp1 from all proposed targets bound in Fig. 1 has been described (Omagari et al., 2008). In side-by-side experiments, all previously published interactions were detected and yet the test failed to identify any new interactions among targets proposed by Ochoa de Alda & Houmard (2000).

To demonstrate cAMP dependence for Crp/DNA binding in our in vitro binding conditions, all oligonucleotides

![Fig. 1](image-url) EMSA demonstrating bound His–SyCrp1/DNA complexes and unbound DNA. The 40 bp radiolabelled dsDNA oligonucleotides surrounding previously proposed Crp binding sequences (Table 1) are indicated. (a) Side-by-side comparison of all targets predicted by Ochoa de Alda & Houmard (2000). Labelled slr1667 and Rndm. oligonucleotides are included as positive and negative controls, respectively. (b) Competitive binding to high-affinity and (c) low-affinity Crp binding sequences. Unlabelled competitor was added as indicated in addition to reaction buffers containing: (a, c) 500.0 nM His–SyCrp1, 20.0 μM cAMP, 500.0 nM unlabelled double-stranded Rndm. oligonucleotide and 1.0 nM of the labelled dsDNA indicated; (b) 100 nM His–SyCrp1, 20.0 μM cAMP and 1.0 nM of the labelled dsDNA indicated. Running buffers and 10 % acrylamide composition were: (a, c) 0.25 X TBE, pH 8.0, at 4 °C, 20 μM cAMP and 50:1 (w/w) acrylamide: bis-acrylamide; (b) 1.0 X TAE, pH 8.0, at 4 °C, 20 μM cAMP and 30:0.8 (w/w) acrylamide: bis-acrylamide.
listed in Table 1 were assayed exactly as in Fig. 1(a), except that cAMP was omitted from the reaction, running buffers and gels. Likely due to a combination of high affinity (even greater than that for the E. coli consensus ICAP) (Omagari et al., 2008) and cAMP carried over from E. coli expression, Crp binding to the proposed murF target was detectable. However, binding was severely reduced to <10 % bound as opposed to 100 % in the presence of 20 μM cAMP. Detectable binding was absent in all other instances (data not shown).

To further demonstrate reproducibility and sequence-specificity for these proposed binding sites, competition assays were performed (Fig. 1b, c). The putative Crp binding sites located upstream of slr0442 and sll1268 were also included. Expression of slr0442 is downregulated in a crp mutant (Yoshimura et al., 2002a); consequently, slr0442 was used as a positive control. The sll1268 target proposed by Omagari et al. (2008) was included because of the high degree of conservation between it and slr0442 in the intergenic and N-terminal coding regions. Our results demonstrated His–Sycrp1 sequence-specific binding to murF, narL, chlA_H, slr0442 and sll1268 proposed targets via competition assays using the sfr1667 target as a specific competitor (Yoshimura et al., 2002a) and a random 40-mer (Rndm.) as a non-specific competitor. In all cases, the unlabelled specific competitor titrated Crp away from the labelled complex in favour of the specific competitor, while unlabelled non-specific competitor did not. Crp does not bind the slr1667 target in the absence of cAMP in vitro as reported by Yoshimura et al. (2002a) and reproduced here (see above). Consequently, titration by competitors further demonstrated the presence of Crp/cAMP complex. Omagari et al. (2008) have established the Crp sequence-specificity to these proposed targets by correlation. Shown in Fig. 1(b, c) is the first verification of specificity by direct competition. These results confirmed that the murF, narL, chlA_H, slr0442 and sll1268 intergenic sites described in Table 1 met sequence-specific binding criteria.

