

Transcription Activation by NtcA and 2-Oxoglutarate of Three Genes Involved in Heterocyst Differentiation in the Cyanobacterium *Anabaena* sp. Strain PCC 7120[∇]

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In *Anabaena* sp. strain PCC 7120, differentiation of heterocysts takes place in response to the external cue of combined nitrogen deprivation, allowing the organism to fix atmospheric nitrogen in oxic environments. NtcA, a global transcriptional regulator of cyanobacteria, is required for activation of the expression of multiple genes involved in heterocyst differentiation, including key regulators that are specific to the process. We have set up a fully defined *in vitro* system, which includes the purified *Anabaena* RNA polymerase, and have studied the effects of NtcA and its signaling effector 2-oxoglutarate on RNA polymerase binding, open complex formation, and transcript production from promoters of the *hetC*, *nrrA*, and *devB* genes that are activated by NtcA at different stages of heterocyst differentiation. Both RNA polymerase and NtcA could specifically bind to the target DNA in the absence of any effector. 2-Oxoglutarate had a moderate positive effect on NtcA binding, and NtcA had a limited positive effect on RNA polymerase recruitment at the promoters. However, a stringent requirement of both NtcA and 2-oxoglutarate was observed for the detection of open complexes and transcript production at the three investigated promoters. These results support a key role for 2-oxoglutarate in transcription activation in the developing heterocyst.

Cyanobacteria are phototrophic prokaryotes that, being the organisms that developed oxygenic photosynthesis, have played a crucial role in the evolution of our planet and of life on it (27). Nowadays, cyanobacteria contribute to an important fraction of the primary production in vast oligotrophic areas of oceans (14, 20). Their contribution to N₂ fixation in oceans and other ecosystems is also remarkable and results in a net input of combined nitrogen into the biosphere (43, 56). To fix atmospheric nitrogen in oxic environments, cyanobacteria have to cope not only with external oxygen but also with that produced intracellularly as a result of water-splitting photosynthesis. To meet this challenge, some filamentous strains differentiate specialized cells called heterocysts when combined nitrogen is not available. Heterocysts exhibit many structural and metabolic differences with regard to vegetative cells (52), which arise during the course of a developmental process that involves activation of the expression of many genes and repression of others (9, 12, 19, 54). At the molecular level, NtcA, the cyanobacterial N control factor, plays a principal role in transcriptional regulation for the triggering and progress of heterocyst differentiation as well as in the mature heterocyst (reviewed in reference 19).

NtcA is a ca. 222-amino-acid protein of universal distribution in cyanobacteria, including strains from very different habitats and various taxonomic groups (18). NtcA belongs to the CRP (cyclic AMP receptor protein)/FNR (fumarate and nitrate reduction regulator) family of transcriptional regulators

and has been shown to bind DNA in specific sites bearing the critical sequence element GTAN₃TAC (32, 47). In most cases studied to date, NtcA acts as a transcriptional activator on promoters constituted by a –10 determinant with the consensus sequence TAN₃T and an NtcA binding box in place of the –35 determinant (i.e., centered at ca. –41.5 nucleotides with regard to the transcription start point [tsp]). This arrangement has been considered the “canonical NtcA-activated promoter” (18) and conforms to the general structure of bacterial class II-activated promoters (7).

NtcA-dependent transcription activation can take place when cells are in the absence of ammonium, the preferred N source. In addition to being autoregulated at the level of gene expression, NtcA is modulated at the activity level as a function of the C-to-N balance of the cells. Thus, NtcA-dependent genes are activated only under conditions that lead to high C-to-N ratios, even in mutant strains that constitutively over-express the NtcA protein from synthetic promoters (34, 40). 2-Oxoglutarate (2-OG), a metabolite that reflects the C-to-N balance of cyanobacterial cells (33, 39), has a positive effect on the expression of NtcA-activated genes (49). *In vitro*, 2-OG has been shown to increase NtcA binding to, and transcription from, the promoters of the *glnA* and *ntcA* genes of the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 (45, 48) and to facilitate NtcA binding to the promoters of *ntcA* (29) and *nrrA* (35) of *Anabaena* sp. strain PCC 7120.

Previously (42), RNA polymerase (RNAP) was isolated from vegetative cells of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, and *in vitro* assays have shown this protein to initiate transcription at a number of vegetative cell promoters but not at a promoter of *glnA* that is used in heterocysts. In this work, we have developed procedures for high-yield purification of the RNAP from *Anabaena* and have

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TABLE 1. Oligodeoxynucleotides used in this study

