DNA-Binding Properties of the *Fremyella diplosiphon* RpbA Repressor

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Mutant strain FdBM1 of the cyanobacterium *Fremyella diplosiphon* is characterized by elevated transcription of the cpcB1A1 gene set due to inactivation of *rpbA* by Tn5469. The predicted RpbA protein contains two regions resembling the characterized helix-turn-helix (HTH) motif involved in DNA recognition by many phage and bacterial transcription regulator proteins. It was therefore hypothesized that RpbA functions as a DNA-binding repressor involved in the control of transcription from cpcB1A1. A histidine-tagged form of RpbA, designated RpbA-His<sub>6</sub>, was examined for its ability to bind to the defined promoter region for cpcB1A1. Gel mobility shift assays showed that RpbA-His<sub>6</sub> specifically binds to a DNA fragment containing the cpcB1A1 promoter and that significant binding can be achieved with equimolar amounts of RpbA-His<sub>6</sub> and the cpcB1A1 promoter probe. DNase I footprint analysis localized the RpbA-His<sub>6</sub> binding site to an asymmetric 21-bp region that overlaps the putative −10 promoter sequence. A mutational analysis suggested that binding by RpbA-His<sub>6</sub> to its cognate DNA may involve both putative HTH motif-like regions. We conclude that RpbA functions as a transcriptional repressor for cpcB1A1 and suggest that binding by RpbA to its cognate DNA may represent an atypical protein-DNA interaction.

Cyanobacteria harvest light energy for photosynthesis with macromolecular antenna complexes termed phycobilisomes (PBS) (for reviews, see references 8 and 22). The PBS consist of two structural domains: a core which peripherally attaches to the photosynthetic membrane and a series of rods that radiate away from the core. Both domains consist of chroomophoric phycobiliproteins and nonchromophoric linker polypeptides. The major cyanoecytorial phycobiliproteins are allophycocyanin, phycocyanin (PC), and phycoerythrin (PE); allophycocyanin is localized to the core in the form of stacked trimers, whereas PC and PE are localized to the rods in the form of stacked hexamers. The linker proteins serve to maintain PBS structure and facilitate energy transfer within the complex and to the photosynthetic apparatus.

The rod PC and PE content for PBS in the cyanobacterium *Fremyella diplosiphon* UTEX 481 is attuned to environmental parameters (for reviews, see references 8, 9, and 24). Three gene sets that encode PC are present in this strain: cpcB1A1 (encodes PC<sub>1</sub>), cpcB2A2 (encodes PC<sub>2</sub>), and cpcB3A3 (encodes PC<sub>3</sub>). In contrast, a single gene set (cpeB) encodes PE. Under nutrient-replete conditions, the rod phycobiliprotein composition is regulated by green and red light via a process termed complementary chromatic adaptation. Green-enriched light promotes synthesis of rods composed of three distal hexamers of PE linked to the core by a PC<sub>1</sub> hexamer, whereas red light promotes synthesis of rods composed of two distal hexamers of PC<sub>2</sub> linked to the core by a PC<sub>1</sub> hexamer. Complementary chromatic adaptation is mediated through differential transcription of the gene sets encoding PE and PC<sub>2</sub> (7) and provides cells an adaptive advantage, as PE absorbs green light and PC<sub>2</sub> absorbs red light. Under sulfate-limiting conditions, cells cease expression of PC<sub>1</sub>, PC<sub>2</sub>, and PE and initiate expression of PC<sub>3</sub> (17). This acclimation response is also mediated at the transcriptional level and is thought to provide the cells an adaptive advantage because PC<sub>3</sub> is significantly reduced in sulfur-containing amino acids.