Owing to complex instability during electrophoresis at room temperature, as evidenced by smearing between bands in the work of Omagari et al. (2008) and reproduced in this work (data not shown), electrophoresis at 4 °C was performed. Despite the strong signal from labelled DNA, increased complex stability at 4 °C was demonstrated, because smearing between bands was minimal to absent. However, at 4 °C, Crp/DNA complexes precipitated in 0.25 × TBE, which rendered them immobile by electrophoresis. Addition of 500 nM Rndm. completely restored solubility and allowed near 100 % binding, as shown by slr1667 and murF targets (Fig. 1a). As little as 0.5 mg l−1 double-stranded polydeoxyinosinic-deoxycytidylic acid (poly-dIdC) added to the reaction buffer also restored solubility, but reduced the fraction of Crp/DNA complex by 60 % (data not shown). Crp/DNA complexes were soluble in 1.0 × TAE at 4 °C, but low-Crp-affinity targets (ΔΔCTotal > 0.7) did not maintain Crp/DNA complexes in this running buffer. Consequently, electrophoresis of narL and chlA_H was performed in 0.25 × TBE. From these results, it is clear that the temperature and ionic strength of electrophoresis buffers greatly affect Crp/DNA complex detection by gel shift.

**The sycrp1 mutant construction was gene-specific, and did not introduce polar effects**

To allow examination of Crp-dependent functions, a crp mutant was constructed by insertional inactivation of sycrp1. Complete segregation was confirmed by PCR. Southern blotting further confirmed that recombination had occurred specifically in the sycrp1 locus and that slr1924 (sycrp2) or slr0593 homologues were not disrupted (data not shown). Additional evidence for gene inactivation was obtained by observing phototactic and crp non-motile phenotypes (Yoshimura et al., 2002b) (data not shown). To discount polar effects of genes surrounding the site of sycrp1 inactivation, transcript abundance of the two genes flanking sycrp1 (sll1370 and sll1372) was quantified in phototaxotrophically growing cultures by RT-QPCR. The quantities of these transcripts in the wild-type did not differ detectably from those in crp mutant strains (Vasquez, unpublished results). To determine whether Crp function was absent in the crp mutant, wild-type and crp crude cell extracts were also assayed for binding to the slr1667 target. Sequence-specific binding was absent in the crp mutant crude extracts but present in wild-type samples (data not shown). Therefore, gene expression differences were ascribed specifically to inactivation of the sycrp1 locus and resultant protein inactivation rather than to polar effects or recombination at non-target sites.

**The shift from dark to light environmental conditions stimulated Crp-dependent transcriptional activation**

To confirm that intracellular cAMP increased under the experimental conditions described, it was quantified by a cell filtration method. Intracellular CAMP increased from 0.046 to 0.92 pmol cAMP (μg Chl a)−1 following a dark (low intracellular cAMP) to light (high intracellular cAMP) transition. These values were in good agreement with those previously reported (see introduction).

To quantify transcript levels, RNA samples were collected in the dark and at 30 and 60 min after illumination. Cultures were sampled over 1 h, because transcriptional profiles are most dynamic during this period (Gill et al., 2002). All target transcripts proposed by Ochoa de Alda & Houmard (2000) were quantified in a low-resolution screen. RNA was sampled from one culture of wild-type cells (Vasquez, unpublished results) to focus effort on non-locus sycrp1 target. Therefore, gene expression differences were ascribed specifically to inactivation of the sycrp1 locus and resultant protein inactivation rather than to polar effects or recombination at non-target sites.
entiate between candidate Crp-dependent and Crp-independent expression. The slr0194 (rpiA) transcript was one of nine proposed targets that did not demonstrate Crp-dependent transcription in this low-resolution screen. Consequently, it was used as a negative, Crp-independent transcription control. Only murF and chlAI demonstrated more than twofold Crp-dependent expression out of the 11 targets proposed by Ochoa de Alda & Houmard (2000) tested in this low-resolution screen.

