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Isolation and Characterization of Multiple Adenylate Cyclase Genes from the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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Adenylate cyclase genes, designated *cyaA*, *cyaB1*, *cyaB2*, *cyaC*, and *cyaD*, were isolated from the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 by complementation of a strain of *Escherichia coli* defective for the presence of *cya*. These genes encoded polypeptides consisting of 735, 859, 860, 1,155, and 546 amino acid residues, respectively. Deduced amino acid sequences of the regions near the C-terminal ends of these *cya* genes were similar to those of catalytic domains of eukaryotic adenylate cyclases. The remaining part of each *cya* gene towards its N-terminal end showed a characteristic structure. CyaA had two putative membrane-spanning regions. Both CyaB1 and CyaB2 had regions that were very similar to the cyclic GMP (cGMP)-binding domain of cGMP-stimulated cGMP phosphodiesterase. CyaC consisted of four distinct domains forming sequentially from the N terminus: a response regulator-like domain, a histidine kinase-like domain, a response regulator-like domain, and the catalytic domain of adenylate cyclase. CyaD contained the forkhead-associated domain in its N-terminal region. Expression of these genes was examined by reverse transcription-PCR. The transcript of *cyaC* was shown to be predominant in this cyanobacterium. The cellular cyclic AMP level in the disruptant of the *cyaC* mutant was much lower than that in the wild type.

Cyclic AMP (cAMP) regulates various biological activities in both prokaryotes and eukaryotes. In prokaryotes, cAMP and cAMP receptor protein regulate gene expression (7). In eukaryotes, cAMP regulates enzyme activity (11), channel activity (42), and gene expression (34) via the cAMP-dependent protein kinase. In cyanobacteria, it has been reported that cellular cAMP content is affected by growth conditions (17, 22), that cAMP concentration changes rapidly in response to light-off and light-on signals (37) or to a pH shift (36), and that cAMP interferes with the pattern formation of heterocysts (43). However, it is still unknown whether cAMP functions as an intracellular signal molecule in cyanobacteria.

Recently, we isolated adenylate cyclase (*cya*) genes from the filamentous cyanobacteria *Anabaena cylindrica* (26) and *Spirulina platensis* (52). Regions near the C-terminal ends of the adenylate cyclases of both cyanobacteria showed significant structural similarity to the catalytic domain of the adenylate cyclase of eukaryotes but showed no homology to that of prokaryotes such as *Escherichia coli*. Furthermore, cyanobacterial adenylate cyclases appeared to be membrane oriented.

To study the physiological functions of these adenylate cyclases, disruption or overexpression of *cya* genes is indispensable. Unfortunately, both *A. cylindrica* and *S. platensis* are not transformable, so they are not suitable for this purpose. In this study, we isolated *cya* genes from a transformable cyanobacterium, *Anabaena* sp. strain PCC 7120, and constructed mutants defective in *cya* genes. Some physiological properties of these mutants were determined.

MATERIALS AND METHODS

Strains and growth conditions. *Anabaena* sp. strain PCC 7120 was supplied by H. Böhme at the Institute for Botany, University of Bonn, Bonn, Germany. Cyanobacterial strains were grown axenically in BG11 liquid medium (39) or BG11 solid medium (39) containing 1% agar in the light (78 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 27°C. *E. coli* Top 10F′ [*mcrA* Δ(*mrr-hsdRMS-mcrBC*) *F80ΔlacZM15* Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*) *7697 galU galK* λ^- *rpsL endA1 nupG* F′] (Invitrogen, Leek, The Netherlands) and a mutant strain of *E. coli*, MK1010 [IN-(*rrmD-rrnE*)1 *cya*::Km^r] (provided by M. Kawamukai, Laboratory of Applied Microbiology, Faculty of Agriculture, Shimane University, Shimane Japan), were used. For *Anabaena* cultures, the following antibiotics were used at the indicated final concentrations; spectinomycin at 1 $\mu g \cdot ml^{-1}$ plus streptomycin at 1 $\mu g \cdot ml^{-1}$ for the liquid medium and spectinomycin at 10 $\mu g \cdot ml^{-1}$ plus streptomycin at 1 $\mu g \cdot ml^{-1}$ for the solid medium.

Construction of an *Anabaena* strain PCC 7120 genomic DNA library. Genomic DNA was isolated and partially digested with *Sau*3AI, and the DNA fragments in the ranges of 2.0 to 6.6 and 6.6 to 23 kb were fractionated as described previously (26). The extracted fragments were ligated to the *Bam*HI site of pBR322 (6). The same fragments were ligated to the *Bam*HI site of pQE11 (Qiagen GmbH, Hilden, Germany), which is an expression vector for *E. coli*. The ligated DNAs were introduced into *E. coli* Top 10F′. A mutant strain of *E. coli* MK1010 was transformed with the libraries and screened, as reported previously (26), for the ability to metabolize maltose or lactose on MacConkey agar plates (Difco Laboratories, Detroit, Mich.) containing 1% maltose or 1% lactose, 50 μg of ampicillin ml^{-1}, and 50 μg of kanamycin ml^{-1}.

