Growth Phase-dependent Activation of Nitrogen-related Genes by a Control Network of Group 1 and Group 2 \( \sigma \) Factors in a Cyanobacterium*

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It has been reported that an RNA polymerase \( \sigma \) factor, SigC, mainly contributes to specific transcription from the promoter PglkB-54,53 under nitrogen-deprived conditions during the stationary phase of cell growth in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Asayama, M., Imamura, S., Yoshihara, S., Miyazaki, A., Yoshida, N., Sazuka, T., Kaneko, T., Ohara, O., Tabata, S., Osanai, T., Tanaka, K., Takahashi, H., and Shirai, M. (2004) *Biotechnol. Biochem. 68, 477–487*). In this study, we further examined the functions of group 2 \( \sigma \) factors of RNA polymerase in NtcA-dependent nitrogen-related gene expression in PCC 6803. Results indicated that SigB and SigC contribute to the transcription from PglkB-54,53 with a \( \sigma \) factor replaced in a growth phase-dependent manner. We also confirmed the contribution of SigB and SigC to the transcription of other NtcA-dependent genes, glnA, sigE, and amtl, as in the case of glnB. On the other hand, the transcription of glnN was dependent on SigB and SigE. In the SigB and SigC-based regulation, the level of SigB increased, but that of SigC was constant under conditions of nitrogen deprivation. Furthermore, it was found that SigC negatively and positively regulates the level of SigB in the log and stationary phase, respectively. SigC also had a positive effect on the level of sigB transcript during the stationary phase. In contrast, SigB acts positively on SigC levels in both growth phases. These results and previous findings indicated that multiple group 2 \( \sigma \) factors take part in the control of NtcA-dependent nitrogen-related gene expression in cooperation with a group 1 \( \sigma \) factor, SigA.

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The RNA polymerase holoenzyme of eubacteria consists of a core enzyme and \( \sigma \) factor (1). The core enzyme is capable of undergoing transcriptional elongation, and the \( \sigma \) factor is required for the initiation of transcription from a specific promoter sequence. Multiple \( \sigma \) factors are usually encoded by a eubacterial genome, and they have been generally classified into three groups (2). Group 1 comprises principal \( \sigma \) factors that are responsible for transcription from a number of housekeeping promoters and are eventually crucial for cell viability. Group 2 and group 3 \( \sigma \) factors are alternative types. Group 2 \( \sigma \) factors are similar to the group 1 types in molecular structure but are nonessential for cell viability. Group 3 \( \sigma \) factors are structurally different from proteins of group 1 and group 2 and are sometimes involved in the transcription of regulons for survival under stress. The cyanobacterium *Synechocystis* sp. strain PCC 6803 used in this study possesses nine species of \( \sigma \) factors assigned to group 1 (SigA), group 2 (SigB, SigC, SigD, and SigE), and group 3 (SigF, SigG, SigH, and SigI) (3, 4). The functions of some of these \( \sigma \) factors have been recently revealed. For example, SigD and SigB are light- and dark-responsive \( \sigma \) factors (4–6). Sigh is also identified as a heat-shock \( \sigma \) factor (4, 6). SigE is a \( \sigma \) factor required for positive regulation of sugar catabolic pathways (7).

Cyanobacteria, blue-green algae, are prokaryotes that perform oxygenic evolving photosynthesis like plants and mainly use inorganic nitrogen sources, ammonium and nitrate. The nitrate is reduced by nitrate reductase and nitrite reductase, and the resulting ammonium is usually incorporated with glutamine synthetase (GS) and glutamate synthase (GOGAT), a pathway commonly known as the GS-GOGAT cycle (8). In this cycle, 2-oxoglutarate (2-OG), which is synthesized by isocitrate dehydrogenase from isocitrate, is used as a carbon skeleton for nitrogen assimilation. A remarkable feature of the intermediary metabolism of cyanobacteria is a lack of 2-OG dehydrogenase (9). Consequently, 2-OG is a main substrate for nitrogen assimilation in cyanobacteria.

The system regulating nitrogen levels is well characterized in enteric bacteria (8, 10). Expression of glnA (encoding glutamine synthetase) and other nitrogen-related genes is required for RNAP-containing RpoN (\( \sigma^{54}\)), an alternative \( \sigma \) factor whose molecular structure and transcriptional mechanism are quite different from those of group 1–3 \( \sigma \) factors (11). The expression is regulated by a two-component regulatory system, NtrB/NtrC, the activity of which is controlled by the uridylylation status of PII (8, 10). The uridylylation or nonuridylylation of PII is coordinated by the ratio of intracellular concentrations of 2-OG and glutamine. PII itself binds 2-OG; therefore, it senses the status of the cells and plays a central role in the assimilation of nitrogen.

The regulation of nitrogen assimilation could differ between enteric bacteria and cyanobacteria, because no homologues of RpoN-type \( \sigma \) factor, NtrB/NtrC, and glutamine synthetase adenylyltransferase have been identified in cyanobacteria. In fact, a cAMP receptor protein family transcription factor, NtcA, plays a central role in nitrogen assimilation in cyanobacteria. A consensus sequence needed for the binding of NtcA to DNA (TGAN\(_T\)TAC) has been reported, and the motif is generally located about 40 bp upstream from the transcription start point (12). The promoters activated by NtcA exhibit a conserved sequence, TAN\(_T\)T, as a ~10 promoter hexamer but do not possess a ~35 hexamer.