Transcription of rpiA, murF, chlAI, and slr0442 were further characterized to determine Crp-dependence following dark to light environmental changes (Fig. 2). Wild-type and crp cells were again cultured, this time in triplicate (n=3), to demonstrate reproducibility, and transcripts were quantified by RT-QPCR more accurately, taking amplification efficiency into account. The positive transcription control slr0442 was not activated in the wild-type either 30 min (grey bars) or 60 min (white bars) following

![Fig. 2](http://mic.sgmjournals.org)
illumination (Fig. 2a). The quantity of detectable transcript was constant for all time points versus the initial low intracellular cAMP condition, and resulted in an expression ratio of 1.0. Conversely, transcript levels in the mutant decreased following illumination (Fig. 2b). After 1 h illumination, wild-type expression of slr0442 was five times greater than that of the mutant (Fig. 2c) and almost twice that reported during moderate intracellular cAMP growth conditions (Yoshimura et al., 2002a). Consequently, it was inferred that the constant slr0442 expression in wild-type cells was due to a steady state achieved by simultaneous transcriptional activation by Crp and post-transcriptional mRNA degradation.

This inference is supported by evidence that both crp and ssr3321 (hfq candidate) single mutants display striking similarity in expression of slr2015–2018, slr1667–1668 and slr0442, which are downregulated approximately four to five-, 40–48- and threefold, respectively, relative to wild-type cells during regular photoautotrophic growth (Dienst et al., 2008; Yoshimura et al., 2002a). Hfq is an RNA chaperone that acts to stabilize transcripts as a RNA chaperone or to facilitate the coupled degradation of sRNA–mRNA duplexes (Dienst et al., 2008). Although the mechanism of Hfq activity has not yet been demonstrated in cyanobacteria, it could explain post-transcriptional modification of slr1667 and slr0442. Under high-light stress, the 3′ mRNA of slr1667 is three to fourfold (clarified by personal communication with A. Singh) more abundant than the 5′ end (Singh et al., 2008). This finding clearly demonstrates strong post-transcriptional mRNA degradation that could be stabilized by a functional RNA chaperone.

Wild-type and mutant transcript levels for all genes in Fig. 2 were approximately equal in dark-adapted cells when intracellular cAMP was low. Transcripts of murF and chlAII were upregulated by illumination, but showed four and ten times, respectively, more transcript expression relative to the mutant following intracellular cAMP increase, thus demonstrating strong Crp dependence for transcription activation. In contrast, transcription from the negative transcription control rpiA did not exhibit Crp dependence, even though it was strongly induced following illumination (Fig. 2a, b). In sum, these results demonstrated that transcription from murF, chlAII and slr0442 met Crp-dependent expression criteria.

Expression driven by SyrJ ‘target’ promoters required Crp in E. coli

To determine whether the transcriptional machinery in E. coli is sufficient to stimulate Crp-dependent transcription from Crp ‘target’ promoters, all target promoters proposed by Ochoa de Alda & Houmard (2000) were oriented to drive transcription of a gfp reporter in wild-type and crp mutant strains of E. coli. Transcripts from cells grown in the high intracellular cAMP condition demonstrated Crp-dependent activation. The glucose effect is well documented (Kolb et al., 1993) and causes a drastic drop in intracellular cAMP. To decrease intracellular cAMP, glucose was added to the culture.

The positive transcription control slr0442 was strongly induced in the wild-type during high intracellular cAMP growth without glucose (Fig. 2d), but not in the mutant (Fig. 2e). Although wild-type expression was four times that of the mutant in the low intracellular cAMP growth condition with glucose (Fig. 2f), expression was 60 times greater in the high intracellular cAMP condition, thereby demonstrating a strong Crp activation dependence. Although not shown, it is interesting to note that the wild-type strain repressed narL transcription in the high intracellular cAMP condition 10 times more than in the crp mutant. The narL reporter was also independently isolated from the Hsp92II genomic reporter library due to similar expression characteristics (data not shown). Otherwise, in general, the E. coli reporter data paralleled results seen in Synechocystis, excepting chlAII. In this case, absolute fluorescence was indistinguishable from background fluorescence, indicating that the chlAII promoter did not drive transcription in E. coli. The background fluorescence between E. coli M182 wild-type and crp strains containing the gfp reporter plasmid but lacking the indicated intergenic regions was indistinguishable (data not shown). All other indicated constructs yielded signals well above this background. Consequently, these data demonstrated that the transcriptional elements in E. coli were sufficient to stimulate Crp-dependent transcription from slr0442 and murF intergenic regions.