Subcloning and sequencing of the *cya* genes. The *Eco*RI (on pBR322)-*Spe*I fragment of p665 was cloned into the *Eco*RI-*Spe*I site of pBluescriptII SK+ (Stratagene Cloning Systems, La Jolla, Calif.) to produce p665SK, the *Eco*RI (on pQE11)-*Hin*dIII fragment of pQ3 was cloned into the *Eco*RI-*Hin*dIII site of pBluescriptII SK+ to produce pQ3SK, the *Eco*RI (on pQE11)-*Eco*RV fragment of pQ2-1 was cloned into the *Eco*RI-*Eco*RV site of pBluescriptII SK+ to produce pQ2-1SK, the *Eco*RV (on pBR322)-*Sal*I (on pBR322) fragment of p667 was cloned into the *Eco*RV-*Sal*I site of pBluescriptII SK+ to produce p667SK, and the *Eco*RI (on pQE11)-*Hin*dIII fragment of pQ6-2 was cloned into the *Eco*RI-*Hin*dIII site of pBluescriptII SK+ to produce pQ6-2SK. Unidirectional deletions were generated with exonuclease III and mung bean nuclease (20). The nucleotide sequences of both strands were determined by the dideoxynucleotide chain termination method with a DNA sequencer (model 373A; Applied Biosystems), with all gaps overlapped.

Since open reading frames (ORFs) on pQ3, pQ2-1, and p667 did not contain 5′ ends, isolation of the 5′ portions of these ORFs was carried out by the following procedure. First, it was confirmed that the 5′ regions of pQ3, pQ2-1, and p667 included in the probe hybridized to the 3.7-kb fragment in *Sca*I digests, the 1.6-kb fragment in *Hin*dIII digests, and the 2.1-kb fragment in *Hin*dIII digests of genomic DNA of *Anabaena* sp. strain PCC 7120, respectively. Then, the size-selected library including the above-described fragments was constructed on pBluescriptII KS+. The clones pQ3N1, pQ2-1N1, and p667N1, which include the 5′ ends of the ORFs on pQ3, pQ2-1, and p667, respectively, were obtained by colony hybridization with the 1.3-kb *Eco*RI (on pQE11)-*Hin*dIII fragment of pQ3, the 0.8-kb (on pQE11)-*Eco*RI fragment of pQ2-1, and the 0.4-kb *Eco*RI (on pBR322)-*Hin*dIII fragment of p667, respectively, as probes (see Fig. 1).

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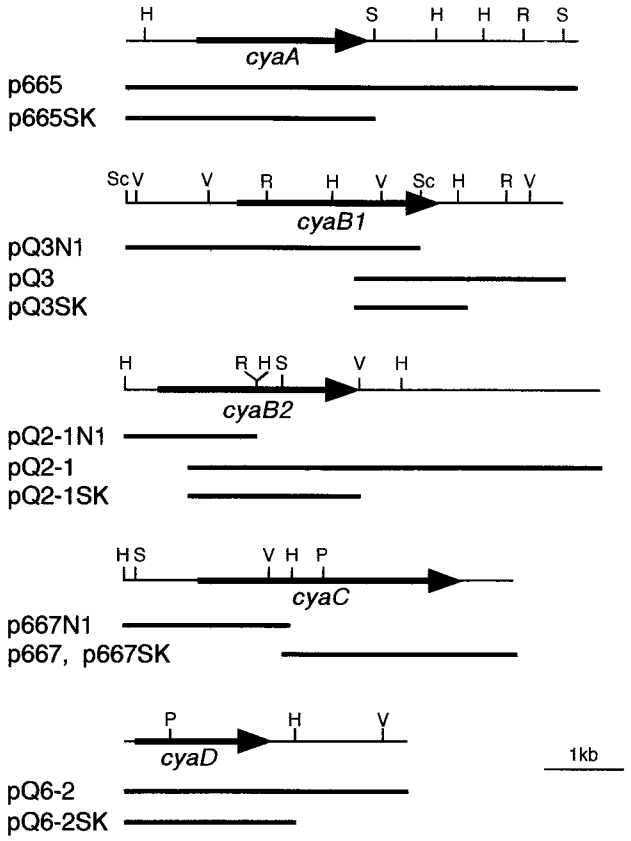

FIG. 1. Restriction maps of the *Anabaena* strain PCC 7120 *cya* gene regions. Abbreviations for restriction sites are as follows: H, *Hin*dIII; P, *Pvu*II; R, *Eco*RI; Sc, *Sca*I; S, *Spe*I; and V, *Eco*RV. Arrows indicate the direction of transcription for the *cya* genes and ORFs. *Sca*I sites are shown in the map of *cyaB1* only. *Hin*dIII sites downstream of the right-end *Hin*dIII site are omitted in the map of *cyaB2*. The restriction maps shown of *cyaB1*, *cyaB2*, and *cyaC* are of the reconstructed genes.

