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Analysis of Expression of the *argC* and *argD* Genes in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

BELÉN FLORIANO,† ANTONIA HERRERO, AND ENRIQUE FLORES*

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Scientíficas, E-41080 Sevilla, Spain

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A cloned DNA fragment from *Anabaena* sp. strain PCC 7120 that complements an arginine auxotrophic mutant from the same organism was found to include an open reading frame encoding a 427-residue polypeptide that is homologous to *N*-acetylornithine aminotransferase from *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae*. The gene encoding *N*-acetylornithine aminotransferase in bacteria has been named *argD*. The expression of *Anabaena* sp. strain PCC 7120 *argD*, as well as of *argC*, was analyzed at the mRNA level. Both genes were transcribed as monocistronic mRNAs, and their expression was not affected by exogenously added arginine. Primer extension analysis identified transcription start points for both genes which were preceded by sequences similar to that of the *E. coli* RNA polymerase σ^{70} consensus promoter. A second transcription start point for the *argD* gene that is not preceded by a σ^{70} consensus promoter was detected in dinitrogen-grown cultures.

The cyanobacteria are a phototrophic group of eubacteria characterized by their ability to carry out oxygenic photosynthesis. Some filamentous cyanobacteria are able to fix molecular nitrogen within specialized cells called heterocysts which differentiate at semiregular intervals along the filament under conditions of nitrogen deprivation and aerobiosis (22). This developmental process has brought much attention to the mechanisms modulating gene expression in cyanobacteria. The RNA polymerase from the vegetative cells of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 has been characterized and found to contain as the principal σ factor a subunit of 52 kDa (20). The sequence of the *sigA* gene encoding that σ factor shows that its deduced polypeptide, SigA, resembles the principal σ factors from *Escherichia coli* and *Bacillus subtilis* (2). The strain PCC 7120 RNA polymerase SigA is able to recognize in vitro promoter sequences similar to those recognized by the *E. coli* RNA polymerase σ^{70} (19, 20). The operation of this type of promoter in *Anabaena* sp. strain PCC 7120 in vivo has recently been shown (7). The transcriptional start points (*tsp*) of only a relatively small number of genes in *Anabaena* spp. have been determined. Some of those genes bear promoters which show some resemblance to the *E. coli* σ^{70} consensus promoter, but others do not (19). In order to extend our knowledge of the structure of cyanobacterial promoters, more genes, both constitutive and subjected to some kind of transcriptional regulation, should be studied. In this report, we present the analysis of expression of two genes that turned out to be constitutively expressed in vegetative cells of *Anabaena* sp. strain PCC 7120. These are the *argC* and *argD* genes involved in the biosynthesis of arginine.

Methods. *Anabaena* sp. strain PCC 7120 and its derivative arginine auxotrophic mutants strains CS335 and CS336 were grown photoautotrophically as described previously (9). Conjugation of strain CS335 with strains of *E. coli* carrying plasmids with cloned strain PCC 7120 DNA was performed by the method of Wolk et al. (23) as described previously (9). The general subcloning strategy used for the *argD* gene was as previously described for the *argC* gene (9). DNA manipulations and construction of plasmids for complementation of strain CS335 and for sequencing were carried out by standard procedures (1, 17). Sequencing was carried out by the method of Sanger et al. (18) with exonuclease III-generated deletions or with the aid of synthetic oligonucleotides. The collection of *E. coli arg* mutants that was used to test complementation by strain PCC 7120 DNA has been reported before (9). Total DNA from strain PCC 7120 was isolated as described by Cai and Wolk (3). Transfer of electrophoretically separated DNA fragments to nylon membranes (Hybond-N$^+$ from Amersham or GeneScreen Plus from DuPont) and hybridization under high-stringency conditions were carried out as recommended by the membrane manufacturers. RNA was isolated from exponentially growing cultures of *Anabaena* sp. strain PCC 7120 as described by Golden et al. (10). After electrophoresis, transfer of RNA to nylon membranes (Hybond-N$^+$ from Amersham or GeneScreen Plus from DuPont) and hybridization were carried out as recommended by the membrane manufacturers. Probes used in the Southern and Northern (RNA) blot analyses were labelled with ^{32}P by using a random primed DNA labeling kit (Boehringer Mannheim) and [α-^{32}P] dCTP. The 5′ ends of the *argC* and *argD* transcripts were identified by primer extension (1) with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). A total of 50 μg of RNA was used in each reaction. The oligonucleotides used as primers, which were labelled with ^{32}P by using polynucleotide kinase and [γ-^{32}P]dATP, are described below.

