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 chro-mophoric phycobiliproteins and nonchromophoric linker polypeptides. The major cyanobacterial phycobiliproteins are allophycocyanin, phycocyanin (PC), and phycoerythrin (PE); 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 (cpeB1A1) 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 PC<sub>3</sub> 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 Fd33, 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.
same enzymes. Plasmid pUMC512 provides for expression of a modified RpbA-His 6 protein with the added peptide sequence Leu-Glu-His 6. PCR-Script (Stratagene) previously digested with NdeI and ligated into vector pET-22b(+) containing intact rpbA coding region; source of RpbA-His 6. Plasmid pUMC520 provides for expression of a modified RpbA-His 6 protein (designated RpbA-His 6, HTH-2) containing the following amino acid substitutions in HTH-2: Tyr51→Ala, Thr57→Ala, and Tyr59→Ala. Construction of native RpbA-His 6 expression vectors. The 416-bp rpbA coding region was amplified by PCR (AmpliTaq polymerase; Perkin-Elmer, Emeryville, Calif.) from pUMC423 with two primers that produce flanking I sites, and rpbA-PCR2 plus rpbA-PTMT-2 (5'-CAAGTACCTCCCGTGATCGAGAGCACG-3') and rpbA-PCR2 plus rpbA-PTMT-3 (5'-GGTAGCTCTCCCAGCCGGTCCGAGA-3'). The resulting construct, designated pUMC485, was verified for an intact rpbA coding region by DNA sequencing. Plasmid pUMC458 provides for expression of a modified RpbA protein, designated RpbA-His 6, that terminates with the added peptide sequence Leu-Glu-His 6. Construction of mutant RpbA-His 6 expression vectors. Specific amino acid substitutions were introduced into the recognition helix of HTH-1 or HTH-2 by using the two-step PCR mutagenesis protocol described by Kless and Vermaas (15). To generate the HTH-1 mutation, two overlapping regions were amplified by PCR (Vent DNA polymerase; New England Biolabs, Beverly, Mass.) from Fd33 genomic DNA with the following primer pairs: rpbA-PCR3 plus rpbA-PTMT-3 (5'-CAAGTACCTCCCGTGATCGAGAGCACG-3') and rpbA-PCR2 plus rpbA-PTMT-4 (5'-GGTAGCTCTCCCAGCCGGTCCGAGA-3'). For each mutation protocol, the overlap- ping PCR products were purified and mixed to serve as template DNA for a subsequent amplification by PCR with the terminal primers rpbA-PCR3 and rpbA-PCR2. The resulting full-length PCR product was ligated into vector PCR-Script (Stratagene) previously digested with Smal. After verification by DNA sequencing, the mutated rpbA coding region was excised by digestion with NdeI and XhoI and ligated into vector pET-22b(+) previously digested with the same enzymes. Plasmid pUMC512 provides for expression of a modified RpbA-His 6 protein (designated RpbA-His 6, HTH-1') containing three amino acid sub- stitutions in HTH-1, Glu51→Ala, Arg54→Gly, and Arg56→Gly. Similarly, plasmid pUMC520 provides for expression of a modified RpbA-His 6 protein (designated RpbA-His 6, HTH-2') containing the following amino acid substitu- tions in HTH-2: Tyr51→Ala, Thr57→Ala, and Tyr59→Ala. DNA preparation for gel mobility shift assays and DNase I footprinting. Plasmid pUMC423 harbors the 973-bp XhoI-EcoRI fragment from pPC4.1 that contains the 5' end of cpc1 and upstream sequences. A 282-bp region encompassing the mapped cpc1 transcription start site was amplified by PCR from pUMC423 with two primers that produce flanking XhoI sites, cpc1-PL2 (5'-GC TTGAGGAAAGTTAAGCGATCGAG-3') and cpc1-PR2 (5'-GCTCTAGA GAAAGTTAAGCGATCGAG-3').
Affiliations

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 cpeB1A1 was examined by gel mobility shift assay. The 232-bp VspI-Bsr98I fragment encompassing the mapped cpeB1A1 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 cpeB1A1 promoter probe.

A modified gel mobility shift assay was used to localize the RpbA-His6 binding site to the promoter region of cpeB1A1. In this experiment, the 291-bp XbaI fragment (encompasses residues 28 to 309 in Fig. 1B) from pUMC485 was cleaved with Ddel or HindIII, and the products were assayed for binding by purified RpbA-His6. Cleavage with Ddel 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 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.