Determination of cAMP contents. Cellular cAMP was extracted with 5% trichloroacetic acid and measured by an enzyme immunoassay (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) as described previously (26).

Inactivation of the *cya* genes. The *cya* genes were inactivated by *sacB*-mediated positive selection for double recombinations (8). A *Sma*I fragment containing the omega Sp^r-Sm^r cassette from pDW9 (19) was inserted into the *Nae*I site of p665SK to produce p665SKΩ, the *Sca*I site of pQ3SK to produce pQ3SKΩ, the *Sna*BI site of p667SK to produce p667SKΩ, and the *Hpa*I site of pQ6-2SK to produce pQ6-2SKΩ. An *Eco*RI fragment containing the omega cassette was inserted into the *Eco*RI site (partially digested) of pQ2-1SK to produce pQ2-1SKΩ. The *Spe*I-*Xho*I fragments of pQ3SKΩ, p665SKΩ, p667SKΩ, and pQ6-2SKΩ were cloned into the *Spe*I-*Xho*I site of pRL271 (5). The *Eco*RV fragment of PQ2-1SKΩ was cloned into the *Eco*RV site of pRL271. Transformation of *Anabaena* strain PCC 7120 was performed by a conjugation method (15), and both single and double recombinations were confirmed by Southern hybridization by enhanced chemiluminescence systems (Amersham).

RT-PCR. Total RNA was extracted from *Anabaena* strain PCC 7120 grown in BG11 medium by the guanidinium thiocyanate-phenol-chloroform method (9). Reverse transcription-PCR (RT-PCR) was performed with an RT-PCR *high* (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. For the RT-PCR experiment, the primers were selected from sequences of *cyaA*, *cyaB1*, *cyaB2*, *cyaC*, and *cyaD* with Primer-Select software (DNASTAR, Inc., Madison, Wis.). Each pair of primers had similar melting temperatures close to 54°C. The pairs of oligonucleotide primers were as follows: 5′-GTCGC TGGGTGGTGGATT-3′ and 5′-GGGCGCGTTTTTGTGAG-3′ for amplification of *cyaA*, 5′-TGCGCCACTACCACTCACAG-3′ and 5′-CCGATCGCAGT ACCTTATGG-3′ for amplification of *cyaB1*, 5′-GGAAATCACACCGTTGGA CA-3′ and 5′-CCATAGGCCGTCCCATTG-3′ for amplification of *cyaB2*, 5′-A TACGCCTGTTGAACTGACCAC-3′ and 5′-AAAGCTAGAAGATTGCGAA CCTC-3′ for amplification of *cyaC*, and 5′-AGTTGTAGGCGTGCTTTATGC T-3′ and 5′-ATTGGCGTTGAGATGTTCC-3′ for amplification of *cyaD*. Thirty cycles of amplification (94°C for 30 s, 54°C for 30 s, and 72°C for 90 s) were performed.

Nucleotide sequence accession numbers. The DNA sequences of the *cyaA*, *cyaB1*, *cyaB2*, *cyaC*, and *cyaD* genes have been submitted to the GenBank database under accession no. D89622, D89623, D89624, D89625, and D89626, respectively.

RESULTS

Isolation of *cya* genes of *Anabaena* sp. strain PCC 7120. An *Anabaena* sp. strain PCC 7120 plasmid DNA library was used to transform an *E. coli* Δ*cya* strain, 1.3×10^6 transformants were screened on MacConkey-lactose plates, and 13 positive transformants were isolated. All positive transformants recovered the ability to metabolize maltose. The plasmids were extracted from these transformants and analyzed with various restriction enzymes. From restriction patterns, these plasmids, p665, pQ3, pQ2-1, p667, and pQ6-2, were classified into five groups. The restriction maps of the inserted DNAs of these positive clones are shown in Fig. 1. Most of the positive clones were isolated from the library that was composed of short DNA fragments (2.0 to 4.0 kb).

Determination of cAMP contents. Cellular cAMP contents were determined in five positive transformants of *E. coli* MK1010. cAMP was detected in all positive transformants when they were subjected to enzyme immunoassay (Table 1). However, the levels of cellular cAMP in these positive clones varied.