Identification of the *Anabaena* sp. strain PCC 7120 *argD* gene. We have cloned previously by complementation of strain PCC 7120 arginine auxotrophs two genes involved in the biosynthesis of arginine in *Anabaena* sp. strain PCC 7120 (9). One of those genes has been identified as *argC* encoding *N*-acetylglutamate semialdehyde dehydrogenase (9). The identification of the second gene cloned is presented here. Starting from cosmid pCSB335-2, which carries ca. 31 kb of strain PCC 7120 DNA (Fig. 1A) and complements the arginine auxotrophic strain CS335, we subcloned DNA fragments that comple-

* Corresponding author. Mailing address: Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-C.S.I.C., Facultad de Biología, Apartado 1113, E-41080 Sevilla, Spain. Phone: 34-5-455.70. 86. Fax: 34-5-462.01.54. Electronic mail address: Flores@CICA.ES.

† Present address: John Innes Institute, Colney Lane, Norwich NR4 7UH, United Kingdom.

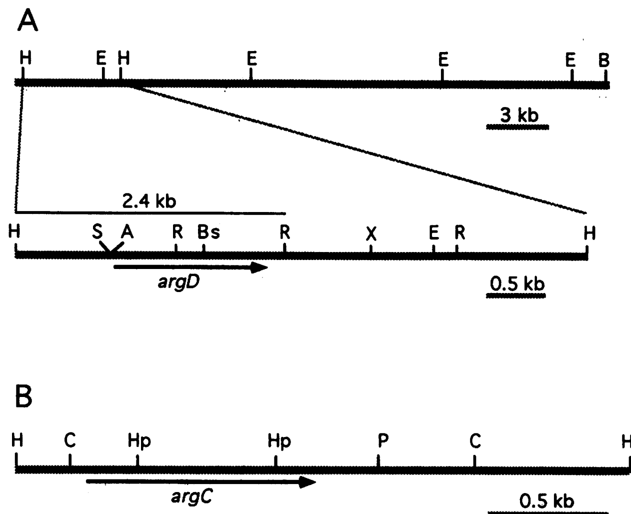

FIG. 1. Restriction maps of the genomic regions of *Anabaena* sp. strain PCC 7120 containing the *argD* (A) and *argC* (B) genes. In the top map of panel A, only the two *Hin*dIII sites that delimit the expanded region are shown. Restriction endonuclease abbreviations: A, *Acc*I; B, *Bgl*II; Bs, *Bst*XI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pvu*II; R, *Eco*RV; S, *Spe*I; X, *Xba*I.

mented this auxotroph. For subcloning, fragments from a partial digestion of pCSB335-2 with *Sau*3AI were ligated to *Bam*HI-digested positive selection vector pRL178 (8). This vector is *bom*$^+$ and therefore can be transferred from *E. coli* to *Anabaena* sp. strain PCC 7120 by conjugation (23), thus permitting the testing of complementation of the auxotrophic recipient strain by cloned DNA fragments. (However, pRL178 is unable to replicate in cyanobacteria; complementation therefore relies upon recombination between the cloned DNA fragment and the cyanobacterial genome.) From a complementing *Sau*3AI fragment, deletions with exonuclease III were generated, and a fragment that included most of the 2.4-kb *Hin*dIII-*Eco*RV fragment shown in Fig. 1A and that was able to complement strain CS335 was identified. The sequence of 2,259 bp of DNA in this region of the strain PCC 7120 genome was determined.

