SigC, the Group 2 Sigma Factor of RNA Polymerase, Contributes to the Late-stage Gene Expression and Nitrogen Promoter Recognition in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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We examined the role of SigC (Sll0184), a sigma factor of RNA polymerase (RNAP), in a unicellular cyanobacterium, *Synechocystis* sp. strain PCC 6803. On the inactivation of sigC, which is an *Escherichia coli* rpoD homolog, cells were viable but had a low survival rate in the stationary phase of growth under normal physiological conditions, indicating that SigC is a group 2 type sigma factor. In analyses of transcript and protein levels using the sigC knockout strain, it was found that expression of *glnB*, a nitrogen key regulatory gene, is controlled by SigC in the stationary phase. Primer extension revealed that the *glnB* nitrogen promoter (P2) was specifically recognized by SigC in the stationary phase under conditions of nitrogen starvation. *In vitro* studies with purified enzymes indicated effective transcription, on supercoiled DNA templates, from P2 by SigC-RNAP with NtcA which is an activator for nitrogen gene transcription. DNase I footprinting also indicated binding and related sites of NtcA and/or RNAP with SigC on the nitrogen promoter. The unique promoter architecture and the mechanism of transcription by RNAP with SigC are also discussed.

Key words: cyanobacteria; RNA polymerase; sigma factor; nitrogen promoter; *glnB*

A unicellular cyanobacterium, *Synechocystis* sp. strain PCC 6803 is a gram-negative prokaryote and non-nitrogen-fixing blue-green alga, and can perform oxygenic photosynthesis. Its entire genome has been sequenced and sigma factor (σ, sig gene products) homologs of RNA polymerase were identified.1) The eubacterial RNA polymerase holoenzyme, composed of a σ subunit and a core enzyme containing the major subunits α2 (RpoA), β (RpoB), and β′ (RpoC), plays a central role in gene transcription.2) It is generally accepted that the ancestors of cyanobacteria gave rise to plant plastids via endosymbiotic events, conferring the photosynthetic ability. In phototrophic bacteria, the β′ subunit diverged into γ (RpoC1) and β′′ (RpoC2), corresponding to β′ (RpoC1) and β′′ (RpoC2) in higher plant chloroplasts.3) The core enzyme functions in mRNA polymerization but requires the sigma subunit for specific transcription initiation at the promoter.4,5) General switching in transcription is mainly due to the modulated promoter selectivity of multiple RNA polymerase holoenzymes combined with the replacement of a common core enzyme with several different sigma factors, responding to environmental or internal cellular change. Heterogeneous sigma factors are classified into three groups. Group 1 comprises principal sigma factors that recognize *Escherichia coli* σ70 (sigma70, RpoD) type promoters of housekeeping genes, and are essential for cell viability. The group 2 sigma factors are highly similar in sequence to the group 1 types, but are nonessential. Group 3 sigma factors are also nonessential, and include alternative sigma, which are structurally different from those of group 1 and 2, required for flagella synthesis, heat shock, or general stress response, and extracellular function (ECF). The cyanobacterial group 1 sigma factors have been cloned and characterized.6–9) In PCC 6803, SigA (Slr0653) might be the principal factor and dark/light-induced SigB/SigD, group 2 sigma factors, were found.10) The group 3 SigF (Slr1564) plays a critical role in motility via the control of pili formation.11) The group 2 and 3 sigma expression, promoter recognition, and stress response have also been characterized.12–20) However, the functional assignments and roles of relevant sigma factors are still not clear in

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cyanobacteria. In cyanobacteria, the transcription of nitrogen-regulated genes is regulated by NtcA, a transcriptional activator of the Crp (cAMP receptor protein) family, the DNA target binding sequence of which is GTA-N8-TAC, upstream of the promoter. The PCC 6803 promoters of the ami1, glnA, glnB, glnV, icd, and sigE (sll1689, rpoD2-V) genes, which affect survival under conditions of nitrogen stress, also possess the NtcA-binding motif. The basic promoters recognized by the E. coli sigma70 family are generally configured around E. coli-supplemented with 15 mM sodium phosphate buffer (pH 7.4), and then put through to transmission electron microscopic observation.