DNA sequencing. We generated several subclones from p665, pQ3, pQ2-1, p667, and pQ6-2 and determined the region required for the complementation of the Lac^- phenotype of *E. coli* MK1010 (p665SK, pQ3SK, pQ2-1SK, p667SK, and pQ6-2SK). These regions included the ORFs encoding the protein moieties that are similar to the putative catalytic domains of adenylate cyclases of *A. cylindrica*, *S. platensis*, *Rhizobium meliloti*, and several eukaryotes. Figure 2 shows the alignment of C-terminal regions of these ORFs and of the putative catalytic domains of adenylate cyclases. The regions near the C termini of these proteins containing about 180 amino acids showed 27.5 to 55.4% identity to each other, 29.7 to 35.2% identity to the adenylate cyclase of *A. cylindrica* (26), 25.9 to 31.4% identity to *S. platensis* CyaA (52), 29.4 to 35.0% identity to *R. meliloti* Cya1 (4), and 19.2 to 20.9% identity to the C1a region of bovine type I adenylate cyclase (28). We concluded that these ORFs in p665SK, pQ3SK, pQ2-1SK, p667SK, and pQ6-2SK are the adenylate cyclase genes of *Anabaena* strain PCC 7120 and designated them *cyaA*, *cyaB1*, *cyaB2*, *cyaC*, and *cyaD*, respectively.

Since pQ3SK, pQ2-1SK, and p667SK did not include the 5′ ends of *cyaB1*, *cyaB2*, and *cyaC*, we isolated the DNA fragments that contained their 5′ ends as described in Materials and Methods. Reconstructed restriction maps are shown in Fig. 1.

cyaA. *cyaA* encodes a polypeptide of 735 amino acids with a predicted molecular mass of 80,760 Da. The CyaA protein

TABLE 1. cAMP levels of a Δ*cya* strain of *E. coli* complemented by *Anabaena cya*

MK1010 strain	Level of cAMP (pmol · mg of protein^{-1})
cya mutant	0
pBR322	0
p665	160
pQ3	370
pQ2-1	1,900
p667	140
pQ6-2	10

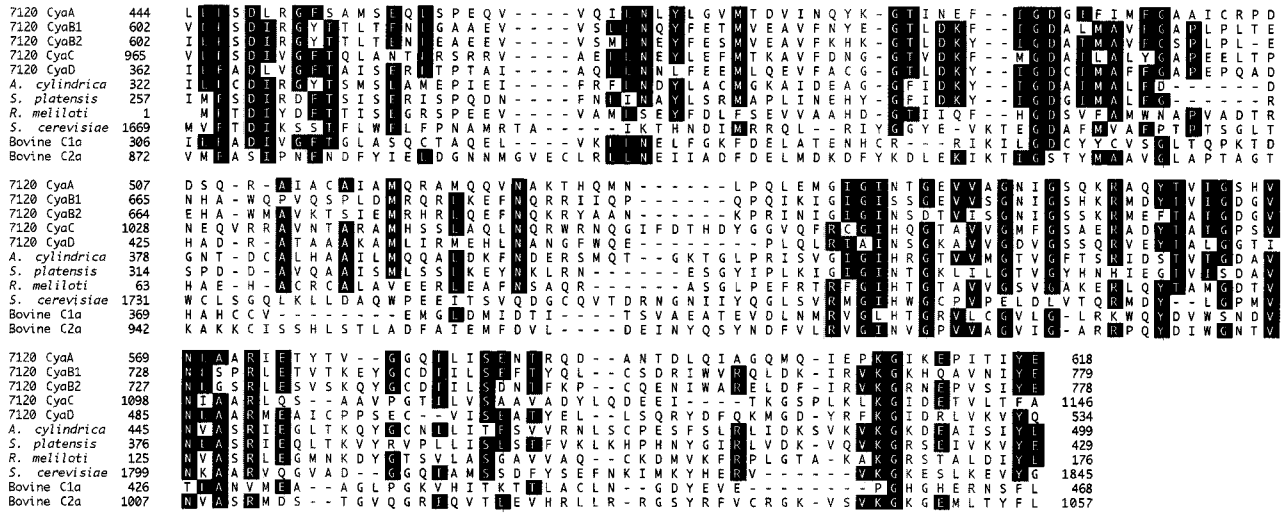

FIG. 2. Alignment of the amino acid sequences of the conserved regions of *Anabaena* strain PCC 7120 (7120), *A. cylindrica*, *S. platensis*, *R. meliloti*, *S. cerevisiae*, and bovine adenylate cyclases. Alignment was carried out by the CLUSTAL method with Megalign software (DNASTAR, Inc.). Amino acid residues in agreement for more than six residues are indicated by filled boxes. Numbers to the left and right of the sequences indicate the amino acid positions relative to the start of each polypeptide sequence.