Oligodeoxynucleotide	Sequence (5'-3')	Positions relative to the translation start of the corresponding gene
RD1	GGAGCATGAGGATCCCGGCATGAACCAG	-19 to +9 (<i>rpoD</i>)
RD2	CCTACATTTCTGGGGCATTC	+1318 to +1299 (<i>rpoD</i>)
RA1	AAGGGAGGCGGATCCGTGGCGCAGTTTC	-15 to +13 (<i>rpoA</i>)
RA2	GACCATGACGGATTAGCTCAG	+1080 to +1060 (<i>rpoA</i>)
RB5	CGGCTAATGACACCCATGGTATGTGCATC	-21 to +8 (<i>rpoB</i>)
RB2	GGGATGAGACTGGCTGAATATG	+500 to +479 (<i>rpoB</i>)
RB3	GTGCGATCGCCTGGAGTTTATTAC	+424 to +447 (<i>rpoB</i>)
RB7	CATCTTGTACCAGTCGTTCCGG	+2134 to +2114 (<i>rpoB</i>)
RB6	GTAGTTGCTGGTCAGGTGC	+1987 to +2005 (<i>rpoB</i>)
RB4	GTGCTGAGTTCTGAGTAAAG	+3524 to +3505 (<i>rpoB</i>)
RC110	CATTAGTGAATAGTCCCGGGTT	+1939 to +1918 (<i>rpoC1</i>)
RC111	CAGCCCATGGGTATGAGACCCGCCAAAC	-12 to +17 (<i>rpoC1</i>)
RC25	GACTAACGAATTCATGATTTTTTCGC	-13 to +12 (<i>rpoC2</i>)
RC26	GTTTCGACGGGATCCCATTTTTTC	+1517 to +1494 (<i>rpoC2</i>)
RC27	GCCAGAGGTGGTTTGATTTG	+1422 to +1437 (<i>rpoC2</i>)
RC28	GATGTCACGACGAAAACTAAGG	+2532 to +2510 (<i>rpoC2</i>)
RC29	GACCTGACCTGAGGATGCAG	+2461 to +2476 (<i>rpoC2</i>)
RC210	GATATAGGATGTTGTCGACGTTGTG	+4173 to +4249 (<i>rpoC2</i>)
DB1	CCCCCTACTCCCTTTCC	-825 to -809 (<i>devB</i>)
DB3	CCTGCTTCTTCTGGTCAGCG	-807 to -788 (<i>devB</i>)
DB4	CATAACATTTCCCAAGTC	-580 to -598 (<i>devB</i>)
DB13	TCCGTCACCCTTGACAT	-3 to +17 (<i>devB</i>)
HC1	TAGTACATCGGTGAGGGGTG	-693 to -674 (<i>hetC</i>)
HC2	TGTGAGCAACATCGACATCTG	-411 to -431 (<i>hetC</i>)
PA1	CAGAGCAGCCGATTGTCTGTTG	+97 to +76 (<i>psbA</i>)
PA2	CTGATAAGTGAGCTATTAC	-145 to -126 (<i>psbA</i>)
NA1	CTGGCGGCTTGATGCACCGG	+104 to +84 (<i>nrrA</i>)
NA2	ATTCAGGCTGAAATGTGAAGGG	-498 to -477 (<i>nrrA</i>)
NA14	CGTAGAGGTAATTGTGGC	-96 to -79 (<i>nrrA</i>)

set up an in vitro system in which we have studied the effect of NtcA and its metabolic effector 2-OG on RNAP binding, open complex formation, and transcription at three regulated promoters activated during heterocyst differentiation.

MATERIALS AND METHODS

Plasmid construction. Total DNA from *Anabaena* sp. strain PCC 7120 was isolated from cells grown in BG11 medium as described previously (8). DNA constructions, sequencing by the dideoxy chain termination method, and PCR were performed by standard procedures (2). The DNA nucleotide sequences of all the cloned genes in all the final constructs were verified.

The 1,321-bp DNA fragment from the *Anabaena* sp. strain PCC 7120 *sigA* region contained in plasmid pCSAV143 was amplified by PCR with oligonucleotides RD1 and RD2 (Table 1) and genomic DNA from strain PCC 7120 as the template, digested with BamHI and PstI, and cloned into BamHI- and PstI-digested expression vector pQE10 (Qiagen). The 1,024-bp DNA fragment from the *rpoA* (encoding the RNAP α subunit) region of *Anabaena* contained in plasmid pCSV25 was amplified by PCR with oligonucleotides RA1 and RA2 (Table 1) and genomic DNA from strain PCC 7120 as the template, digested with BamHI and PvuII, and cloned into BamHI- and HincII-digested expression vector pQE9 (Qiagen). The 3,527-bp DNA fragment from the *rpoB* (encoding the RNAP β subunit) region of *Anabaena* sp. strain PCC 7120 included in plasmid pCSAV155 was amplified in three segments by PCR with oligonucleotides RB5 and RB2, RB3 and RB7, and RB6 and RB4 (Table 1) and genomic DNA from strain PCC 7120 as the template. These fragments were cloned in pGEM-T Easy vector (Promega), producing plasmids pCSAV150, pCSAV151, and pCSAV152, respectively. An EcoNI-ScaI fragment from pCSAV151 was ligated to the EcoNI-ScaI fragment from pCSAV152, generating plasmid pCSAV153, which was cut with ClaI and SalI enzymes and ligated to a ClaI-SalI fragment from pCSAV150, producing plasmid pCSAV154, which contains the complete *rpoB* gene in vector pGEM-T Easy. The NcoI fragment from pCSAV154 was finally cloned into NcoI-digested expression vector pTrc99A, rendering pCSAV155. The 1,924-bp DNA fragment from the *rpoC1* (encoding the RNAP γ subunit) region of *Anabaena* sp. strain PCC 7120 included in

plasmid pCSAV158 was amplified by PCR with oligonucleotides RC110 and RC111 (Table 1) and genomic DNA from strain PCC 7120 as the template, digested with NcoI and SmaI, and cloned into NcoI-SmaI-digested expression vector pTrc99A. Plasmid pCSAV157 contains a 4,164-bp DNA fragment from the *rpoC2* (encoding the β' subunit) region of *Anabaena* sp. strain PCC 7120. This gene was amplified in three segments by PCR with oligonucleotides RC25 and RC26, RC27 and RC28, and RC29 and RC210 (Table 1) and genomic DNA from strain PCC 7120 as the template. The DNA product amplified with oligonucleotides RC25 and RC26 was digested with EcoRI and BamHI and cloned in the expression vector pTrc99A cut with the same restriction enzymes, producing plasmid pCSAV156. The DNA fragment amplified with oligonucleotides RC27 and RC28 was first cloned in pGEM-T Easy and then excised with DraIII and SalI and cloned in the same restriction sites of pCSAV156, producing pCSAV156B. Finally, the product of the amplification with oligonucleotides RC29 and RC210 was cut with Eco4VII and SalI and inserted into pCSAV156B digested with Eco4VII and SalI, producing pCSAV157.