**DNA and RNA techniques.** Genomic DNA and total RNA was prepared from PCC 6803 cells (Kazusa DNA Research Institute) as described previously. Hybridization using DNA or RNA was done using methods reported elsewhere.

**Transcriptome and proteome analyses.** For the proteome analysis, wild-type and knockout sigma strains were cultivated until a stationary phase, total protein was isolated from the PCC 6803 cells, and a two-dimensional SDS-polyacrylamide gel electrophoresis (2D-SDS-PAGE) was done. For the transcriptome analysis, total RNA was also prepared from the cells, and ribosomal RNA was removed from the RNA with anti-rRNA oligo magnetic beads (Dynabeads, Dynal), labeled with fluorescein (Minus Label KIT, Pan Vera Corp.), and hybridized with high density gene filters (HDF).

**Quantitative real-time PCR.** Total RNA (100 μg), prepared by the hot-phenol method, was treated with 15 U of DNase I (Takara Shuzo Co., Ltd., Tokyo, Japan) and purified with an RNasy Mini kit column (QIAGEN, Hilden, Germany). The purified RNA (10 μg) was again treated with the DNase I (15 U) for removal of DNA contamination, and the total RNA was recovered by ethanol precipitation following phenol/chloroform/isooamyl alcohol treatment. The obtained total RNA (1 μg) was used for cDNA synthesis with glnB-RT-R primer (5’-TACCAAACCTTGCAACAC-3’). After the cDNA synthesis, the mixture was treated with 2 U of RNase H (Takara Shuzo Co., Ltd.) to preserve cDNAs in cDNA-RNA hybrids. Real-time PCR was done with an iCycler (Bio-Rad, Hercules, CA, USA) in a 30 μl reaction mixture containing 1 μl of cDNA, 20 pmol of primers [glnB-RT-F (5’-AGTAGAAGGGAT TTCG-3’) and glnB-RT-R], and 15 μl of iQ SYBR Green Supermix (Bio-Rad). Amplifications were done by incubating reaction mixtures at 95°C for 30 sec prior to 40 cycles of 30 sec at 95°C (for melting) followed by 30 sec at 61°C (for annealing) and 30 sec at 72°C (for extension). Data were collected and analyzed at each 72°C stage. After the amplification, a melting curve was examined to confirm specific amplification of the products, in a program consisting of 80 cycles, starting at 55°C, with 0.5°C increments/cycle at 10-sec intervals. A standard curve was also constructed with several serial dilutions (1 to 1 x 10^-4) of cDNA, synthesized with total RNA extracted from wild-type cells under nitrogen starvation conditions at mid-log phase. The relative abundance of glnB transcripts was calculated with the standard curve.
Assays without cDNA were examined for every experiment as a negative control. All assays were done in triplicate.