shows no sequence homology to other known proteins except in a region near the C terminus that is conserved among eukaryotic adenylate cyclases. A hydropathy profile shows that CyaA contains two hydrophobic regions that putatively pierce a membrane. One is near the N-terminal end (Trp-13 to Thr-33), and the other is in the middle part (Thr-328 to Ser-406) of the protein. According to the algorithms of M. G. Claros and G. von Heijne (10), the latter hydrophobic region is predicted to be divided into three segments (Thr-328 to Gln-346, Pro-363 to Ile-383, and Val-386 to Ser-406). The hydrophobic segment near the N terminus shows an essential property of the signal peptide of prokaryotes (18). The whole structure of CyaA is similar to those of adenylate cyclases of *A. cylindrica* (26) and *S. platensis* (CyaA) (52). The region between the two hydrophobic segments shows no homology to the corresponding region of the adenylate cyclase of *A. cylindrica* or CyaA of *S. platensisis.*

The adenylate cyclases of eukaryotes such as *Dictyostelium discoideum* (ACG) (38), *Trypanosoma brucei* (GRESAG4s and ESAG 4) (2), and *Leishmania donovani* (RAC-A and RAC-B) (40) have domain organizations similar to that of CyaA. It has been pointed out that the structures of these adenylate cyclases are similar to that of the transmembrane receptor protein (38). It has also been noted that in *T. brucei*, adenylate cyclase pierces the membrane twice and that the region between these two transmembrane spans is supposed to function as a sensor of a certain exogenous signal (2). Although it is unknown whether the transmembrane region near the N terminus is processed, the domain lying between the two transmembrane spans seems to be exposed to the periplasmic space or thylakoid lumen. This region is not conserved within the cyanobacterial adenylate cyclases, suggesting that this region functions in accepting particular signals.

The adenylate cyclases of mammalian cells commonly have transmembrane spans, though their whole structures are very different from those of cyanobacteria. The enzymes of mammals have two sets of six transmembrane spans and two sets of conserved catalytic domains (47), while the enzymes of cyanobacteria have only one catalytic domain.

cyaB1. *cyaB1* encodes a polypeptide of 859 amino acids with a predicted molecular mass of 96,810 Da that has no hydrophobic region. The region near the N terminus of CyaB1 protein is similar to the N-terminal regions of the cyclic GMP (cGMP)-stimulated cGMP phosphodiesterases. This region of CyaB1, which contains 270 amino acids, shows 21.6% identity to the bovine cGMP-stimulated phosphodiesterase (44), 19.3% identity to the bovine cGMP-binding, cGMP-specific phosphodiesterase (32), and 16.7% identity to the beta subunit of the human photoreceptor cGMP phosphodiesterase (27). In mammals, this region is proposed to be an allosteric cGMP-binding region of phosphodiesterases (32).

cyaB2. *cyaB2* encodes a polypeptide of 860 amino acids with a predicted molecular mass of 96,780 Da that has no hydrophobic region. The whole primary structure of CyaB2 is similar to that of CyaB1. The identity between CyaB1 and CyaB2 is 32.6%. The region near the N terminus of CyaB2 is similar to those of cGMP-stimulated cGMP phosphodiesterases. CyaB1 and CyaB2 possibly have the same function in *Anabaena* strain PCC 7120. Since cGMP was detected in cyanobacteria (21, 37) the regions near the N termini of CyaB1 and CyaB2 may interact with intracellular cGMP.

cyaC. *cyaC* encodes a polypeptide of 1,155 amino acids with a predicted molecular mass of 128,740 Da that has no hydrophobic region. It has been noted that the CyaC protein contains domains homologous to those of proteins of the bacterial two-component regulatory system (45). CyaC consists of four distinct domains: two response regulator-like domains, one histidine kinase-like domain, and a catalytic domain of adenylate cyclase (Fig. 2). Two response regulator-like domains of CyaC, r1 (Met-1 to Glu-137) and r2 (Ala-789 to Glu-925), show 20.0 and 36.8% identity to the OmpR protein of *E. coli* (51), 17.5 and 30.2% identity to the PhoB protein of *E. coli* (31), and 20.5 and 29.5% identity to the CheY protein of *E. coli* (35). The histidine kinase-like domain of CyaC (Leu-511 to Leu-729) is similar to the transmitter domain of histidine kinase proteins. This region of CyaC shows 27.9% identity to the LemA protein of *Pseudomonas syringae* (23), 32.4% identity to the PhoR protein of *Shigella dysenteriae* (30), 26.0% identity to