We are examining the molecular mechanisms involved in environmental control of phycobiliprotein gene expression in *F. diplosiphon*. Earlier, we characterized pigmentation mutant strain FdBM1, which exhibits elevated constitutive transcription of cpcB1A1 due to Tn5469 inactivation of the rpbA gene (12, 13). The predicted RpbA protein contains two regions resembling the characterized helix-turn-helix (HTH) motif involved in DNA recognition by many bacterial and phage transcription regulator proteins (10), suggesting that it functions as a DNA-binding repressor involved in transcriptional control of cpcB1A1. To examine this possibility, a histidine-tagged form of RpbA, designated RpbA-His<sub>6</sub>, was purified and assayed for its ability to specifically interact with the mapped promoter region for cpcB1A1. Gel shift and DNA footprint analyses support the hypothesized repressor role for RpbA and suggest that it binds as a monomer to its cognate DNA sequence. A similar analysis of mutant forms suggests that both of the putative HTH motifs on RpbA-His<sub>6</sub> may be involved in DNA binding.

**MATERIALS AND METHODS**

**Strains and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Strain Fd33 is a short filament mutant of *F. diplosiphon* UTEX 481 (5). Cyanobacteria were cultured in liquid or on solid BG-11 medium (1) as described previously (4).

*Escherichia coli* DH5α was purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and used as the host for plasmids. *E. coli* BL21(DE3) was purchased from Novagen (Madison, Wis.) and used as the host for expression of native and mutant RpbA-His<sub>6</sub> forms. *E. coli* strains were propagated in liquid or on solid Luria-Bertani medium (LB) with antibiotics at standard concentrations (2).

**DNA methods.** DNA restriction endonucleases and modifying enzymes were purchased from Promega (Madison, Wis.). [α-<sup>32</sup>P]dCTP and [α-<sup>32</sup>P]dATP were purchased from ICN (Irvine, Calif.). DNA manipulations including restriction digests, gel electrophoresis, ligations, PCR amplification, transformation of *E. coli*, and plasmid minipreparations were performed by established procedures (2, 21). Double-stranded DNA sequencing templates were prepared with a kit from Promega.
Construction of native RpbA-His6 expression vectors. The 416-bp rpbA coding region was amplified by PCR (AmpliTaq polymerase; Perkin-Elmer, Emeryville, Calif.) with primers rpbA-PCR3 (5'-CATCAGGACATATGCCCAAGGTCGCAAATAAAGC-3') and rpbA-PCR2 (5'-CTGGTACTCCGAGAGGCCCTG-3'), which produce a 5' flanking NdeI site, and rpbA-PCR2 (5'-CCATGGGACATATGGAGGCCCTG-3'), which produces a 3' flanking XhoI site. The PCR product was digested with NdeI and XhoI and ligated into vector pET-22b (+) previously digested with the same enzymes. The resulting construct, designated pUMC485, was verified for an intact rpbA coding region by DNA sequencing. Plasmid pUMC485 provides for expression of a modified RpbA protein, designated RpbA-His6, that terminates with the added peptide sequence Leu-Glu-His6.

Construction of mutant RpbA-His6 expression vectors. Specific amino acid substitutions were introduced into the recognition helix of HTH-1 or HTH-2 by substitutions, I sites, and rpbA-PCR1 plus rpbA-PCR2. The resulting full-length PCR product was ligated into vector pET-22b (+) previously digested with the same enzymes. Plasmid pUMC520 provides for expression of a modified RpbA-His6 protein (designated RpbA-His6, HTH-2) containing the following amino acid substitutions in HTH-2: Tyr17→Ala, Thr20→Ala, and Tyr57→Ala.

Isolation and purification of native and mutant RpbA-His6 forms. Crude soluble protein extracts from RpbA- His6 expression strain UMC60 and control strain UMC462 were used for the initial gel mobility shift assays. With the exception of cell propagation, all procedures were carried out at 4°C. For each strain, a mid-log 500-ml culture was induced by addition of isopropyl- b-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 3 h, the cells were harvested by centrifugation at 6,400 × g for 10 min, resuspended in 20 ml of breakage buffer (50 mM Tris [pH 8.0], 500 μM phenylmethylsulfonyl fluoride [PMSF], 1 mM benzamidine), and disrupted by passage through a French press at 20,000 lb/in². The crude extract was isolated as the supernatant following centrifugation of the French press lysate at 16,000 × g for 20 min.