**RNA polymerase, sigma factors, and NtcA.** The *E. coli* core and holoenzymes were purified. Overproduction and purification of SigA and SigC were done by previous procedures. For NtcA (Sll1423: the position of the gene in the genome is from 1,590,061 to 1,589,384), a DNA fragment was amplified by PCR, digested with *Sph*I and *Sal*I, then inserted into the *Sph*I and *Sal*I sites of pQE30 to create an expression vector, pNTCA, which allows one to produce NtcA 6×His-tagged in the N-terminal region. Overproduction and column chromatography purification of NtcA from the host *E. coli* M15[pREP4] cells harboring pNTCA were done as follows. When the turbidity of the 250 ml of cell culture reached 0.5 absorbance units at A660, IPTG was added to the culture at a final concentration of 0.2 mM. After incubation at 20°C for 6 hours, the cells were harvested by centrifugation and stored at −80°C until use. After thawing of the overexpressed cells on ice, they were suspended in 3 ml of lysis buffer [50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl] containing 10 mM imidazole. After that, the cells were disrupted by a sonication (TOMY UD-201: OUT-PUT level of 3.5, 1 min, four times). This suspension was centrifuged for 10 min at 15,000 × g, and the supernatant involving overproduced NtcA was collected. The supernatant was applied to an Ni²⁺-nitrilotriacetic acid column (Qiagen, 4 ml of column volume), equilibrated in the lysis buffer containing 10 mM imidazole. The column was washed with 10 ml of the lysis buffer containing 10 mM imidazole and 30 ml (3 times 10 ml) of the lysis buffer containing 20 mM imidazole. The recombinant protein was eluted with 10 ml of the lysis buffer containing 250 mM imidazole. The eluted NtcA protein was dialyzed against storage buffer [50 mM Tris-HCl (pH 7.9), 50% glycerol, 0.1 mM EDTA-2Na, and 0.1 mM DTT] and stored at −80°C.

**Polyclonal antibody and Western blot.** The preparation of rabbit antiserum against the purified GlnB (PII) protein and Western blot analysis using the polyclonal antibodies were based on previous methods. The signal intensities corresponding to target proteins (Fig. 3A) were measured by BIO-1D V. 96 SOFTWARE (Vilber Lourmat, Marne la Vallée, France).

**In vitro mRNA synthesis.** A multiple-round run-off transcription assay was done as described previously. The assay mixture (40 μl) comprised 50 mM Tris-HCl (pH 8.0), 0.05 mM EDTA-2Na, 0.5 mM DTT, 10 mM MgCl₂, 0.1 mM each of ATP/CTP/GTP/UTP, template pGLN9B DNAs carrying the PCC 6803 *glnB* (ssl0707) upstream region (−290 to +139, +1 as the starting codon), and RNA polymerases. The mixture was incubated at 30°C for 10 min, and then the reaction was stopped by the addition of 60 μl of stop solution (40 mM EDTA-2Na, 300 μg/ml of *E. coli* tRNA and 300 mM LiCl). The RNA products were precipitated with 2-propanol and put through primer extension.

**Primer extension.** Primer extension analyses were done as described with total RNA, prepared from the PCC 6803 cells, and a glnB-R2 ([−GGGGCGGATTAA–TGGCTTC-3')) primer at an annealing temperature of 42°C. Products of the extension were dissolved in 7 μl of dye solution, and denatured at 95°C for 5 min. Then a portion of 3 μl was resolved on a 6% polyacrylamide gel containing 8 M urea. For primer extension using synthesized mRNA, the mRNA products synthesized in vitro (see the section above) were dissolved with 10 μl of water, then subjected to the reaction mentioned above. The signal intensities corresponding to target transcripts (Fig. 5) were measured by BIO-1D V. 96 SOFTWARE.

**DNase I footprint.** Primer glnB-R2 (40 pmol) was 5′-end labeled with [γ-32P]ATP (6,000 Ci/mmol, Amer sham), and then diluted with water to a final concentration of 2 pmol per μl. A DNA fragment containing the *glnB* promoter region was synthesized by PCR amplification using non-labeled glnB-F ([−ACCCGGGTTCGATTCCCGCGCTTAGAC-3]) primer on the non-transcribed strand, italic: *Sma*I linker) and labeled glnB-R2 (on the transcribed strand) primers (each 20 pmol), and plasmid pGLN9B as template DNA. The resultant PCR fragment, of which one strand was only labeled at the 5′-end, was purified on a G-50 spin column (Pharmacia), then prepared as a final concentration of 1 pmol per μl. Binding of RNAP to the RNA fragment was done as follows. Relative RNA polymerase and the labeled DNA (1 pmol, 1 μl) were incubated in 25 μl of a reaction mixture containing 250 mM Tris-HCl (pH 8.0), 2.5 mM EDTA-2Na, 10 mM DTT, 50% (w/v) glycerol, 150 mM KCl, 15 mM MgCl₂, 0.5 mM ATP, and 50 μg/ml bovine serum albumin. After the DNA-binding reaction for 10 min at 30°C, 5 μl of DNase I (0.1 units) was added to the mixture, and then incubation proceeded for 1 min at room temperature. After that, the reaction was stopped by adding 75 μl of stop solution [10 mM EDTA-2Na, 0.1% (w/v) SDS, and 25 μg/ml *E. coli* tRNA]. This mixture was put through 2 rounds of phenol extraction followed by precipitation with 2-propanol, and a wash with 80% ethanol. After centrifugation, the pellet was resuspended in 10 μl of sample buffer [95% (w/v) formamide, 20 mM EDTA-2Na, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol], incubated for 5 min at 95°C, and then the sample (3 μl) was electrophoresed on a 6% sequencing gel.