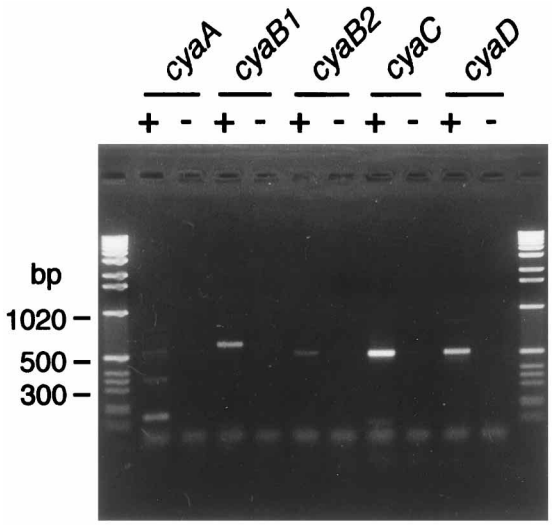

FIG. 3. Expression of adenylate cyclase genes in *Anabaena* strain PCC 7120. Total RNA was extracted from the cells grown in BG11 medium and reverse transcribed, and PCR was performed with specific primers as described in Materials and Methods. The reactions were performed in the absence (−) or the presence (+) of reverse transcriptase.

the RcsC protein of *E. coli* (24), and 33.3% identity to the PleC protein of *Caulobacter crescentus* (50). The highly conserved histidine residue of histidine kinase proteins and the aspartate residue of response regulator proteins, both of which are responsible for protein phosphorylation, exist in regions of CyaC (His-524, Asp-59, and Asp-847). Phosphorylation of these residues may regulate catalytic activity of this adenylate cyclase.

cyaD. *cyaD* encodes a polypeptide of 546 amino acids with a predicted molecular mass of 60,880 Da. CyaD protein has a weak hydrophobic stretch (Val-85 to Ile-106); however, it is unknown whether this protein pierces the membrane or not.

The N-terminal region of CyaD (Phe-24 to Ile-86) shows sequence similarity to the forkhead-associated (FHA) domain. This domain was found in several unrelated proteins, as well as in some transcription factors and kinases, and is supposed to be involved in a nuclear signaling. This region of CyaD shows 41.4% identity to the FHA domain of human antigen KI-67, which is thought to be required for maintaining cell proliferation (41), and 38.6% identity to the FHA domain of kinase-associated protein phosphatase of *Arabidopsis thaliana* (46).

Expression of the *cya* genes of *Anabaena* strain PCC 7120. To identify the transcripts of the *cya* genes of *Anabaena* strain PCC 7120, we carried out an RT-PCR experiment. The oligonucleotide primers that are specific to each *cya* gene were selected. All transcripts of *cya* genes were detected in *Anabaena* strain PCC 7120 grown in BG11 medium as shown in Fig. 3. These amplified products had the expected sizes (547 bp in *cyaA*, 592 bp in *cyaB1*, 515 bp in *cyaB2*, 514 bp in *cyaC*, and 520 bp in *cyaD*), and the DNA fragments obtained after digestion with appropriate restriction enzymes corresponded to those predicted from the restriction map. In the lanes containing *cyaA*, several small PCR products were detected, which was probably due to a nonspecific binding of the primer to genomic DNA since these products were not hybridized with the DNA fragment containing *cyaA* (data not shown). The transcripts of *cyaA* were usually very small and scanty. Thus, it is unclear why *cyaA* was expressed constitutively under our culture conditions.

Inactivation of *cya* genes. To determine the functions of adenylate cyclases in *Anabaena* strain PCC 7120, we constructed disruptants of the *cya* genes by insertion of an Sp^r-Sm^r cassette (omega cassette) into five *cya* genes. The omega cassette was inserted into the putative catalytic domains of CyaA (corresponding to Ala-550) and CyaD (corresponding to Leu-377), the regions that precede the putative catalytic domains of CyaB2 (corresponding to Glu-418) and CyaC (corresponding to Val-812), or the regions just behind the putative catalytic domain of CyaB1 (corresponding to Ser-785). All *cya* genes with inserted omega cassettes lost the ability to produce cAMP in *E. coli* MK1010. Then, the inactivated *cya* genes were cloned into the *sacB*-carrying vector pRL271. The sensitivity to sucrose provided by the *sacB* vector pRL271, which does not bear any cyanobacterial origin of replication, allows for the positive selection of double recombinations in *Anabaena* sp. (8). pRL271, which includes inactivated *cya* genes, was introduced into *Anabaena* strain PCC 7120 by conjugation. Single and double recombinants, confirmed by Southern hybridization (data not shown), were obtained on plates with BG11 supplemented with appropriate antibiotics.

The cellular cAMP levels of *cyaA*, *cyaB2*, and *cyaD* mutant strains in the light were similar to that in wild-type cells (2.4 pmol · mg of chlorophyll^{-1}). However, in *cyaC* mutant cells, the cAMP level (0.6 pmol · mg of chlorophyll^{-1}) was lower than that of wild-type cells. On the contrary, the cAMP level (3.9 pmol · mg chlorophyll^{-1}) of *cyaB1* mutant cells was slightly higher than that of wild-type cells. As for growth rates, no difference was found between wild-type cells and all *cya* mutant cells in both nitrogen-replete and nitrogen-free media (data not shown). From microscopic observations, it was found that the sizes of *cyaB1* mutant cells were slightly smaller than those of wild-type cells, while the *cyaD* mutant cells appeared to be larger than wild-type cells. The shape of the *CyaD* mutant cells on BG11 solid medium became fuzzier in outline than that of wild-type cells.