Cloning and purification of recombinant *Anabaena* RNAP. Overproduction and purification of recombinant *Anabaena* sp. strain PCC 7120 RNAP was performed as described for the *E. coli* enzyme (44), with modifications. Histidine-tagged α and SigA subunits were purified from isopropyl- β -D-thiogalactopyranoside (IPTG)-induced cultures of *E. coli* containing plasmids pCSV25 and pCSAV143, respectively. Extracts from the inclusion bodies of those cultures solubilized in buffer A (50 mM Tris-HCl [pH 8.0], 6 M guanidine hydrochloride, 0.3 M NaCl, 10% glycerol) were chromatographed through a 1.25-ml Ni²⁺-charged His-Select cartridge (Sigma) by using a fast protein liquid chromatography system (Pharmacia). Samples containing α or SigA were supplemented with 50% (final concentration) of glycerol and stored at -20°C.

β , β' , and γ subunits were purified from inclusion bodies of IPTG-induced *E. coli* strains containing plasmids pCSAV155, pCSAV157, and pCSAV158, respectively. Inclusion bodies were solubilized in buffer B (50 mM Tris-HCl [pH 8.0], 6 M guanidine hydrochloride, 10 mM MgCl₂, 10 μ M ZnCl₂, 1 mM EDTA, 10 mM dithiothreitol [DTT], 10% glycerol) and used as crude preparations.

For preparation of RNAP, a mixture of approximately 80 μ g of hexahistidine-tagged α , 300 μ g of crude preparation of β , 600 μ g of β' , and 600 μ g of γ and, separately, approximately 300 μ g of hexahistidine-tagged SigA were dialyzed

against buffer C (50 mM Tris-HCl [pH 8.0], 200 mM KCl, 10 mM MgCl₂, 1 mM DTT, 5 mM 2-mercaptoethanol, 20% glycerol), containing decreasing amounts of urea (1 h with 6 M, 1 h with 5 M, 1 h with 4 M, 1.5 h with 3 M, 1.5 h with 2 M, and 3 to 6 h with 1 M), and finally against buffer D (50 mM Tris-HCl [pH 8.0], 200 mM KCl, 20% glycerol). Following dialysis, the two preparations were combined, incubated for 45 min at 30°C, cleared by centrifugation (16,000 × g, 15 min at 4°C), and chromatographed through a 1.25-ml Ni²⁺-charged His-Select cartridge (Sigma). Contaminating proteins were eluted by washing the column with 25 volumes of buffer D plus 3.5 mM of imidazole. Bound RNAP was eluted with a linear gradient of imidazole (3.5 mM to 460 mM) in buffer D. Samples containing the complete RNAP holoenzyme were concentrated by centrifugal ultrafiltration (Amicon 100000 MWCO; 2,000 × g at 4°C), mixed with glycerol (50% final concentration), and stored at -20°C. Just before use, the RNAP preparation was supplemented with an ~3-fold molar excess of the purified and reconstituted SigA protein and incubated for 15 min at 32°C.

Proteins were analyzed by standard electrophoresis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by staining with Coomassie brilliant blue R. Protein concentration was estimated by a dye-binding assay (Bio-Rad) based on that of Bradford (5).

Overproduction and purification of recombinant *Anabaena* NtcA protein. NtcA was purified from crude extracts of *E. coli* containing plasmid pCSAM61 (37), which were chromatographed through a 1-ml HiTrap heparin HP column (Pharmacia Biotech). Bound NtcA was eluted with a linear gradient of NaCl (500 mM to 2 M) in 20 mM sodium phosphate buffer (pH 7.0) supplemented with 10% glycerol. Samples containing NtcA were desalted by centrifugal ultrafiltration (Amicon 10000 MWCO; 7,000 × g at 4°C) and stored in 20 mM sodium phosphate buffer (pH 7.0) containing 250 mM NaCl and 10% glycerol at -20°C.

Preparation of DNA fragments. The double-stranded DNA fragments used in electrophoretic mobility shift assays (EMSA) or potassium permanganate footprinting and in vitro transcription experiments were obtained by PCR amplification, followed by purification of the corresponding products with a GFX gel band purification kit (Pharmacia Biotech). The fragment of the *hetC* region was obtained with primers HC1 and HC2 (positions -121 to +162 with regard to the *tsp*) (Table 1) and plasmid pCSAM83 (36) as the template. The *devB* fragment (positions -103 to +125 with regard to the *tsp*) was amplified using primers DB3 and DB4 (Table 1) and plasmid pCSAM155 (which contains the *devB* promoter region, amplified with oligonucleotides DB1 and DB13 and cloned in pMBL-T vector) (A. M. Muro-Pastor, unpublished data) as the template. The *nrrA* fragment used in in vitro transcription and potassium permanganate footprinting assays was amplified using oligonucleotides NA1 and NA2 (positions -471 to +131) (Table 1), and the fragment used in EMSA was amplified with primers NA14 and NA1 (positions -69 to +131) (Table 1) and plasmid pCSAM113 (35) as the template. The *psbA* fragment encompasses positions -145 to +97 with regard to the translation start of the gene, amplified using primers PA1 and PA2 (Table 1) and plasmid pRL278 (4) as the template.

EMSA. EMSA was carried out using 5 to 10 fmol of the specific DNA fragment end labeled with T4 polynucleotide kinase (Boehringer) and [γ -³²P]ATP, and 0.07 mg/ml poly(dI-dC) as nonspecific competitor DNA, in a final volume of 20 μ l. The reaction mixtures with the corresponding promoter fragment were incubated with purified NtcA for 5 min at 32°C and then with the reconstituted RNAP holoenzyme supplemented with purified SigA (see above). The protein-DNA complexes were separated on native 5% polyacrylamide gels in low-ionic-strength buffer (40 mM Tris [pH 8.0], 40 mM boric acid, 0.8 mM EDTA) at 200 V for 3 to 5 h at 4°C with a prerun of 1 h. Images of radioactive gels were obtained and quantified with a Cyclone storage phosphor system (Packard).