Purified native or mutant RpbA-His6 forms were used for later gel mobility shift assays. Overnight shaker cultures of strains UMC460, UMC514, or UMC522 were diluted 1:60 with LB and returned to the shaker-incubator. At mid-log phase (440 = 0.7 to 0.9), IPTG was added to a final concentration of 1 mM and the cells were cultured for an additional 3 h. The cells were harvested by centrifugation at 6,400 × g for 10 min and resuspended in nondenaturing lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, 1 mg of lysozyme per ml). After incubation on ice for 30 min, the suspension was sonicated and the subsequent lysate was cleared by centrifugation at 16,000 × g for 15 min at 4°C. The RpbA-His6 form was purified from the supernatant by using a Ni-nitrilotriacetic acid spin kit from Qiagen (Valencia, Calif.) as instructed by the manufacturer. Typically, the eluted protein from two preparations was pooled, brought to 40% (vol/vol) glycerol and 1 mM PMSF, and stored at −20°C. Protein concentration was determined by using a bicinchoninic acid kit from Pierce (Rockford, Ill.).

DNA preparation for gel mobility shift assays and DNAase I footprinting. Plasmid pUMC423 harbors the 973-bp XbaI-EcoRI fragment from PC4.1 that contains the 5' end of cpcB1 and upstream sequences. A 282-bp region encompassing the mapped cpcB1 transcription start site was amplified by PCR from plasmid pUMC423 with two primers that produce flanking XbaI sites, cpc1-PL2 (5'-GC TCTAGAGGAAAGTAAAGCGATCAG-3') and cpc1-PR2 (5'-GCTCTTAGCAGGAAAGTAAAGCGATCAG-3').
ACTGAAACCTTCACCGCTTATTC-3')]. The PCR product was digested with Xbal and ligated into vector pGEM3zf(-) previously digested with the same enzyme, creating plasmid pUMC485. For initial gel mobility shift assays, plasmid pUMC423 was digested with VspI and BsrFII, and the products were radioactively labeled at both ends with \([\alpha-^{32}P]dATP\). Unincorporated radionucleotides were removed from the latter probe preparations by gel filtration on a G-25 spin column from Pharmacia (Piscataway, N.J.). A 70-bp competitor DNA fragment corresponding to the putative RpbA binding site was annealed to the labeled probe and used in competition experiments.

**Gel mobility shift assays**. Gel mobility shift assays were performed essentially as described by Ausbel et al. (2). Protein samples were incubated with 30,000 cpm of end-labeled DNA probes in the presence of 2 \(\mu\)g of poly(dI-dC) - poly(dI-dC) (Pharmacia) and 5 \(\mu\)g of bovine serum albumin in a final volume of 15 to 30 \(\mu\)l. Incubations were carried out at 30°C for 15 min in a solution of 6 mM HEPES, 60 mM KCl, 10% (vol/vol) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 40 \(\mu\)M PMSF, and 20 \(\mu\)M benzamidine. Samples were loaded onto low-ionic-strength 4% (wt/vol) polyacrylamide (acrylamide:bisacrylamide, 80:1) gels which were preelectrophoresed at 4°C for 90 min at 150 V in 0.5× TBE buffer, consisting of 45 mM Tris [pH 8.0], 45 mM borate, and 1 mM EDTA. The gels were electrophoresed at 4°C until the bromophenol blue migrated to the bottom of the gel, transferred to Whatman paper, dried, and analyzed by autoradiography.