In wild-type cells of *Anabaena* strain PCC 7120, cAMP levels

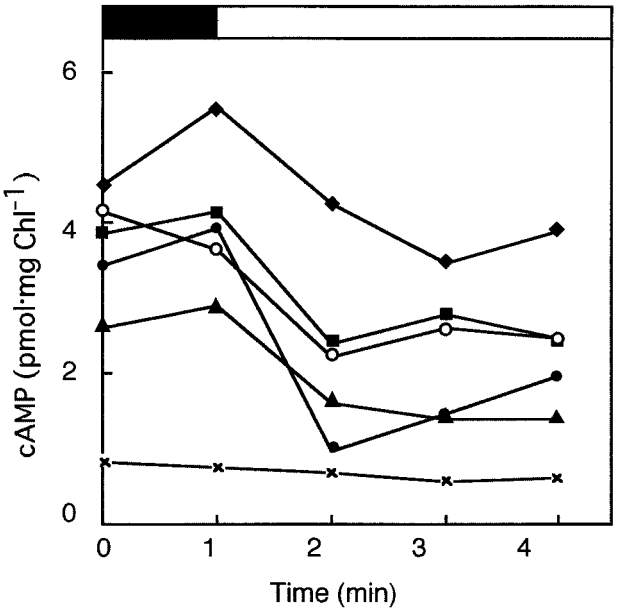

FIG. 4. Changes in cellular cAMP levels in *Anabaena* strain PCC 7120 wild-type cells (open circles), *cyaA* mutant cells (filled squares), *cyaB1* mutant cells (filled diamonds), *cyaB2* mutant cells (filled circles), *cyaC* mutant cells (x's), and *cyaD* mutant cells (filled triangles) during dark-to-light transition. Cells were incubated in darkness with starling for 40 min before the first sampling at 0 min (darkness is represented by the filled bar). Then the light was switched on, and cells were incubated in the light. Chl, chlorophyll.

decreased in response to a light-on signal (Fig. 4). This was true for the cAMP levels of *cyaA*, *cyaB1*, *cyaB2*, and *cyaD* mutant cells (Fig. 4). On the other hand, cAMP levels of *cyaC* mutant cells were about one-fourth of levels in wild-type cells in the dark and were scarcely affected by the light-on signal (Fig. 4). CyaC seems essential to maintain the level of cellular cAMP of *Anabaena* strain PCC 7120.

We examined glycogen contents, which are known to decrease with a light-to-dark transition (16); however, no difference in the patterns of decrease in glycogen content between wild-type cells and *cya* mutants was found with a light-to-dark transition. The glycogen content of wild-type cells was 5.5 mg · mg of chlorophyll^{-1} and decreased at the rate of 0.4 mg · mg of chlorophyll^{-1} · h^{-1} when cells were transferred from light to darkness. The *cya* mutant strains contained almost the same amount of glycogen as wild-type cells (5.4 to 7.2 mg · mg of chlorophyll^{-1}), and the rate of decrease in glycogen after *cya* mutant cells were transferred to darkness was similar to that of wild-type cells (0.4 to 0.7 mg · mg of chlorophyll^{-1} · h^{-1}).

DISCUSSION

Anabaena strain PCC 7120 was shown to have at least five different adenylate cyclase genes, *cyaA*, *cyaB1*, *cyaB2*, *cyaC*, and *cyaD*. It has never been reported that a prokaryote possesses so many different kinds of *cya*. Most bacterial adenylate cyclases have been isolated from members of the enterobacterial family, such as *E. coli*, which has a single *cya* gene (1, 13, 14, 33, 49). As exceptions, it has been reported that *R. meliloti* possesses at least three *cya* genes (3) and that *Stigmatella aurantiaca* possesses two *cya* genes (13). We have obtained several DNA fragments that putatively contain *cya* genes from another filamentous cyanobacterium, *S. platensis* (52).

An adenylate cyclase having two transmembrane regions, like CyaA, was found in cyanobacteria (26, 52) and seems to be common in them. This adenylate cyclase is similar to those found in the lower eukaryotes *Dictyostelium* and *Trypanosoma*. In these organisms, adenylate cyclase is considered to function as a receptor or a sensor (2, 38). Recently, the adenylate cyclase of *Dictyostelium* that is expressed in spores was shown to be involved in sensing extracellular osmolarity (48). It is strongly suggested that the CyaA-like adenylate cyclase of cyanobacteria functions as a receptor or sensor.