DNase I footprint assays. Protein-DNA complexes were formed in a final volume of 70 μ l of buffer [10 mM Tris-HCl (pH 8.0), 30 mM KCl, 10 mM MgCl₂, 5 mM CaCl₂, 2 mM DTT, 0.07 mg/ml bovine serum albumin, 0.07 mg/ml poly(dI-dC)], with 10 fmol of ³²P-end-labeled (with T4 polynucleotide kinase and [γ -³²P]ATP) DNA fragment, 1 mM ATP, and the reconstituted RNAP holoenzyme. The reaction mixtures were incubated for 30 min at 32°C. Then, DNase I (1 U; Roche) was added in a buffer containing 50 mM sodium acetate and 6 mM MgSO₄, and the reaction was immediately stopped with 320 μ l of stop solution (0.5 mM EDTA, 0.125% SDS, 12.5 mM ammonium acetate [pH 5.2]). The products were resolved in 6% polyacrylamide-4.68 M urea gels next to the corresponding sequencing ladder. Images of radioactive gels were obtained as described above.

Potassium permanganate footprinting assays. Potassium permanganate footprinting was performed with minor modifications of the procedure described by Jiang and Gralla (22). Complexes were formed under the same conditions as those described for EMSA, with 5 to 10 fmol of nonlabeled DNA template and 1 mM ATP (*psbA* and *devB*) or 1 mM ATP plus 1 mM GTP (*hetC*). After 30 min of incubation, 4 mM potassium permanganate was added and then incubated for

1 min at 31°C. Potassium permanganate-hypersensitive sites were detected by asymmetric PCR using end-labeled primers (HC1, NA1, DB4, and PA1) (Table 1) and EcoTaq polymerase (Ecogen). PCR products were analyzed on 6% polyacrylamide-4.68 M urea gels next to the corresponding sequencing ladder. Images of radioactive gels were obtained as described above.

In vitro transcription assays. The runoff transcription assays were performed in a total volume of 30 μ l of buffer (40 mM Tris-HCl [pH 8.0], 50 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin, 5% glycerol). Complexes were formed under the same conditions as those described for EMSA. Transcription was started by the addition of 3 μ l of a substrate solution of 0.15 mM each of ATP, GTP, and UTP, 20 μ M CTP, and 3 μ Ci [α -³²P]CTP. After 30 min of incubation at 37°C, the reaction was terminated by the addition of 30 μ l phenol, 10 mM EDTA, and 3 μ l of 200-ng/ μ l glycogen prepared in 3 M sodium acetate (pH 5.2). The products were precipitated with ethanol and fractionated by electrophoresis on 6% polyacrylamide-4.68 M urea gels. Images of radioactive gels were obtained as described above.

RESULTS

***Anabaena* RNAP.** As a first step to study the relations of NtcA and the cyanobacterial RNAP at the regulated promoters, purification of RNAP of *Anabaena* sp. strain PCC 7120 was addressed in order to set up a fully defined in vitro assay system and to avoid artifacts that could arise from the use of a heterologous RNAP. The core cyanobacterial RNAP has a subunit composition different from that of the typical proteobacterial $\alpha_2\beta\beta'$, as found in, e.g., *E. coli*. The cyanobacterial core is constituted of $\alpha_2\beta\beta'\gamma$ subunits, in which the two domains found in the *E. coli* β' subunit are split into two subunits in cyanobacteria: the γ subunit corresponds to the amino-terminal half of the *E. coli* β' subunit, and the β' subunit corresponds to the carboxy-terminal half of the *E. coli* β' subunit (3, 42).

Besides the principal, group 1 σ factor SigA, multiple alternative group 2 σ factors have been identified in the several cyanobacteria investigated to date (6, 26, 55). In the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, the expression of some NtcA-dependent genes has been shown to take place principally with SigA, whereas the group 2 factors SigB and SigC could contribute to expression under particular cellular conditions (21). Because NtcA-activated promoters encompass a standard -10 determinant (18, 32), we first chose to set up in vitro assays with RNAP bearing the principal *Anabaena* type 1 σ factor, SigA.

Purification of *Anabaena* RNAP was accomplished through two different approaches. First, it was isolated, by chelating affinity chromatography, from a derivative of strain PCC 7120 that expresses a modified version of the β' subunit carrying a polyhistidine tail fused to its carboxy terminus (E. Olmedo-Verd, A. M. Muro-Pastor, E. Flores, and A. Herrero, unpublished data). Second, it was reconstituted from the single subunits cloned and expressed in *E. coli*, as described in Materials and Methods (Fig. 1). The two preparations of *Anabaena* RNAP were tested in the different types of in vitro assays used throughout this study, rendering similar results. Thus, because higher purification yields were obtained with the enzyme expressed in *E. coli*, this was the source of the enzyme that was used subsequently and in the experiments described below. Figure 2 shows the performance of the cloned *Anabaena* RNAP in a consensus-type promoter, that of the *psbA* gene from *Amaranthus hybridus*, which includes standard -10 and -35 determinants and is utilized in *Anabaena* sp. strain PCC 7120 (51). Binding of RNAP to the promoter region leading to