**RESULTS**

**Binding by RpbA-His6 to the promoter region of cpcB1A1**. The predicted RpbA protein contains two HTH motif-like regions: HTH-1, corresponding to residues 30 to 49, and HTH-2, corresponding to residues 59 to 78 (Fig. 1A). To investigate potential DNA binding by RpbA, RpbA-His6 was expressed in \(E.\ coli\). Binding by RpbA-His6 to the promoter region of \(cpcB1A1\) was examined by gel mobility shift assay. The 232-bp VspI-BsrFII fragment encompassing the mapped \(cpcB1A1\) transcription start site (Fig. 1B) was incubated with a soluble protein extract from strain BL21/pET22b (control) or BL21/pUMC460 (expresses RpbA-His6) and assayed for the formation of a protein-DNA complex. No complex was detected following incubation of the probe with the BL21/pET22b extract (Fig. 2, lane 2). In contrast, a single, slower-migrating complex was detected following incubation of the probe with the BL21/pUMC460 extract (Fig. 2, lane 3), supporting binding by RpbA-His6 to the \(cpcB1A1\) promoter probe.

A modified gel mobility shift assay was used to localize the RpbA-His6 binding site to the promoter region of \(cpcB1A1\). In this experiment, the 291-bp XbaI fragment (encompasses residues 28 to 309 in Fig. 1B) from pUMC485 was cleaved with \(DdeI\) or HindIII, and the products were assayed for binding by radioactively labeled RpbA-His6. Cleavage with \(DdeI\) yielded fragments of 188 and 103 bp; in the assay, only the 188-bp fragment formed a protein-DNA complex with RpbA-His6 (Fig. 3A, compare lanes 3 and 4). Cleavage with HindIII yielded fragments of 174

![FIG. 2. Gel mobility shift analysis of binding to the promoter region of cpcB1A1 by RpbA-His6.](http://jb.asm.org/)
and 117 bp, of which only the 174-bp fragment formed a protein-DNA complex with RpbA-His$_6$ (Fig. 3B, compare lanes 3 and 4). These data indicate that RpbA-His$_6$ recognizes and binds to sequences within the 70-bp region flanked by the $Dde$I and HindIII sites; this region contains the putative promoter sequences and mapped transcription start site for *cpcB1A1* (Fig. 1B). DNA sequence analysis did not reveal any obvious inverted repeated sequences in the 70-bp region bound by RpbA-His$_6$.

**Specific binding by RpbA-His$_6$ to the promoter region of *cpcB1A1***. The specificity of binding by purified RpbA-His$_6$ to the promoter region of *cpcB1A1* was examined by gel mobility shift assay. In one experiment, different amounts of unlabeled, linearized pGEM3zf(+) or pUMC470 were added as competitor DNA to the binding reaction mixture. The addition of up to a 10-fold molar excess of pGEM3zf(+) DNA had no discernible effect on binding by RpbA-His$_6$ to the 232-bp *cpcB1A1* promoter probe (data not shown). In contrast, binding by RpbA-His$_6$ to the same probe was abolished by the addition of a fivefold molar excess of pUMC470 DNA (data not shown). Given that the difference between the two competitor DNAs is that pUMC470 harbors the 70-bp region flanked by the $Dde$I and HindIII sites described above, these data support specific binding by RpbA-His$_6$ to the promoter region of *cpcB1A1*.

A more quantitative gel mobility shift analysis of specific DNA binding by RpbA-His$_6$ was also performed. In this experiment, the competitor DNA was an unlabeled 70-bp PCR-generated fragment (encompasses residues 125 to 195 in Fig. 1B) containing the *cpcB1A1* promoter region described above. Increasing amounts of competitor DNA were added to binding reaction mixtures containing molar equivalents of purified RpbA-His$_6$ and the 232-bp *cpcB1A1* promoter probe. In the absence of competitor DNA, a significant fraction of the probe DNA formed a complex with RpbA-His$_6$ (Fig. 4, lane 2). Among replicate experiments, the degree of protein-DNA complex formation with molar equivalents of RpbA-His$_6$ and the *cpcB1A1* promoter probe was consistently near absolute (data not shown). In contrast, the addition of one molar equivalent of competitor DNA significantly reduced the formation of the protein-DNA complex (Fig. 4, lane 3), and addition of 5 or 10 molar equivalents of competitor DNA completely abolished complex formation (Fig. 4, lanes 4 and 5).