Analysis of the sequenced segment showed the presence of an open reading frame (ORF) of 1,284 nucleotides starting with a GTG codon. A putative ribosome binding site (AG GAGG) is found 7 nucleotides (nt) upstream of the putative translation start site (Fig. 2A). Downstream of the stop codon, an inverted repeat that is capable of forming a stem-loop structure and that bears several T (U in mRNA) residues in its 3′ end is found (Fig. 2A). The free energy of formation of this structure, which could act as a rho-independent transcription terminator, is −26.3 kcal/mol (−110.0 kJ/mol). The polypeptide deduced from that ORF consists of 427 amino acids and

A

(5' region)

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CTTCTGTAAACCTGTTGACAATGTAGCCAAGATTGACTACACCCAACAAAATTAAGGCAATAGTAAATAAGCGTCCACGACTACCAAGAGGGTTGGTTTCG

CCATATCTACGGTAGCTAAGGTAATAACTGTCATGTAAGCTGCATCTTCCCATGACCAACCCTCAAACAAAGCGATACCACAAAGTACCGAGAATTAATAC
                                                                        tsp (BG11₀)            -35
ACCACCGAGAGCGATCGCCCCGGCCATTAACTCTTTTTGGATGCGTTGGTATTTCTGTTCAAGAGTTGAATACAAAATTATTTTCCACTATTGCCACAGG
             -10      tsp (BG11 and BG11₀)
TTTGAGGATATATAATGTTAAGCTCAAGAGAAAGTAAGAAACACAATAAATAAAAATTTATTTTTAAGAAACTCTAGGAAACTAGTAAGTAAGTATACAA

TCAAAAATCCGCAGGAGGAGCGTTA GTG AGC CTA CAA ACT CTC ATT GAG CAA GCC ACG AAC CCC CCA GAG TCA GGT TCT GCT
             RBS           Met ser leu gln thr leu ile glu gln ala thr asn pro pro glu ser gly ser ala
```

(3' region)

```
GAA ATC AAC ACC GCC TTA AAA TTA CTG GAA AAA GCA TTA GCA ACT GTG ACA GCG TAA ATCATAGACTGCTGGCAAAAATAGCG
glu ile asn thr ala leu lys leu leu glu lys ala leu ala thr val thr ala OCH

AAAATAATCACCGCCGATACACACCAATGAACGCTGATAAATGTCTGTGTTCATCTACTGAAATCGGCGGCTTATTTTTATCAAAAATCTTCACTACTTGAG
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B

(5' region)

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AAGCTTGTGTGTTTTCCATCTGTGAATAAAAATTTTATTTTTTATTGGGAAAATTGTCTGGGATTAGCATTAAGCACTCTATCATTTTTGTCCTATTGCCCT

AAAAAATATCCCAAATGTAGGGTGCGTCAGTGTGATATACCCTAACTATAGGCAGAAATTATTCATGCTGACGCAACCTACCCAACTACCAAACCATCGATT
         -35                  -10      tsp (BG11 and BG11₀)
TCGGATAATTTATTTTTTGGTGTTCCCTAAGGTAAAATTCAGTATACAAATGCTGAAGCCATT ATG AAT AAA CCG AAA ATT TTT ATT GAT
                                                           RBS      Met Asn Lys Pro Lys Ile Phe Ile Asp

GGG GAA GCG GGA ACT ACA GGC TTA CAG ATT TAC TCA CGC CTC AAC GAG CGA GAT GAT ATT GAG CTA GTT AGT ATT GCA
Leu Asn Glu Arg Asp Asp Ile Glu Leu Val Ser Ile Ala Gly Glu Ala Gly Thr Thr Gly Leu Gln Ile Tyr Ser Arg
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FIG. 2. Nucleotide sequences of the 5′ ends of the *argD* (A) and *argC* (B) genes of *Anabaena* sp. strain PCC 7120, the regions upstream of these genes, the 3′ end of the *argD* gene, and the region downstream of this gene (A). Some putative transcription start points (*tsp*) found with RNA isolated from cultures grown on BG11 and/or $BG11_0$ medium are indicated (boldface and underlined). Putative −35 and −10 promoter sequences found upstream from the *tsp*s used in both BG11 and $BG11_0$ medium are indicated in boldface. Putative ribosome binding sites (RBS) are underlined. Within the coding sequence of the genes, the segments complementary to the oligonucleotides used for primer extension analysis are underlined. In the region downstream of the 3′ end of the *argD* gene, sequences that may be involved in the formation of a stem-loop structure are underlined.