The other adenylate cyclases found in *Anabaena* strain PCC 7120 contain different structures in their sequences. CyaB1 and CyaB2 have a domain that is similar to the cGMP-binding domain. CyaC has domains that are similar to the proteins of the bacterial two-component regulatory system. CyaD has a domain that is similar to the FHA domain.

Proposed domain structures of adenylate cyclases of *Anabaena* sp. strain PCC 7120 are summarized in Fig. 5. These enzymes commonly have a domain that is homologous to the putative catalytic domain of eukaryotes (13) near the C-terminal region. It is highly probable that this domain functions as a catalytic domain of adenylate cyclase. Plasmid pQ3 contains merely the C-terminal region of CyaB1 (the region from Asp-505) and confers the ability to maintain a high level of cAMP (380 pmol · mg of protein^{-1}) in *E. coli* MK1010. The insertion of omega cassettes into this conserved region led to a loss of ability to maintain the levels of cAMP (0.012 pmol · mg of protein^{-1}).

On the other hand, other domains including the N-terminal region are characteristically different in five adenylate cyclases. It is probable that the upstream regions of catalytic domains include regulatory domains of these adenylate cyclases. The difference in these regions may represent the difference in regulatory properties of enzyme activity. The domain organizations of adenylate cyclases of some lower eukaryotes, such as that of CYR1 of *Saccharomyces cerevisiae* (25), that have single catalytic domains are roughly similar to those of cyanobacterial enzymes. In CYR1 of *S. cerevisiae*, it was shown that the upstream region of the catalytic domain contained the region that interacts with the RAS protein, the activator protein of CYR1 (12).

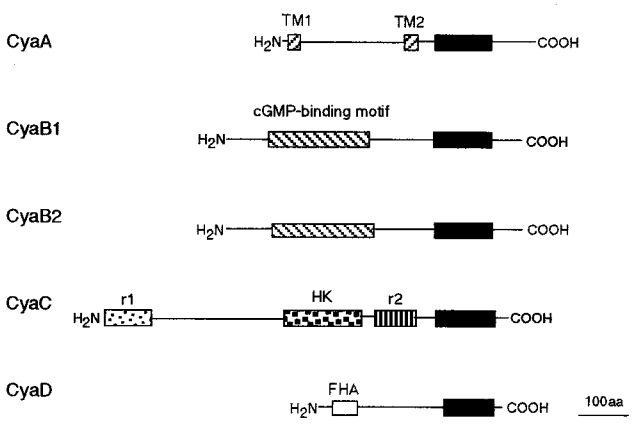

FIG. 5. Proposed domain structures of the adenylate cyclases of *Anabaena* strain PCC 7120. Conserved domains among adenylate cyclases are indicated by filled boxes. Regions labeled TM1 and TM2 in the map of CyaA indicate hydrophobic regions that putatively span a membrane. The sequences of domains r1 and r2 of CyaC are similar to those of the response regulator proteins. HK indicates the region that is similar to sequences of histidine kinase proteins. aa, amino acids.

Recently, A. Danchin classified the adenylate cyclases of many organisms, including prokaryotes and eukaryotes, into three classes based on their primary structures (13). Adenylate cyclases of prokaryotes such as *E. coli* and several enterobacteria were placed in class I because they share a domain organization possessing the N-terminal catalytic domain and the C-terminal glucose-sensitive regulatory domain. Adenylate cyclases of two pathogenic bacteria, *Bordetella pertussis* and *Bacillus anthracis*, were placed in class II. These enzymes were activated by calmodulin of host cells. Adenylate cyclases of all eukaryotes investigated and a few bacteria, such as *R. meliloti* and *Streptomyces aurantiaca*, were placed in class III because they share a putative catalytic domain. Cyanobacterial adenylate cyclases have homology to the catalytic domain of eukaryotic adenylate cyclases and apparently belong in class III.

As for the functions of cyanobacterial adenylate cyclases, it is highly probable that CyaC is involved in the maintenance of the steady-state level of cellular cAMP in *Anabaena* strain PCC 7120. It is possible that the decrease of cellular cAMP in response to light is brought about by a repression of a major adenylate cyclase, such as *cyaC*, and/or by activation of cAMP phosphodiesterase. Investigation of the regulation of enzyme activities involved in cAMP turnover in response to a light signal will be the next target of our research.

From the results of the RT-PCR experiment, it was found that all *cya* genes investigated in this study were expressed in *Anabaena* strain PCC 7120, though the level of transcription of a *cya* gene seems to be different for each gene. A large amount of the transcript of *cyaD* was detected in wild-type cells; however, there was no difference in cAMP levels between *cyaD* mutant cells and wild-type cells. Adenylate cyclase activity of CyaD may be repressed under ordinary growth conditions, and a certain signal may be required for the activation of this enzyme activity.

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