DNaSe 1 footprinting. DNase 1 footprinting was performed essentially as described by Ausubel et al. (2). For each reaction mixture, 30,000 cpm of end-labeled DNA probe (65 ng) was added to 180 μl of assay buffer (10 mM Tris-HCl [pH 7.0], 200 mM KCl, 2.5 mM MgCl2, 1 mM CaCl2, 0.1 mM EDTA, 100 μg of bovine serum albumin per ml, 2 μg of salmon sperm DNA per ml). Increasing amounts of purified native RpbA-His6 (0.42, 0.84, 1.26, 2.1, and 4.2 μg) were added to individual reaction mixtures, and protein-DNA binding was carried out at 30°C for 30 min. A control reaction mixture lacked RpbA-His6. Each reaction mixture was supplemented with 0.4 U of DNase I and incubated at 30°C for 2 min. DNase I activity was terminated by addition of 700 μl of stop solution (650 μl of 100% ethanol, 45 μl saturated ammonium acetate, 5 μl of a 1.0-mg·ml-1 tRNA stock), and the reaction mixtures were incubated in an ethanol-dry ice bath for 30 min. The DNA samples were pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in 5 μl of formamide loading buffer. DNA samples were loaded onto a standard 7% (wt/vol) polyacrylamide (acrylamide: bisacrylamide, 80:1) sequencing gel, electrophoresed, transferred to Whatman paper, dried, and analyzed by autoradiography.

RESULTS

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FIG. 1. (A) Predicted amino acid sequence of RpbA. Residues corresponding to HTH motif-like regions HTH-1 and HTH-2 are underlined. Boldface letters in HTH-1 and HTH-2 identify residues characteristic of HTH motifs (10). Letters in parentheses correspond to the carboxyl-terminal residues of RpbA-His6. Letters below the sequence indicate the amino acids substituted in HTH-1 and HTH-2 to generate RpbA-His6 mutant forms RpbA-His6-THH-1 and RpbA-His6-THH-2, respectively. (B) DNA sequence of the cpeB1A1 promoter region. The determined (16) transcription start site (+1) and putative promoter sequences (−35 and −10) are shown in boldface. Restriction sites used in generation or cleavage of DNA probes are double underscored. The predicted amino-terminal residues of the C peptide polyprotein.
and 117 bp, of which only the 174-bp fragment formed a protein-DNA complex with RpbA-His6 (Fig. 3B, compare lanes 3 and 4). These data indicate that RpbA-His6 recognizes and binds to sequences within the 70-bp region flanked by the DdeI 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-His6.

**Specific binding by RpbA-His6 to the promoter region of cpcB1A1.** The specificity of binding by purified RpbA-His6 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-His6 to the 232-bp cpcB1A1 promoter probe (data not shown). In contrast, binding by RpbA-His6 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 DdeI and HindIII sites described above, these data support specific binding by RpbA-His6 to the promoter region of cpcB1A1.

A more quantitative gel mobility shift analysis of specific DNA binding by RpbA-His6 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-His6 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-His6, (Fig. 4, lane 2). Among replicate experiments, the degree of protein-DNA complex formation with molar equivalents of RpbA-His6, 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-His6 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-His6 protected a single 21-bp region from digestion by DNase I (Fig. 5). The protected region lies between the DdeI 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-His6 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-His6 forms were assayed for the ability to bind the 276-bp cpcB1A1 promoter probe. One mutant form, designated RpbA-Arg9, contains three substituted amino acids (Gln 41 → Ala, Thr75 → Ala, and Tyr78 → Gly) in the putative recognition helix of HTH-1, whereas the other mutant form, designated RpbA-His6, HTH-2’, contains three substituted amino acids (Tyr74 → Ala, Thr75 → Ala, and Tyr78 → Ala) in the putative recognition helix of HTH-2 (Fig. 1A). Incubation of the probe with native RpbA-His6 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-His6, HTH-1’ or RpbA-His6, HTH-2’ (Fig. 6, lanes 3 and 4).

**DISCUSSION**

Previous work with mutant strain FdB1 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-His6 binding site overlaps the putative −10 promoter sequence recognized by RNA polymerase.
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...
The precise role of RpbA in the regulation of PBS biosynthesis in *F. diplosiphon* remains to be determined. As a repressor for the *cpcB1A1* 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 *cpcB1A1* is constitutive, regardless of light quality. However, in response to sulfate deprivation, transcription from *cpcB1A1* (as well as *cpcB2A2* and *cpcB4A4*) is repressed while transcription from *cpcB3A3* 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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