has a predicted molecular weight of 46,110. A search for proteins with similar sequences in the available databases revealed that the deduced polypeptide had homology to numerous aminotransferases from very diverse biological sources. The proteins most similar to the strain PCC 7120 polypeptide were the *N*-acetylornithine aminotransferase enzymes from *B. subtilis* (16), *Saccharomyces cerevisiae* (12), and *E. coli* (12). These proteins are of a size similar to that of the deduced strain PCC 7120 polypeptide and show identities of 44% (*B. subtilis*), 41% (*S. cerevisiae*), and 41% (*E. coli*) with that polypeptide. The gene encoding *N*-acetylornithine aminotransferase (EC 2.6.1.11) in bacteria has been named *argD*. The ArgD protein catalyzes the transformation of *N*-acetylglutamate semialdehyde into *N*-acetylornithine by means of a transamination. This is the fourth step in the pathway of biosynthesis of arginine from glutamate (4). The identification of the sequenced gene from *Anabaena* sp. strain PCC 7120 as *argD* is consistent with the phenotype of mutant strain CS335, which was able to grow on ornithine (which is synthesized from *N*-acetylornithine [4]) but not on glutamate (9). Aminotransferases catalyze the reversible transfer of amino groups from amino acids to oxo acids by using pyridoxal phosphate as a cofactor. Pyridoxal phosphate interacts with the ε-amino group of a Lys residue to form a Schiff base. A search for functional domains in strain PCC 7120 ArgD by using the program MOTIFS of the Genetics Computer Group package (6) identified a putative pyridoxal phosphate binding site in which Lys-277 would be involved in Schiff base formation.

The *argC* and *argD* genes are not clustered in the genome of *Anabaena* sp. strain PCC 7120. Southern blot analysis of total DNA isolated from strain PCC 7120 digested with *Hin*dIII showed a single strong hybridization band of about 5 kb when the *Hin*dIII fragment carrying the strain PCC 7120 *argD* gene (Fig. 1A) was used as a probe and a single hybridization band of 2.6 kb when a probe of the *argC* gene (the 1.6-kb *Cla*I fragment [Fig. 1B]) was used (data not shown). An additional, slightly hybridizing band of 2.6 kb that might correspond to sequences encoding another aminotransferase was observed with the *argD* probe. It appears, therefore, that *argC* and *argD* are single-copy genes in *Anabaena* sp. strain PCC 7120, which is consistent with the fact that strain PCC 7120 mutants altered in those genes have been readily isolated (9).

No evidence for clustering of the *argC* and *argD* genes in the strain PCC 7120 genome has been found. Thus (i) none of the cosmids carrying strain PCC 7120 DNA that were able to complement the *argC* mutant (strain CS336) was able to complement the *argD* mutant (strain CS335) and, conversely, the CS335-complementing cosmids were unable to complement strain CS336 (9); and (ii) no cross-hybridization between pCSB335-2 and CS336-complementing cosmids was observed (not shown). This data together with a comparison of the restriction map of the insert of cosmid pCSB335-2 (Fig. 1A) with those of the inserts of cosmids isolated from complemented exconjugants of the *argC* mutant (strain CS336) (9) suggest that the *Hin*dIII fragments containing the *argC* and *argD* genes are at least 9 kb apart in the genome of strain PCC 7120.

With regard to other arginine biosynthesis (*arg*) genes, the DNA sequences flanking *argD* that were determined (ca. 400 bp 5′ of the gene and ca. 500 bp 3′ of the gene) do not seem to encode any protein related to arginine biosynthesis, and an ORF that is found 60 bp downstream of *argC* (accession number X65511 in the EMBL/GenBank/DDBJ nucleotide sequence data libraries) would code for a product unrelated to arginine metabolism. (This ORF, of which only 178 codons were sequenced, has 56% identity in an overlap of 174 amino acids with *o389* of *E. coli*, an ORF which is located between two genes involved in the biosynthesis of enterobacterial common antigen [5].) Additionally, in complementation assays of a collection of strains of *E. coli* mutated in different arginine biosynthesis genes, we did not observe complementation of any *E. coli* mutant by the *argD*-carrying cosmid pCSB335-2, whereas the *argC*-carrying cosmid pCSB336-24 (9) was able to complement only an *E. coli argC* mutant (data not shown). (In this study, no *argD* mutant of *E. coli* was tested because *E. coli argD* mutants are leaky.) These results suggest that in the genome of *Anabaena* sp. strain PCC 7120, neither *argC* nor *argD* is flanked by other *arg* genes.