**Results**

**Knockout of sigC and cell viability**
Knockout of sigC, Growth and Survival.

(A) Gene disruption in the PCC6803 genome. Ba, BamHI; Bg, BglII; Bs, BsiI; E, EcoRI; H, HindIII; P, PstI; X, XhoI. (B) Genomic Southern. The wild-type strain (WT) and transformants (lanes 1 to 4) were examined by the blot analysis. Probes of a 1.0-kb EcoRI-Xhol fragment of sigC and a 0.8-kb PstI-PstI fragment of the kanamycin resistance gene (KmR) for Southern blotting are shown in panel A. The sizes (kb) are indicated.

Identification of a gene controlled by sigC.

A proteome analysis using two-dimensional SDS-PAGE was done with total protein prepared from the sigC knockout strains in the stationary phase under white-light illumination. We could confirm about 200 different spots of protein on the gel (http://www.kazusa.or.jp/zyano/zyano2D), and evaluated the signal intensities between the wild-type and ΔsigC. Among spots reducing the signal intensity (data not shown), we were very interested in a spot corresponding to the nitrogen regulatory gene (glnB, ssl0707) product, PII, in the ΔsigC cells. It has been reported that the glnB expression elevates under nitrogen starvation. Therefore, the level of PII was examined by Western blot (Fig. 3A). The expression was markedly reduced in the ΔsigC cells under nitrogen depletion at the stationary phase but not at logarithmic phase. This result might show that sigC contributes to PII expression. Of note, we could not identify other possible transcriptional regulatory proteins in proteome analysis, since about 200 spots of proteins correspond to only 6 to 7% of total potential proteins in PCC 6803. Under this situation, we therefore did further experiments for obtaining functional evidences of the sigma factor in nitrogen gene expression, because no comprehensive reports have appeared concerning its effect in the cyanobacterium which does not have &sigma; type sigma factor. To obtain evidence that sigC expression directly affects glnB transcription, transcrip-
tome analysis was done using high density gene filters (HDF, macroarray filter) and labeled mRNAs prepared from cells grown under the same conditions as in Figs. 1 and 2. The two HDFs are a set containing 5,177 dots of DNA fragments (1- to 1.5-kb long) with minimal overlap, which were amplified by PCR, that cover the entire Synechocystis genome. The \textit{glnB} gene is located at position 2,152,629 to 2,152,291 in the genome (http://www.kazusa.or.jp/cyano/). The transcription start points of P2 or P1 are 2,152,682/2,152,683, or 2,152,662, respectively. The size of transcript is 0.68-kb. Five spots (2A11#12, #13, #14, #15, #16) on HDF contain DNAs for DNA probes covering the \textit{glnB} region (2,149,546 to 2,152,098; 2,150,131 to 2,152,599; 2,150,946 to 2,153,634; 2,151,580 to 2,153,917; 2,152,324 to 2,155,074, respectively). The total signal intensities of their \textit{glnB} spots were reduced about 50% in the /C1\text{ sigC} cells (data not shown). QRT-PCR analyses were further done and we could also confirm the decrease of the \textit{glnB} transcripts in the /C1\text{ sigC} cells at stationary phase (Fig. 3B), strongly showing that \textit{glnB} transcription directly depends on \textit{sigC} expression.