Transcription start sites were determined

All presented transcription +1 start sites for murF, narL, chlAII, slr0442 and slr1268 were determined by RACE in this work (Fig. 3). These promoters are accordingly labelled in Fig. 3, and the most distal from the gene is assigned P1. We are unaware of any other studies mapping start sites for these genes. It should be noted that RACE requires much less transcript than primer extension due to its high sensitivity. However, RACE is not a quantitative method; consequently, the relative strengths of these promoters as affected by Crp activation were not inferred.

RNA from both wild-type and crp strains experiencing both low and high intracellular cAMP all yielded the same start sites, although ±1 base chatter between samples was observed at murF P2 (Fig. 3a). P1 TGGTAAGATACA-CCCTG (transcriptional start site in italicized bold type) is not shown and lies 136 bases upstream of P2. Crocosphaera watsonii and Cyanophora sp. CYY 0110 MurF whole-protein BLAST scores were 9e-136 and 1e-129, respectively, relative to Synechocystis MurF. The intergenic and non-conserved N-terminal murF regions from these closely related cyanobacteria were aligned to highlight other conserved elements because of an apparent conservation of the proposed Crp site. A total of 30 and 31 base gaps were observed for Crocosphaera and Cyanophora, respectively in

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the region between the conserved Crp core binding sequence and the strongly conserved protein-coding region. These deletions corresponded almost exactly to three 10.5 bp turns of the α-helix. In the alignments shown, a 12 bp gap that corresponds to approximately one helical turn is located between P3 and the putative Crp binding site.

The intergenic chlAII region contained only one putative transcriptional start site (Fig. 3b). Similar sequence alignments of intergenic regions from chlA orthologues of these closely related cyanobacteria were uninformative due to low sequence homology and the absence of readily identifiable Crp site core sequences. Consequently, an alignment is not shown.

The slr0442 and sll1268 intergenic sequences were aligned, because the encoded proteins of these genes bear 75% identity within the first 58 N-terminal amino acids, and the proposed Crp sites were roughly equidistant from the common, not the annotated, ATG start codon (Fig. 3c). Only one base-spacing difference was observed between the transcription start and the proposed Crp sites for these two gene promoters.

**DISCUSSION**

Some proposed ‘target genes of Sycrp1’ may not have been detected

The utility of dark to light conditions for elucidating Crp-dependent activation as proposed by Omagari *et al.* (2008) has been demonstrated by the results reported here. Predicted targets were upregulated in a Crp-dependent manner only when predictions made by Ochoa de Alda & Houmard (2000), Xu & Su (2009), and Omagari *et al.* (2008) overlapped (bold and underlined type in Table 1). In all cases, excluding narL, those intergenic regions containing Crp core binding sites <3.1 ΔΔG<p><sub>total</sub> were bound by His–Sycrp1 in vitro and demonstrated
Crp-dependent activation in vivo. Consequently, murF, chlA_H and slr0442 meet the ‘target gene of Syrcp1’ criteria used to annotate the Kazusa Cyanobase.

It is, however, conceivable that the low-resolution expression screen overlooked subtle expression differences such as those of the divergently transcribed regulatory genes narL and slr1805 (hik16 subunit). The possible protein–protein interactions suggested by yeast two-hybrid experiments in which NarL interacts with Hik16 and MurC (Sato et al., 2007) suggest protein-level regulation in the first step of the peptidoglycan biosynthetic pathway three enzymic reactions upstream of MurF-catalysed ligation. Such regulation is expected to be involved in modulating the balance of intracellular carbon and nitrogen (Singh et al., 2008).