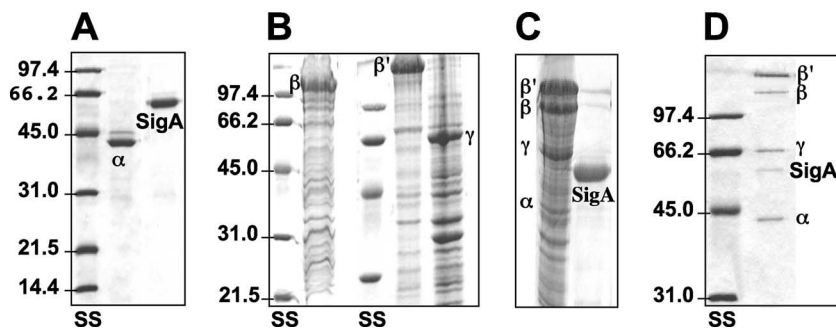


FIG. 1. Preparation of recombinant RNAP from *Anabaena* sp. strain PCC 7120. Preparations of His-tagged α and σ (SigA) subunits of *Anabaena* RNAP expressed in *E. coli* and purified (A) and of solubilized inclusion bodies from *E. coli* containing the β , β' , and γ subunits (B), all of them obtained under denaturing conditions, were subjected to SDS-PAGE and stained with Coomassie brilliant blue R. Portions of the preparations of His-tagged α , β , β' , and γ subunits were mixed and renatured together. The resulting preparation was mixed with a preparation of renatured His-SigA, and the obtained reconstituted RNAP holoenzyme was purified by chromatography through chelating Sepharose. Preparations obtained after renaturation (C) and holoenzyme purification (D) were also analyzed by SDS-PAGE. See Materials and Methods for further details. Size standards (SS) in kDa are shown.

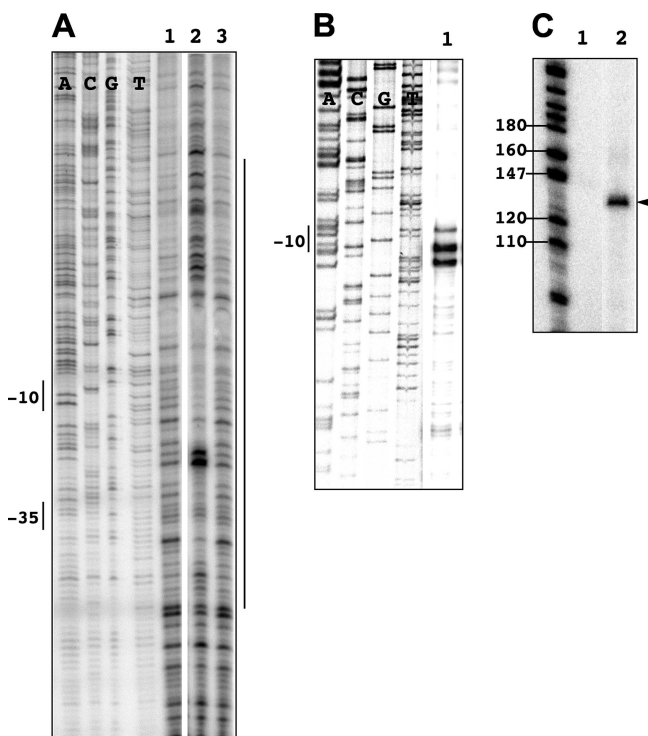


FIG. 2. Activity of the cloned *Anabaena* RNAP on the promoter of the *psbA* gene. (A) Results of DNase I footprinting. The DNA fragment of the promoter region of the *psbA* gene was incubated without (lanes 1 and 3) or with (lane 2) purified RNAP holoenzyme (11 nM plus 40 nM SigA) before DNase I treatment (noncoding strand represented). The positions of the -10 and -35 determinants as well as the footprinted region are indicated by vertical lines. (B) Results of potassium permanganate footprinting. The DNA fragment encompassing the promoter of the *psbA* gene was incubated with purified RNAP holoenzyme (60 nM plus 100 nM SigA) before potassium permanganate treatment (lane 1, the coding strand subjected to primer extension). The -10 promoter element is indicated in the sequence ladder. (C) Results of the in vitro runoff transcription assay. The *psbA* DNA fragment was incubated without (lane 1) or with (lane 2) RNAP (15 nM plus 48 nM SigA) before the addition of nucleoside triphosphates (see Materials and Methods for details). The arrowhead indicates the full transcript corresponding to the DNA fragment used. Size markers (in nucleotides) are also indicated.

footprinting signals from ca. -55 to $+20$ bp with respect to the tsp of the gene (Fig. 2A; see also reference 10), melting of the promoter exposing T's to the reaction with potassium permanganate (Fig. 2B; see below), and production of full transcripts for the DNA fragment used (Fig. 2C) took place efficiently. These results show an adequate performance of the obtained RNAP preparations.

Binding of *Anabaena* RNAP to promoters activated by NtcA during heterocyst differentiation. Binding of purified RNAP and the effect of NtcA and 2-OG were tested with DNA fragments containing a promoter of *hetC*, *nrrA*, or *devB*. *hetC* encodes an ABC-type exporter required at an early stage of heterocyst development (25), *nrrA* encodes a response regulator proposed to mediate NtcA-dependent activation of *hetR*, the first-acting regulatory gene specific for heterocyst differentiation (11, 12), and the *devBCA* operon encodes an ABC-type exporter involved in the maturation of the heterocyst envelope (15). The expression of *hetC* (36), *nrrA* (35), and *devBCA* (16) is activated upon combined nitrogen deprivation from NtcA-dependent promoters. Binding of RNAP and NtcA to the promoters of these genes was studied by EMSA. As described previously, NtcA is able to bind to the three tested promoters in the absence of any effector (16, 35, 36), although, as in other regulated promoters, binding affinity might increase to some extent in the presence of 2-OG (35; our unpublished results).