\textbf{sigC controls \textit{glnB} P2 transcription induced by nitrogen deprivation}

To address the function of \textit{sigC} in \textit{glnB} transcription, specific promoter recognition was verified by primer extension in the \textit{Delta sigC} strain (Fig. 4). Results clearly showed that \textit{glnB} transcription was induced from P2 (-54, -53) on nitrogen starvation in a mid-log phase of
growth in both the wild-type and \( \Delta \text{sig}C \) strains (lanes 3 and 4) as previously reported.\(^{40}\) There were almost no differences in the signal patterns of these cells (lanes 1 vs 2, 3 vs 4). On the other hand, the signal intensities were relatively reduced in the stationary phase (lanes 5 to 8), however, significant induction was observed in the wild-type cells (lane 7) but not (no signal) the \( \Delta \text{sig}C \) strain (lane 8) under conditions of starvation. The inactivation of \( \text{sig}C \) did not affect the transcription of P1 (odd vs even lanes). These results show that SigC takes part in the P2 transcription induced by nitrogen depletion in the stationary phase, and the P1 transcript is expressed constitutively. It was also clear that a sigma factor other than SigC was required for the transcription of P2 induced by nitrogen depletion, in the exponential phase of cell growth (Fig. 4, lane 4).

In vitro \( \text{gln}B \) transcription by RNAP with SigC

We examined whether SigC can directly and specifically recognize the \( \text{gln}B \) P2 promoter, and characterized the \( \text{gln}B \) transcription based on the DNA template architecture, RNAP (+ sigma), and a nitrogen regulator, NtcA. Since the −10 region of the P1 (−33) and P2 (−54, −53) promoter has an \( \text{E. coli} \) consensus sequence recognized by \( \sigma^{70} \), we used PCC 6803 SigA principal sigma factor to compare with SigC function in vitro. The results are shown in Fig. 5. We used \( \text{E. coli} \) core enzyme, which has a high degree of amino acid sequence similarity to that of PCC 6803 core subunits, for the reconstitution of RNAP with PCC 6803 sigma factors, since neither core enzyme from the PCC 6803 cells nor core enzymes reconstituted using each recombinant PCC 6803 core subunit from \( \text{E. coli} \) have been obtained. Specific recognition by RNAP holoenzyme reconstituted with an \( \text{E. coli} \) core and cyanobacterial sigma factors has been reported.\(^{26,36,38,42–44}\) When an \( \text{E. coli} \) RNAP holoenzyme with PCC6803 NtcA was used, a strong signal was observed from the −35 site, which is more than 2 bases upstream from the −33 site, identified in vivo as the position of P1, using linear template DNAs (Fig. 5A, lane 2). No transcript from P2 (−54, −53) and very little transcript from the −56 position also appeared (Fig. 5A, lane 2). This indicates that the principal sigma of \( \text{E. coli} \) better recognizes the P1 than P2 type promoter, however, \( \text{E. coli} \) Esx cannot drive \( \text{gln}B \) transcription from the precise positions of P1 and P2 from the linear DNA template. When holoenzymes, reconstituted with \( \text{E. coli} \) core enzyme plus PCC 6803 SigA, were used, significant amounts of transcripts from P1 and P2 were observed, as well as large amounts of pseudo transcripts from the position −35 or −56 (Fig. 5A, lane 3). This means that the promoter recognition between PCC6803 SigA and \( \text{E. coli} \) \( \sigma^{70} \) on the promoter is similar, but the transcription efficacy of SigA is higher than that of \( \sigma^{70} \). When SigC was used, the transcripts from P1 and P2 were detected, and pseudo transcripts from −35 and −56 were markedly reduced compared with the case of SigA (Fig. 5A, lane 4). These findings might show that in amount of transcription, SigA > SigC, but in the specificity of transcription, SigC > SigA, when linearized DNA template was used. On the other hand, when supercoiled templates were used, larger and more specific transcripts from the correct start points of P1 and P2 were observed by RNAP with SigC than SigA (Fig. 5A, lanes 5 to 8). When NtcA was added, the amount of transcript increased significantly (lanes 6 and 8). Interestingly, the amounts of specific transcripts (P1 and P2) also increased significantly when supercoiled DNA templates (lanes 6 and 8) were used instead of linearized DNA (lanes 3 and 4). This showed that an efficient and accurate \( \text{gln}B \) transcription in vitro requires DNA with a supercoiled architecture. Furthermore, the transcript from P2 was more abundant with SigC than SigA, indicating that the nitrogen promoter P2 might be more efficiently transcribed by RNAP with SigC than SigA (lanes 8 vs 6), even without the addition of NtcA (lanes 7 vs 5). These results also indicated the functional similarity and distinction of promoter recognition among \( \sigma^{70} \), SigA, and SigC. Of note, a relative weak signal at −47 was observed in vitro when PCC 6803 SigA or SigC was used but not \( \text{E. coli} \) \( \sigma^{70} \) (Fig. 5A). No obvious
or consensus promoter sequences from the position have been previously reported. To confirm the role of SigC, we further did a stoichiometric competition analysis for transcription (Fig. 5B). When increasing amounts of SigA were added to a constant amount of SigC, the amount of transcript from P2 gradually decreased in vitro (lanes 1 to 5), whereas it increased in the opposite case (lanes 6 to 10), reinforcing the idea that RNAP with SigC rather than SigA efficiently and specifically drives transcription from the P2 promoter. The reason for the decrease in transcript from P2 on addition of SigA (Fig. 5B, lanes 1 to 5) will be discussed later.