The RpbA-His$_6$ binding site in the promoter region for *cpcB1A1* was defined by DNase I footprint analysis. Preincubation of the 276-bp *cpcB1A1* promoter probe with increasing amounts of purified RpbA-His$_6$ protected a single 21-bp region from digestion by DNase I (Fig. 5). The protected region lies between the $Dde$I and HindIII sites upstream of the mapped *cpcB1A1* transcription start site and includes the putative −10 promoter sequence for the gene set (Fig. 1B). DNA sequence analysis did not reveal any form of direct or inverted repeated sequences within the protected and flanking regions.

**Potential involvement of both HTH motif-like regions for binding by RpbA-His$_6$ to the promoter region of *cpcB1A1***. To examine whether both HTH motif-like regions on RpbA are involved in the binding interaction, two mutant RpbA-His$_6$ forms were assayed for the ability to bind the 276-bp *cpcB1A1* promoter probe. One mutant form, designated RpbA-His$_6$ HTH-1', contains three substituted amino acids (Gln$_{41}$→Ala, Arg$_{64}$→Gly, and Arg$_{68}$→Gly) in the putative recognition helix of HTH-1, whereas the other mutant form, designated RpbA-His$_6$ HTH-2', contains three substituted amino acids (Tyr$_{74}$→Ala, Thr$_{75}$→Ala, and Tyr$_{78}$→Ala) in the putative recognition helix of HTH-2 (Fig. 1A). Incubation of the probe with native RpbA-His$_6$ produced the characteristic protein-DNA complex (Fig. 6, lane 2). In contrast, no protein-DNA complex was observed following incubation of the probe with either RpbA-His$_6$ HTH-1' or RpbA-His$_6$ HTH-2' (Fig. 6, lanes 3 and 4).

**DISCUSSION**

Previous work with mutant strain FdBMI showed that inactivation of *rpbA* by endogenous transposon Tn5469 resulted in a twofold increase in the steady-state level of transcripts from the *cpcB1A1* gene set (13). The identification of two HTH motif-like regions in the predicted RpbA sequence led us to hypothesize that RpbA functions as a repressor of transcription from *cpcB1A1*. In this work we have demonstrated that a histidine-tagged form of RpbA specifically recognizes and binds to a 21-bp sequence in the determined (16) promoter region for *cpcB1A1*. The RpbA-His$_6$ binding site overlaps the putative −10 promoter sequence recognized by RNA polymer-
ase during initiation of transcription. On the basis of these data, we conclude that RpbA functions as a repressor in the control of transcription from cpcB1A1. In this capacity, RpbA represents the first documented DNA-binding transcription regulator involved in the regulation of cyanobacterial PBS biosynthesis.

Several lines of evidence suggest that RpbA binds as a monomer to the cpcB1A1 promoter region. First, the majority of the cpcB1A1 promoter probe formed a protein-DNA complex in the presence of a molar equivalent of RpbA-His6, in the gel mobility shift assay (Fig. 4). In some replicate experiments, the degree of this complex formation was near absolute. Presuming that the RpbA-His6 molecule is incapable of simultaneously binding two target DNA molecules, we predict that such extensive complex formation would require at least two molar equivalents of RpbA-His6, if dimerization was necessary for efficient binding. Second, the 21-bp sequence protected from DNase I digestion by bound RpbA-His6 lacks a direct or inverted repeated sequence (Fig. 5). This contrasts with the situation for most structurally characterized bacterial repressors, which are composed of two monomeric subunits, each possessing a single HTH motif (10, 19, 23). In the dimeric state, the two HTH motifs are spaced to interact with the symmetrical DNA sequence, often with the cognate bases separated by one helical turn of the DNA (3). For such repressors, the twofold symmetry of the dimer is matched by the twofold symmetry of the recognized DNA sequence.