Figure 3 shows that RNAP was able to bind to the three tested promoters both in the presence and in the absence of NtcA, alone or together with 2-OG (upper parts of Fig. 3). The addition of NtcA, preferably with 2-OG, prior to the addition of RNAP had some positive effect on the amount of RNAP-containing complexes detected. Quantification of the results shown in Fig. 3 gave the following relative amounts of DNA bound to RNAP for the assays containing RNAP, RNAP plus NtcA, and RNAP plus NtcA and 2-OG, respectively: *hetC*, 1:1.1:2.0 (Fig. 3A, lanes 3 to 5); *nrrA*, 1:1.5:1.9 (Fig. 3B, lanes 4 to 6); and *devB*, 1:1.4:1.7 (Fig. 3C, lanes 3 to 5). Although unspecific interactions of RNAP with the ends of DNA fragments can lead to misleading interpretations of binding features in EMSA, we are confident that the observed binding was specific because site-directed mutation of the *hetC* promoter fragment changing the -10 determinant from TAGTAT to

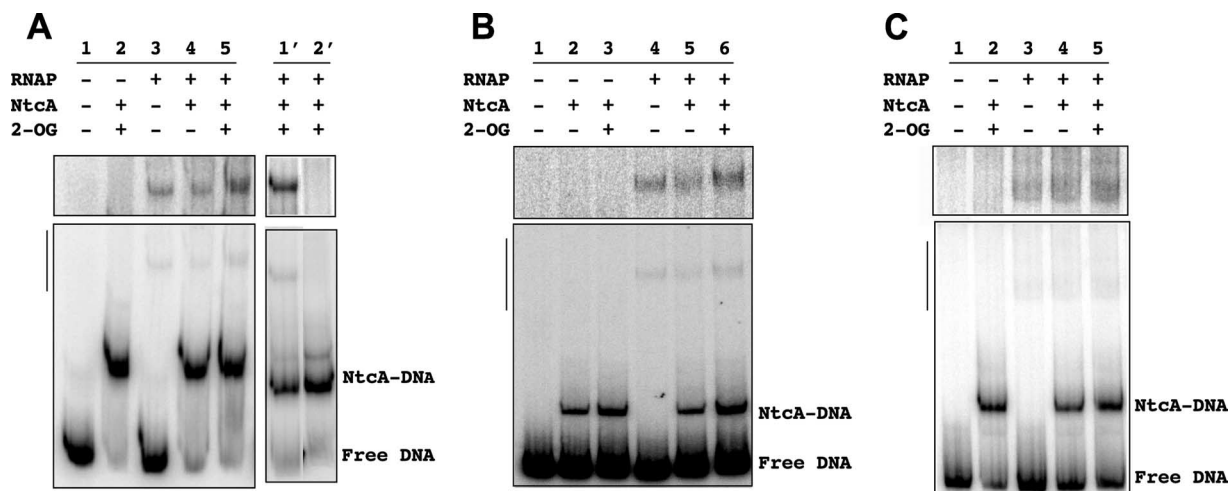


FIG. 3. Results of EMSA of the binding of *Anabaena* RNAP and NtcA to promoters of the *hetC*, *nrrA*, and *devB* genes. The DNA fragment of the *hetC* (A), *nrrA* (B), or *devB* (C) promoter region was incubated, as indicated, with purified NtcA without or with 2-OG (0.6 mM), then supplemented with purified RNAP holoenzyme, and further incubated before being loaded onto PAGE gels (protein concentrations: NtcA, 142 nM for *hetC* and *nrrA* and 130 nM for *devB*; RNAP, 48 nM for *hetC* and 60 nM for *nrrA* and *devB*, plus 110 nM SigA in the three cases [see Materials and Methods for details]). Lanes 1' and 2' of panel A show the results of an independent assay in which a DNA fragment bearing a mutated -10 box with the sequence GGGTAG instead of the native TAGTAT one was used in lane 2' (protein concentrations: NtcA, 130 nM; RNAP, 60 nM plus 110 nM SigA). The upper panels are overexposed images of the top portions (indicated with vertical lines) of the bottom gels showing the RNAP-containing complexes. The positions of the binary NtcA-DNA complexes and of the free DNA are indicated.

GGGTAG completely abolished RNAP binding but not NtcA binding (Fig. 3A, lanes 1' to 2').

NtcA-dependent transcription. Given that both RNAP and NtcA were able to independently bind to the DNA of the tested *Anabaena*-regulated promoters, we wanted to assess the requirement of NtcA and 2-OG for transcription from those promoters. Runoff in vitro transcription was tested with *Anabaena* RNAP in the presence and absence of NtcA and 2-OG. The bands corresponding to the *hetC* (Fig. 4A), *nrrA* (Fig. 4B), and *devB* (Fig. 4C) transcripts (162, 131, and 125 nucleotides, respectively [distance from the *tsp* to the

end of the corresponding fragment; see Materials and Methods]) were readily produced in the presence of NtcA plus 2-OG but not when RNAP was tested alone or in the presence of NtcA without 2-OG. With the three promoters, the requirement of NtcA and 2-OG was observed irrespective of the order of the addition of RNAP and NtcA (not shown). No effect of NtcA or 2-OG in transcript production from the constitutive *psbA* promoter described above was observed (not shown). Remarkably, these results show that, in spite of independent binding of NtcA and RNAP to DNA in the presence and absence of 2-OG (Fig. 3), transcription from