Binding sites of NtcA and RNAP with SigC
To address the DNA binding sites of NtcA and RNAP in the glnB promoter regions, we did a DNase I protection assay (Fig. 6). The NtcA-binding site to the upstream region at Synechococcus glnA has been characterized, however no data have been published on the PCC 6803 glnB gene by NtcA and/or RNAP. When we used NtcA, a clear protective site from −10 to −35 was observed with hyper-sensitive sites (single arrowheads). This finding shows that an NtcA-binding site at the NtcA box (GTA-N8-TAC) causes a change resulting in the hyper-sensitive sites for DNase I (left panel, lane 2). When a holoenzyme with SigC plus NtcA was used, the hyper-sensitive band intensities at −79 and −69 were increased (left panel, lane 5; right panel, double arrow heads), indicating that NtcA might interact more with SigC in an RNAP just upstream from the −10 region of the P2 promoter (Fig. 6, right panel). When a holoenzyme with SigA plus NtcA was used, a similar pattern was observed but slight decreasing signal intensities at the positions of −79 to −69 or around −10 region (−55 to −38) of the P1 promoter was detected on an original X-ray film (left panel, lane 4 vs lanes 3 and 5). This suggests that RNAP with SigA can interact at the P1 promoter.

Discussion
The evidence obtained in this study indicates that SigC directly recognizes the P2 promoter under con-
ditions of nitrogen starvation during the stationary phase of growth. Of note, the expression of the sigC transcript itself also increased following nitrogen deprivation and the cell viability was apparently reduced by the starvation (data not shown). We observed no significant reduction in the transcription of P2 in knockout strains lacking other group 2 sigmas, SigB, SigD, and SigE, at the stationary phase (in preparation for publication).