Binding by RpbA to its cognate DNA may involve both HTH-1 and HTH-2. The prototypical HTH motif consists of a 20-residue segment in which the first 7 residues form an α helix (helix 1), the next 4 residues form a turn in the polypeptide, and the remaining 9 residues form a second α helix (helix 2) (10). In the protein-DNA interaction, helix 1 lies across the major groove of DNA while helix 2, often referred to as the recognition helix, lies within the groove and contributes important base pair contacts. The HTH motif is not defined by a consensus sequence, but certain amino acids are characteristic for the substructures; the residues at positions 4, 8, 10, 16, and 18 are often hydrophobic, and the residues at positions 5 and 9 are usually alanine and glycine, respectively. The noncharacteristic residues of helix 2 provide the side groups important for specific contacts with nucleotide bases in the cognate DNA. In our analysis of binding by the mutant RpbA-His6 forms, three amino acid substitutions in the putative recognition helix of either HTH-1 or HTH-2 abolished formation of the characteristic protein-DNA complex (Fig. 6). For both HTH-1 and HTH-2, at least some of the three residues targeted for substitution were predicted to form side group contacts with specific nucleotide bases in the cognate DNA; all of the targeted amino acids possessed potentially interactive side groups, and none was characteristic for the helix 2 substructure. The binding deficiency of RpbA-His6, HTH-1′ and RpbA-His6, HTH-2′ is consistent with a protein-DNA interaction that requires both HTH-1 and HTH-2, although we cannot rule out the possibility that either mutant protein was rendered nonfunctional due to a related structural alteration.

The HTH-1 and HTH-2 regions on RpbA may represent a novel bipartite HTH motif. The first such motif was determined for the POU region of the eukaryotic Oct-1 transcription factor (14). Additional eukaryotic transcription regulators containing two HTH motifs per subunit have been reported (11, 18, 25). A prokaryotic bipartite HTH motif was recently determined for the monomeric E. coli MarA transcriptional activator (20), which belongs to the AraC/XylS family of prokaryotic transcriptional regulators (6). A feature common to these regulators is two HTH motifs separated by a flexible linker that allows the motifs to bind the DNA in various orientations relative to one another with a parallel or antiparallel...
arrangement of the two recognition helices. Presuming that HTH-1 and HTH-2 comprise a bipartite HTH motif, the RpbA protein-DNA interaction would necessarily differ from the determined eukaryotic and prokaryotic bipartite HTH structures. Given the spatial constraint of the nine-residue linker separating HTH-1 from HTH-2, it is likely that the two corresponding recognition helices would tandemly interact with the major groove on perpendicular or opposite sides of the DNA double helix.

The precise role of RpbA in the regulation of PBS biosynthesis in *F. diplosiphon* remains to be determined. As a repressor for the *cpeB1A1* gene set, RpbA functions in controlling the synthesis of PC1, which plays a critical role in PBS structure and function as the phycobiliprotein component of the invariant core-proximal hexamer of each rod. Under nutrient-replete conditions, transcription from *cpeB1A1* is constitutive, regardless of light quality. However, in response to sulfate deprivation, transcription from *cpeB1A1* is repressed while transcription from *cpeBA* is induced (17). One possibility is that RpbA plays a role in this sulfate acclimation response. Alternatively, RpbA may function in coordinating PC1 synthesis to the cellular demand for light-harvesting capacity. Such a regulatory mechanism is demonstrated by cyanobacterial strains that respond to a decrease in light availability by increasing their cellular PBS content (24).

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P.M. and R.P.N. contributed equally to this work, and both should be considered first authors.

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