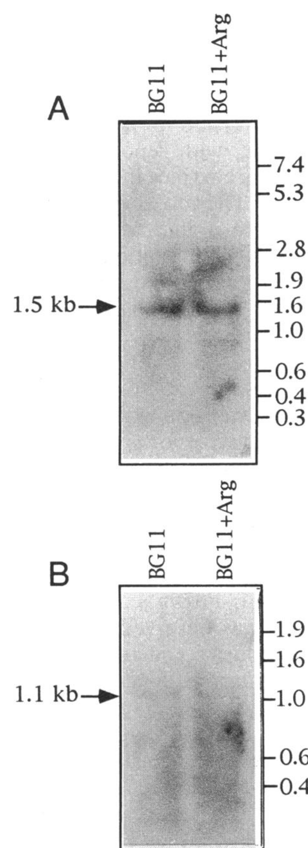

FIG. 3. Northern blot analysis of the expression of the *argD* (A) and *argC* (B) genes in *Anabaena* sp. strain PCC 7120. Total RNA isolated from cells grown in medium BG11 supplemented or not with 5 mM L-arginine was used (25 μg in each lane). The position and sizes (in kilobases) of some RNA molecular weight standards are indicated.

Analysis of *argC* and *argD* mRNAs. RNA was isolated from cultures of *Anabaena* sp. strain PCC 7120 grown on BG11 medium supplemented or not with 5 mM L-arginine, which is an amino acid that is readily taken up by cells of strain PCC 7120 (13). Northern blot analysis was performed by using as a probe an *Eco*RV fragment of 0.9 kb that contains the 3′ region of *argD* (Fig. 1A) or an *Hpa*I fragment of 0.6 kb internal to the *argC* gene (Fig. 1B). Single hybridizing RNAs of about 1.5 kb (Fig. 3A) and 1.1 kb (Fig. 3B) were observed with the *argD* and *argC* probes, respectively. Because the *argD* and *argC* genes consist of 1,284 and 966 bp, respectively, both genes appear to be transcribed as monocistronic mRNAs. No difference between the RNA preparations isolated from cultures supplemented with arginine and those from cultures not supple-

mented with arginine was observed. In the case of the *argC* mRNA, the lack of effect of exogenously added 5 mM L-arginine was corroborated by primer extension analysis (data not shown).

The lack of repression of the *argC* and *argD* genes by arginine is consistent with reported data on the activities of arginine biosynthesis enzymes in *Anabaena variabilis* and other cyanobacteria (15). Therefore, in the cyanobacteria, the only regulatory mechanism found to date to operate for arginine biosynthesis is feedback inhibition of *N*-acetylglutamate phosphotransferase (14), a regulatory mechanism that is typical in organisms that, like the cyanobacteria, exhibit the cyclic pathway of ornithine synthesis (4).

Promoter structure. The 5′ endpoints of mRNAs of the *argC* and *argD* genes in *Anabaena* sp. strain PCC 7120 were determined by primer extension analysis. The primers used are depicted in Fig. 2A and B for *argD* and *argC*, respectively. An extension product that can represent a transcription start point (*tsp*) was found 103 bp upstream from the start of the *argD* gene when RNA isolated from cultures grown in BG11 (in the presence of nitrate) or $BG11_0$ (with no source of combined nitrogen) was used (Fig. 4A; see also Fig. 2A). With RNA isolated from $BG11_0$-grown cultures, an additional, larger extension product was observed (Fig. 4A; see also Fig. 2A). This extension product could represent a *tsp* located 158 bp upstream from the start of the *argD* gene, though the possibility that it originated from degradation of a larger RNA cannot be ruled out. With regard to *argC*, an extension product that can represent a *tsp* was found 20 bp upstream from the start of the gene (Fig. 4B; see also Fig. 2B). Similar results of primer extension assays of *argC* were obtained with RNA isolated from cultures grown in either BG11 or $BG11_0$ medium (Fig. 4B). Several 5′ endpoints corresponding to smaller RNAs are noticeable for the reactions shown in Fig. 4A and B. They could be due to partial RNA degradation or incomplete extension of the primers.