Taking all these things into consideration, we conclude that SigC is a cyanobacterium group 2 type sigma factor of which may be required in a stage-specific manner and which controls at the very least the promoter for a nitrogen-related gene. The results also suggested that a good candidate for a long-life saver is sigC since the cell viability of the sigC mutants was low in the stationary phase (Fig. 1). Furthermore, natural changes to the sigC mutant clearly indicate significant sigC function (Fig. 2).

A summarized schema is shown in Fig. 7. The activity of SigC and/or other trans-acting factors is elevated in the stationary but not exponential phase, since sigC expression only affects the glnB P2 promoter in the stationary phase (Fig. 4, lane 8). This suggests that another sigma factor recognizes the P2 promoter in the exponential phase, therefore, there might be a “sigma-switch” for the nitrogen-promoter recognition during the exponential to stationary phase. A recent report showed that a group 2 type sigma factor, sigE (sll1689, rpoD2-V), promoter has a NtcA-box and its transcription was dependent on nitrogen deprivation. If sigE directly controls the glnB P2 promoter, a deletion of sigE will result in no induction by nitrogen starvation in the exponential phase. In addition, another unsolved thing is

Fig. 6. The Binding Specificity of NtcA and RNAP for SigA or SigC.

[Left] DNase I protection assay. E. coli RNAP core enzyme (2.9 pmol), PCC 6803 SigA or SigC (10 pmol each), NtcA (10 pmol), labeled glnB DNA fragments (1.7 pmol), DNase I (0.5 units: lanes 1 to 5, 1 min; lane 6, 2 min). A schema of the binding site is shown in the right margin. The position of the glnB start codon (+1) is indicated at the left. Non, no protein; Ec, E. coli core enzyme of RNAP; 1 or 2 min, reaction times with DNase I. [Right] The glnB 5'-upstream region. NtcA binding and hypersensitive sites (arrowheads) for DNase I are shown. The hyper-sensitive bands (−79 and −69) for RNAP with SigC are shown as double arrowheads. The promoter sequences of SigA (constitutive type, P1) or SigC (nitrogen-starvation induced type, P2), a glnB start codon, and the position of the glnB-R2 (= glnB6) primer are presented.

Fig. 7. Model for glnB Transcription.

P1, constitutive expression; P2 nitrogen-starvation induced transcripts. See text for details.
whether SigC can preferentially recognize nitrogen promoters, e.g. sigE, glnA, glnN, ntcA, and ntcB, containing (or not containing) the NtcA-box sequence. We are currently testing these points. Because SigA, a potential principal sigma factor, can recognize and increase in vitro transcription from P2 on addition of NtcA (Fig. 5A, lane 6), there is also a possibility that SigA confers the ability for transcription to the P2 promoter in a redundancy with other sigma factors in the exponential phase. We cannot exclude the possibility that sigmas other than SigA and SigC can specifically recognize the P2 promoter under conditions of nitrogen-starvation in the exponential phase. It has been generally accepted that a principal sigma factor is indispensable for cell growth and the binding affinity to the core enzyme is stronger than that to alternative sigma factors in E. coli. The excess of SigA in vitro might cause an occupation of the core enzyme, resulting in the decrease of transcription by RNAP with SigC (Fig. 5B, lanes 1 to 5). The molar ratio and the timing of the activation of sigma factors may also be important to glnB P2 transcription.