Consistent with the only microarray data, to our knowledge, published using the motile glucose-sensitive Synechocystis exposed to similar environmental conditions (Gill et al., 2002), the target transcripts in Fig. 2 that were proposed by Ochoa de Alda & Houmard (2000) were strongly upregulated following illumination. Such upregulation is also consistent with the functions of these gene products. Specifically, murF upregulation is expected, because its protein product is essential for the peptidoglycan synthesis required for cell division during periods of growth (Malakhov et al., 1995). Also, chlA_H upregulation upon illumination is expected, because cultures that are dark-adapted for prolonged periods and attain oxygen equilibrium with the air are micro-oxic relative to actively photosynthesizing cultures. This micro-oxic state is achieved via the same mechanisms that cause the diurnal dissolved oxygen cycles observed in lakes and cyanobacterial mats (Jorgensen et al., 1979). Under micro-oxic illuminated conditions, chlA_H is transcribed in a putative operon containing ho2 (Sugishima et al., 2005; Xu & Su, 2009; Zhang et al., 2005) and hemN1, which catalyse three steps in the chlorophyll biosynthetic pathway (Minamizaki et al., 2008). The Crp-dependent chlA_H activation observed suggests coordination between motility and photosynthetic acclimation, but requires further characterization of both pilin and chlA_H Crp-dependent expression at these promoters.

Analysis of elements in Syrcp1 class I and II promoters

Although mutagenesis was not used to demonstrate that the proposed Crp binding sites in Fig. 3 are required for gene activation, in vivo evidence exists to support such a conclusion. First, the conserved spacing of proposed Crp binding and +1 transcriptional start sites between these promoters and extensively characterized promoters of E. coli was observed. Second, in vivo transcriptional activation under conditions stimulating high intracellular cAMP required Crp in both E. coli and Synechocystis, thus demonstrating that transcription elements in E. coli are sufficient to stimulate Crp-dependent transcription from murF and slr0442 intergenic regions (Fig. 2). Together these data indicate that the E. coli Crp mechanisms can be compared with those in Synechocystis.

E. coli Crp promoters are classic model systems that have been thoroughly reviewed recently (Borukhov & Lee, 2005) and in the past (Busby & Ebright, 1999). By definition, Crp and RNA polymerase (RNAP) must be on the same side of the DNA strand to make contacts that stimulate transcription via the readily describable mechanisms of class I, class II and class III promoters. Consequently, intervals of 10.5 bp z-helical turns must be maintained from the middle of the −10 sigma factor binding site to the middle of the Crp site for Crp to contact RNAP. Five or more turns is defined as class I, and three turns as class II. Four-turn spacing does not occur, because several Crp–RNAP interactions would be impeded. In Synechocystis, transcription start site mapping of the slr1667–1668 operon has revealed that the proposed Crp binding site is 15.5 helical turns upstream from the middle of the −10 region, thereby placing Crp on the opposite side of the DNA strand relative to RNAP (Yoshimura et al., 2002a). In this case, Crp cannot contact RNAP via the readily describable mechanisms outlined here. Consequently, the Crp activation mechanism at this locus is unclear. As opposed to class I and class II promoters, class III promoters require two or more activator molecules and RNAP for full transcription activation. A major difference between E. coli and cyanobacterial promoters is the frequent absence of a −35 sigma factor binding site (Curtis & Martin, 1994); however, the −10 region TATAAT is conserved and TANNNT is most frequently observed (Su et al., 2005; Vogel et al., 2003). The proposed class I and class II promoters described below are inferred based on this spacing, until the involvement of an additional element is demonstrated to define class III organization.