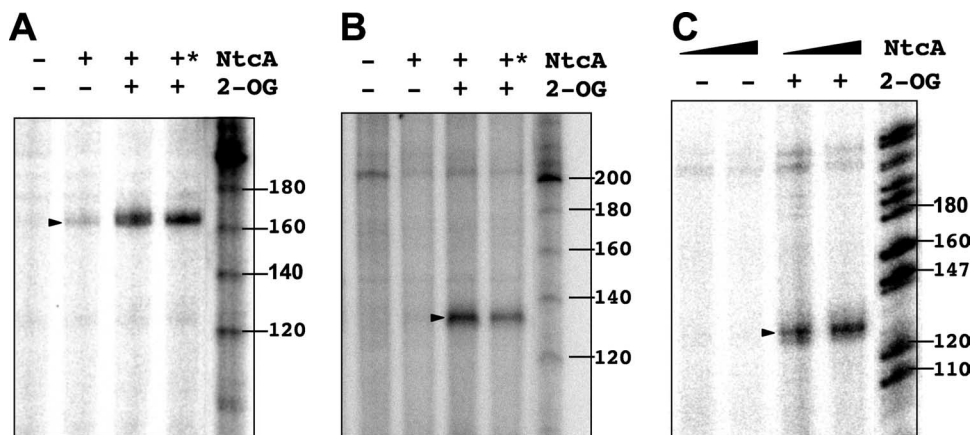


FIG. 4. Effect of NtcA and 2-OG on transcription from promoters of the *hetC*, *nrrA*, and *devB* genes. In vitro runoff transcription assays were performed with DNA fragments of the *hetC* (A), *nrrA* (B), or *devB* (C) genomic region in the presence or absence of NtcA and 2-OG as indicated. NtcA (130 nM [A], 170 nM [B], and 41 or 100 nM [C]), supplemented with 2-OG (0.6 mM) when indicated, was incubated with the DNA fragment before the addition of RNAP (27 nM plus 88 nM SigA [A and B] and 29 nM plus 96 nM SigA [C]; see Materials and Methods for details). Where indicated with *, RNAP was first added to the DNA preparation and, after incubation, supplemented with NtcA and 2-OG. Arrowheads indicate full transcripts encompassing positions from the corresponding *tsp* to the end of the *Anabaena* fragment used. Size markers (in nucleotides) are also indicated.

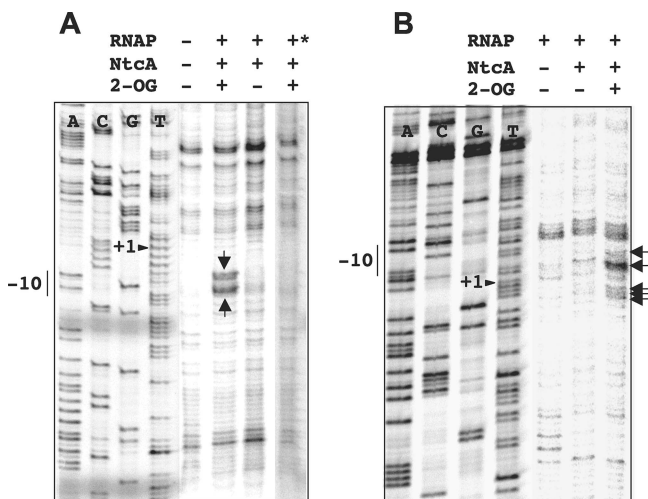


FIG. 5. Effect of NtcA and 2-OG on permanganate footprinting in promoters of the *hetC* and *devB* genes. The DNA fragments of the genomic regions of *hetC* (the fragment with the mutated -10 box in the lane marked with *; see the text for details) (A) and *devB* (B) genes were incubated, as indicated, with purified NtcA (188 and 130 nM, respectively) without or with 2-OG (0.6 mM) and then with purified RNAP holoenzyme (100 nM plus 390 nM SigA and 60 nM plus 110 nM SigA, respectively) and further incubated before potassium permanganate treatment (see Materials and Methods for details). Arrows point to the reactive T's (the noncoding strand of *hetC* and the coding strand of *devB* were subjected to primer extension). The -10 promoter element and the *tsp* (+1) are indicated in the corresponding sequence ladder.

the tested *hetC*, *nrrA*, and *devB* promoters requires both NtcA and 2-OG.

Open complex formation. To trace back the requirement of NtcA and 2-OG in the sequence of events leading to transcription activation at the tested promoters, permanganate footprinting assays were performed to detect open complex formation in the presence and absence of NtcA and 2-OG. Opening of the DNA strands, which during transcription initiation takes place at the promoter, can render T's in the melted region exposed to potassium permanganate so that the detection of reactive T's in the region from ca. -12 to +2 is indicative of the presence of promoter open complexes (for details, see reference 10). Reactive T's can be detected as points of ter-

mination in primer extension reactions performed with the treated DNA. In the *hetC* promoter (Fig. 5A), extension product ends mapping at the T's in the -10 box of the coding strand were specifically detected when the promoter DNA was incubated with RNAP and NtcA plus 2-OG but not when 2-OG was omitted (Fig. 5A and 6). Note that when the DNA was incubated in the absence of RNAP (Fig. 5A), or simply subjected to primer extension without previous treatment (not shown), all the other extension products shown in Fig. 5A were produced. These products should therefore correspond to non-specific stops during the extension reaction. No specific ends were detected when the DNA fragment of the *hetC* promoter bearing a mutated -10 box (Fig. 3A) was used (Fig. 5A). Specific reactivity to potassium permanganate was also apparent for T's between positions -12 and -3 in the noncoding strand of the *devB* promoter (Fig. 5B and 6). Although only weakly labeled, T's at the -10 box in the noncoding strand of the *nrrA* promoter could also be detected (not shown). Remarkably, in all three studied promoters, reactive T's were detected only when both NtcA and 2-OG were present in the assay. These results indicate that the concurrence of the transcriptional activator NtcA and its effector 2-OG is required for an open complex to be formed in the assayed promoters.