The glnB promoter architecture is unique (Fig. 6). It has a tandem hexamer of canonical −10 sequences (P1, TATATA; P2, TAAAAG) but a possible −35 hexamer (TTAAAA) only from P1.40 The distance between each −10 hexamer is only 14 base pairs and the NtcA-box lies 23 base pairs upstream from the −10 hexamer of the P2 promoter. The results on NtcA- and RNAP-binding to the promoter regions (Fig. 6) may indicate that the NtcA-binding recruits an RNAP containing SigC upstream of the P2 promoter (Fig. 7). It was actually observed that the hypersensitive signal intensity (−79 and −69) of DNase I was increased more, when NtcA coexisted with SigC (Fig. 6, left panel, lane 5). The NtcA-binding sites (−110 to −82) and the area just downstream have several deoxy adenine and/or thymine tracts which have been known to cause an intrinsic DNA curvature, which is often important for transcription, in cyanobacteria.26,42,47 The NtcA-binding further results in a change in the DNA architecture and the hypersensitive sites appear (Fig. 6) up- and downstream of the binding region. These three-dimensional DNA structures are important for glnB transcription, as supported by the finding that supercoiled DNA templates are required for effective transcription (Fig. 5). On the other hand, the RNAP with SigA can bind to the P1 promoter (Fig. 6) which has the −35 and −10 hexamers as the consensus promoter. Transcripts from P1 were constitutively expressed in the ΔsigC cells (Fig. 4). These findings suggest that the P1 promoter is a SigA-type.

The PCC 6803 group 2 sigma, sigE (sll1689, rpoD2-V), transcripts were induced by nitrogen starvation and the gene disruption caused a reduction in the transcripts of glnN (type III glutamine synthetase).40 In that report, glnB (P1 signaling protein) and amt1 (ammonium/methylammonium permease) were not significantly altered in the cells with a disrupted sigE gene. From that and the results shown in this study, the nitrogen-regulated gene expression therefore might be controlled by several sigmas. This seems to be a difference for nitrogen gene expression involving a regulatory system between sigma54 in E. coli and group 2 sigma in cyanobacteria. Synechocystis PCC 6803 is a unicellular, non-nitrogen-fixing cyanobacterium, in which signal transduction to sense changes in the environmental condition of a nitrogen source is involved in the phosphorylation and dephosphorylation at a seryl-residue on GlnB (PIIser).48 This phospho-PII may act as a sensor kinase.49 NtcA-dependent gene expression requires the GlnB signal transduction protein.50 On the other hand, a recent study revealed that cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate (2-OG) levels51 and that the binding of NtcA to glnA and ntcA promoters of Synechococcus PCC 7942 in vitro has been shown to be increased by 2-OG.52 The possibility of an activity increase by 2-OG on the glnB P2 promoter is being evaluated in vitro with SigC-RNAP and NtcA in our laboratory. The structures of Synechocystis PCC 6803 SigC, Anabaena PCC 7120 SigC, and Synechococcus PCC 7002 SigE are similar in the cyanobacterial group 2 sigma factors.17,38 In the nitrogen-fixing cyanobacterium PCC 7120, sigC mRNAs are detectable only under nitrogen-limiting conditions.13 However, the inactivation of sigC indicates that neither gene alone is essential for nitrogen fixation or heterocyst differentiation. Three group 2 sigma factors, sigD, sigE, and sigF (sigD, sigB, and sigE homologue of PCC 6803) also have been identified in PCC 7120. Insertional inactivation of these genes did not affect growth on nitrate under standard conditions but did transiently impair the abilities of sigD and sigE mutant strains to undergo diazotrophic growth, and the appearance of heterocysts was delayed.20,49 In another non-nitrogen-fixing cyanobacterium, PCC 7002, sigE transcripts were specifically expressed in the post-exponential phase,15 as in the case of PCC 6803 sigC.38 A functional PCC 7002 SigE is required for the expression of the starvation-induced protein DpsA/PexB.16

We could identify the gene, glnB, controlled by SigC in PCC 6803, using approaches with transcript and protein-level analyses. Overall, the in vivo and in vitro results coincide well in this study. In high density gene filter (HDF) analysis, the profile of sigC gene disruption revealed the signal intensity to be relatively weak, suggesting that SigC affects gene transcription at a global level in the stationary phase (data not shown). However, it is of interest that there were a lot of up- and down-spots on the 2D-SDS-PAGE gel for proteome analysis (data not shown). These findings indicate that gene expression by SigC might be regulated at the transcriptional, post-transcriptional, and translational levels. These approaches will be also useful for functional analyses of other sigma factor genes.
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