Upstream from the putative *argC tsp* and from the putative main *tsp* of *argD*, sequences that resemble the consensus sequence for promoters recognized by the *E. coli* RNA polymerase σ^{70} (TTGACA[16 to 18 nt]TATAAT[5 to 9 nt]-*tsp*) are found. These sequences read as follows: TTTATT(18 nt)TA AAAT(5 nt)-*tsp* (*argC*) and TTGCCA(16 nt)TAATGT(4 nt)-*tsp* (*argD*) (Fig. 2). (Overlapping with the putative −10 box of the *argD* promoter, the sequence TATAAT, which represents a perfect Pribnow box, is found; however, this hexamer is separated by only 14 nt from the putative −35 box of the promoter.) Therefore, these *argC* and *argD* promoters, which are constitutive, may be recognized by the *Anabaena* sp. strain PCC 7120 RNA polymerase carrying the principal σ factor, SigA, which has been shown to recognize promoters similar to the *E. coli* RNA polymerase σ^{70} consensus promoter (19).

It is interesting that an RNA species more abundant in dinitrogen-fixing ($BG11_0$ medium) than in nitrate-grown (BG11 medium) cultures of *Anabaena* sp. strain PCC 7120 was detected with the *argD* primer (Fig. 4A). We cannot exclude that a $BG11_0$-specific RNA can also exist for *argC*, since the *argC* mRNA was difficult to detect and a low-abundance extension product could have been unnoticed. In strain PCC 7120, some genes, e.g., *sigA* or *glnA*, have been shown to be transcribed from several promoters (2, 21). It is possible that genes that are expressed in both heterocysts and vegetative cells are transcribed from different promoters in the two cell types. Although a number of genes that are expressed specifically in heterocysts have been investigated (e.g., nitrogen fixation genes), a consensus sequence for promoters functional in heterocysts has not yet been derived. Arginine biosynthesis

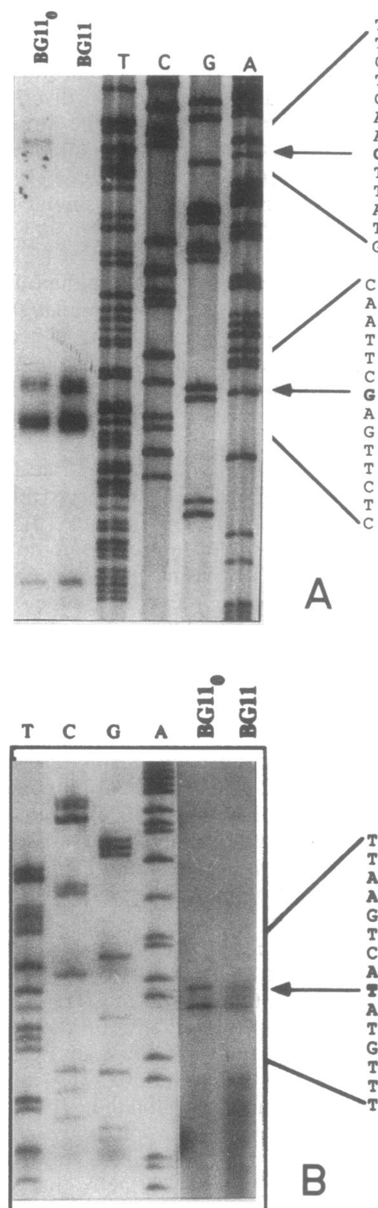

FIG. 4. Identification of the 5′ ends of the *Anabaena* sp. strain PCC 7120 *argD* (A) and *argC* (B) transcripts. RNA prepared from cells grown on BG11 or $BG11_0$ medium was used in primer extension experiments. The primer-extended products were coelectrophoresed with a sequencing ladder generated with the same oligonucleotide primer. The arrows on the right of each panel indicate the positions of putative *tsps*. The nucleotide sequences around these putative *tsps* are also presented (note that the sequence of the strands complementary to those presented in Fig. 2 are shown).

enzymes, including *N*-acetylornithine aminotransferase (ArgD), are present in *Anabaena* spp. in heterocysts as well as in vegetative cells (11). We do not know, however, whether the upper band found with RNA preparations from cultures grown in $BG11_0$ medium corresponds to heterocyst-localized mRNA molecules. This RNA species was also detectable, although in reduced amounts, with RNA preparations from BG11-grown cultures, but it should be noted that BG11-grown cultures of

Anabaena sp. strain PCC 7120 are not completely devoid of heterocysts.

Nucleotide sequence accession number. The sequence reported in this note of 2,259 bp of DNA of the strain PCC 7120 genome will appear in the EMBL/GenBank/DDBJ data libraries under accession number X78854.

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