DISCUSSION

NtcA, a cyanobacterial transcription factor exerting N control, is essential for the developmental process leading to heterocyst-containing filaments. Thus, *ntcA* mutant strains are unable to initiate heterocyst differentiation (17, 46, 50, 53). Besides that, a substantial number of genes are expressed from NtcA-dependent promoters in differentiating cells or in mature heterocysts, among them the *hetC*, *nrrA*, and *devB* genes (19, 35). Here, we have investigated the requirement of NtcA and the metabolite 2-OG during transcription activation of *hetC*, *nrrA*, and *devB* promoters from the cyanobacterium *Anabaena* sp. strain PCC 7120, using a defined in vitro system that includes the RNAP of the same organism. In the three cases, a stringent requirement of NtcA and 2-OG is observed for transcription. However, RNAP is able to bind the DNA of the three promoters in the absence of NtcA, with only a ≤2-fold positive effect of NtcA plus 2-OG on the amount of DNA-RNAP-containing complexes formed. Thus, the strong re-

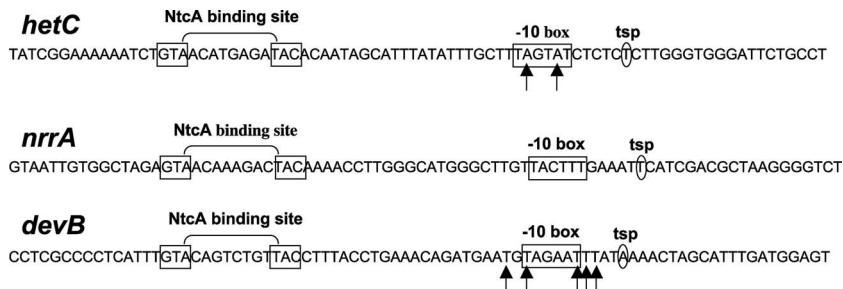


FIG. 6. Features of the NtcA-regulated *hetC*, *nrrA*, and *devB* promoters used in this study. The depicted sequences (noncoding strands) are included in the DNA fragments of the corresponding promoters used throughout this study (see Materials and Methods for details). The locations of the sequence signature of the NtcA binding site, the -10 box, and the *tsp* are as described by Muro-Pastor et al. (36) for *hetC*, Muro-Pastor et al. (35) for *nrrA*, and Fiedler et al. (16) for *devB*. Arrows point to the positions marked in potassium permanganate footprinting in the promoters of *hetC* and *devB* (Fig. 5).

quirement of the regulator at the level of transcript production does not seem to respond only to an effect on RNAP recruitment at these promoters.

Concerning the effect of 2-OG, this metabolite has been shown to exert a moderate positive effect on the affinity of NtcA for the NtcA-regulated promoters, whose magnitudes vary from one to another (see above). However, because NtcA can bind DNA to a substantial degree in the absence of 2-OG and because the amounts of DNA-bound RNAP do not vary significantly when different ratios of free DNA versus NtcA-bound DNA are used (not shown), it is unlikely that the observed requirement of 2-OG for transcription activation responds solely to an effect on NtcA binding. Instead, NtcA and 2-OG would be required at a later step during the formation of the active transcriptional complex. Because DNA melting is detected only in the presence of NtcA and 2-OG at the three investigated promoters, an action of the regulator, which would require 2-OG, at the step of open complex formation could be considered. In this respect, the role of NtcA as a transcriptional activator resembles that of CRP, which at class II-activated promoters increases both the binding of RNAP to DNA for the formation of the RNAP-promoter closed complex and the rate of isomerization of the closed to the open complex (30). However, whereas CRP, as well as its homologous regulators FNR (24) and CooA (23), is unable to specifically bind its DNA targets in the absence of its effector (28), NtcA is capable of specific binding to DNA in the absence of additional factors.

As mentioned above, a positive effect of 2-OG on *in vitro* binding of NtcA to some NtcA-regulated genes of *Anabaena* has already been shown, and 2-OG added externally to a derivative of strain PCC 7120 expressing a cloned 2-OG transporter has been reported to promote differentiation (likely nonfunctional) heterocysts in the presence of nitrate but not of ammonium (31). However, our observations of a stringent requirement of 2-OG for the expression of the *Anabaena hetC*, *nrrA*, and *devB* genes provide strong evidence in support of a key regulatory role of this metabolite at transcription activation in the developing heterocyst. Unfortunately, little is known about the metabolic features of the *Anabaena* cells that are undergoing differentiation, including information about metabolite levels. At the whole-filament level, the *icd* gene, which encodes the isocitrate dehydrogenase that synthesizes 2-OG, has been shown to be expressed at higher levels in diazotrophic cultures of *Anabaena* sp. strain PCC 7120 than in cultures using nitrate or ammonium as the nitrogen source (38). On the other hand, in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, the expression of the *icd* gene is activated by NtcA (38). Given that the *ntcA* gene is induced in proheterocysts (41), activation by NtcA of the *icd* gene during heterocyst differentiation could be a mechanism contributing to high 2-OG levels in the differentiating cell.

Finally, it is worth pointing out that open complex formation was more readily detected with the *psbA* promoter than with the *hetC*, *nrrA*, or *devB* promoter used here, even in the presence of NtcA and 2-OG, consistent with higher levels of *in vitro* transcription of the *psbA* gene (compare Fig. 2 with Fig. 4 and 5). This might reflect the *in vivo* situation, where low levels of expression have been detected at least for the *hetC* (36) and *devB* (16) genes. However, once the requirement of

NtcA activation has been met, the expression of these regulated genes could be further modulated *in vivo*. A number of other protein factors have been identified, mainly by genetic studies, as regulators of heterocyst differentiation (for details, see references 19 and 54), although in most cases neither their precise mechanisms of action nor their molecular targets have been identified. Also, the protein PipX has been described as a coactivator of NtcA in *Synechococcus* sp. (13). Possible roles of many candidates as transcriptional regulators can be tested in the *in vitro* system that we have set up here, as can the promoter specificities of the different alternative sigma factors found to be upregulated in the differentiating cells (1). Additionally, this system should permit the study of the regulation of other promoters that, being activated during heterocyst differentiation in an NtcA-dependent manner, do not exhibit NtcA binding sites and are thus apt to be activated by NtcA indirectly (11, 19).

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