The murF P3 contains class I promoter spacing relative to the transcriptional start site

The murF P3 bears class I promoter structure only in that the proposed Crp binding site is 7.0 z-helical turns from the transcriptional start site (Fig. 3a). However, the −10 region is not readily discernible within four to seven bases of the transcriptional start. The only conserved TNNNNT sequence places Crp centred 5.7 helical turns away in suboptimal positioning but close to the same side of the DNA strand as the proposed sigma factor binding site. However, the spacing of these elements is not conserved among these freshwater cyanobacteria. Instead, deletions totalling three helical turns seem to have occurred independently because the deletions are not of identical lengths. One is 30, the other 31 bp long. This keeps the elements that are retained on either side of the deletions in similar helical orientation and on same side of the DNA strand in the Crocosphaera and Cyanothece sequences shown. Therefore, the observed conservation of helical spacing may be significant for regulation. Xu & Su (2009)
predicted a −10 region, TAACAT, located 32 bp downstream from the proposed Crp binding site. This −10 region is not properly positioned to initiate transcription from any of the +1 sites identified by RACE. Retention of the proposed Crp core binding sequences in these closely related cyanobacteria suggests that Crp regulation of \textit{murF} is also conserved.

The \textit{chlA}_{II} promoter class is unclear

The \textit{chlA}_{II} promoter class is unclear because the proposed Crp site is very distant and on the opposite face of the DNA strand relative to the transcriptional start site (Fig. 3b). The proposed Crp site is 28.6 helical turns from the transcriptional start site. Furthermore, a plausible −10 region is not apparent; thus, we cannot support the validity of this proposed transcriptional start site by relation to other conserved elements. Xu & Su (2009) predicted that a −10 region, TCGATT, is 29 bp downstream of the proposed Crp site; however, no +1 sites were identified by RACE in this region.

The \textit{slr0442} \textit{P}_{2} contains class II promoter spacing

The \textit{slr0442} \textit{P}_{2} bears class II promoter structure maintaining the characteristic three \textit{x}-helical turn spacing between the centre of the near-consensus \textit{P}_{2} −10 region TAAAT and the proposed Crp site (Fig. 3c). Crp binding would repress transcription from \textit{P}_{2} via steric hindrance of RNAP, thereby switching most initiation to \textit{P}_{2}. The proposed intimate proximity with RNAP strongly suggests interactions between Crp and RNAP; however, the analysis of activating regions 2 and 3 described for \textit{E. coli} (see reviews cited above) by primary sequence alignment is insufficient to address the possibility of these interactions. The perfect alignment of the proposed Crp and −10 sites in both \textit{slr0442} and \textit{sll1268} was also predicted by Xu & Su (2009), and strongly suggests conservation of function as class II Crp promoter regulation. A second Crp site that was proposed by Omagari \textit{et al.} (2008) (boxed with dashes in Fig. 3c) has not been confirmed by binding studies. It is optimally positioned on the same side as RNAP, thereby potentially implicating class III promoter structure.

The genes \textit{slr0442} and \textit{sll1268} are homologous within the N-terminal domain. This homology defines a large set of hitherto uncharacterized cyanobacterial proteins. The significance of this conserved region suggests coordinated regulation of \textit{slr0442} and \textit{sll1268} by Crp.

As has been discussed above for \textit{murF} and \textit{slr0442}, promoter mapping revealed the same well-characterized class I and class II promoter organization in \textit{Synechocystis} as in \textit{E. coli}. When intergenic regions containing these promoters were oriented to drive \textit{gfp} transcription in \textit{E. coli}, the results paralleled the regulatory effects observed in \textit{Synechocystis}. These results thereby illustrate structure and function associations \textit{in vivo} (Fig. 2), and strongly suggest that cyanobacterial Crp-dependent promoter mechanisms can function similarly to those in \textit{E. coli}. Furthermore, we provide the first experimental evidence, to our knowledge, to support the validity of bioinformatic predictions based on class II spacing of −10 and Crp site elements in cyanobacteria (Xu & Su, 2009).

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