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2 The abbreviations used are: 2-OG, 2-oxoglutarate; RNAP, RNA polymerase; QRT-PCR, quantitative real-time PCR; IS, integration site; ppGpp, guanosine 3,5-(bis)pyrophosphate; \( \Delta \), \( \Sigma \), and \( \Delta E \), sigB, sigC, sigD, and sigE knock-out strain, respectively; Log, midexponential phase; Sta, stationary phase; ~N and +N, without and with nitrogen, respectively; GS, glutamine synthetase; GOGAT, glutamate synthase.
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Under nitrogen-deprived conditions, 2-OG directly binds to NtcA and increases the DNA binding affinity of NtcA (13, 14). NtcA with 2-OG activates the expression of nitrogen assimilation-related genes. Although 2-OG and NtcA play key roles in the assimilation of nitrogen, a molecular study of NtcA-dependent transcription has been performed only in a few cases in cyanobacteria. For example, Muro-Pastor et al. (15) have reported that the group 2 σ factor gene, sigE (rpoD2-V), possesses the NtcA-binding motif upstream of its promoter, and its transcription is induced under nitrogen-deprived conditions. Expression of glnN, a type-3 glutamine synthase gene, was impaired in strains bearing an inactivated copy of the sigE gene of Synechocystis sp. PCC 6803 (15).

Our recent study revealed that transcription from the glnB (encoding PI) (16) promoter (PglnB-54, -53) is due to specific recognition by a PCC 6803 group 2 σ factor, SigC, in the stationary (postexponential) growth phase under nitrogen-deprived conditions (17). This raised the possibility that another σ factor recognizes the glnB promoter in the logarithmic (exponential) growth phase and that a “σ-switch” for the nitrogen-promoter recognition occurs during the log to stationary phase. However, which σ factor recognizes the glnB promoter under the log phase has remained to be elucidated. Here, we presented data for resolving this issue. We also characterized the specificity with which PCC 6803 group 2 σ factors recognize other NtcA-dependent promoters, glnA, sigE, amt1, and glnN. We summarize these results and present a possible regulatory network of group 1 and group 2 σ factors for the transcription of NtcA-dependent nitrogen-related genes.

**EXPERIMENTAL PROCEDURES**

Strains and Growth Conditions—*Synechocystis* sp. strain PCC 6803 (Kazusa strain) was grown at 30 °C with shaking (120 rpm, NR-30, TAITEC, Tokyo, Japan) under white light (35 μmol m⁻² s⁻¹ photon flux) in BG11 medium (18), supplemented with 15 μg/ml kanamycin sulfate and/or 40 μg/ml spectinomycin if required. For nitrogen-starved conditions, PCC 6803 was cultivated in BG11 medium without NaNO₃ for 6 h, as described previously (17).

Isolation of RNA and Primer Extension Analysis—Procedures were performed as described previously (19). The oligonucleotides used in the primer extension for glnB, glnA, amt1, glnN, sigE, and sigC were glnB-R2 (17), glnR-R (5′-CGTGGATATCAGCCATGAGGCTTC-3′), amt1-R4 (5′-CTACATGGTTCACAGCAAG-3′), glnN-R2 (5′-GCCAGAAGATAGGCTGCAG-3′), sigB-R2 (4), and 0184V-R3 (5′-TCGGTCTCGTGTATGTC-3′), respectively. The products of reverse transcription were dissolved in 7 μl of a dye solution and denatured at 95 °C for 3 min. Then an aliquot of 3 μl was resolved on a 7% polyacrylamide gel containing 8M urea followed by autoradiography.

Quantitative Real Time PCR Analysis—Quantitative real time PCR (QRT-PCR) was performed as described previously (17). The set of oligonucleotides used for glnB, glnA, glnN, sigE, amt1, and rnr166Sa was as follows: glnB-RT-F/glnB-RT-R (17), glnA-RT-F (5′-CCCAACCCGACCAACATCTC-3′)/glnA-RT-R (5′-GCTGGATATCAGCCATGAGGCTTC-3′), glnN-RT-R (5′-CTCGGAAATATGTTGCGTGCT-3′)/glnN-RT-F (5′-CTGGTCTCGTGTATGTC-3′), sigE-RT-F (5′-AAAGAAATGAGGCCTCTACCC-3′)/sigE-RT-R (5′-TTGGTCTACAGGGGTACGTC-3′) and 16srRNA-RTF (5′-CTGGAAGATGCGGCTGCTC-3′)/16srRNA-RT-R (5′-CTCATTGACCGGGCCTGACGCT-3′), respectively. Standard curves for each gene were also constructed with serial dilutions (1 to 1 × 10⁻⁵) of cDNA, synthesized with total RNA extracted from wild-type cells under nitrogen-deprived conditions at the log phase. Relative relative levels of transcripts were calculated with the relevant standard curve. Assays without cDNA were conducted for each experiment as a negative control. All assays were done in triplicate.

Plasmids and a Strain for Complementation Tests—The plasmid DNA, pOXL6803-COMP-B, used in this study was constructed as follows. A plasmid, pOXL6803-2 (20), was digested with SmaI and KpnI followed by MungBean treatment, and then a resultant 10.9-kb fragment was self-ligated to create pOXL6803-3. Fragments annealed with oligonucleotides, GATCCCCGGGAGGTCACCA and GGCGCCATGCTC (double underlines, underlines, and italic type indicate a sequence that can unite with a BglII site, an SmaI site, and a KpnI site, respectively) were restricted with BglII and cloned into the same restriction enzyme cutting site of pOXL6803-3 to yield pOXL6803-4. A PCR-amplified BglII-SacII 1538-bp segment containing the PCC 6803 sigg gene and its promoter region (−500 to +1038, +1 as the initiation codon) was cloned into the same restriction enzyme site of pOXL6803-4-6 to make pOXL6803-COMP-B. For the construction of pOXL6803-COMP-C, a PCR-amplified KpnI-SacII 1715-bp segment containing the PCC 6803 sigg gene and its promoter region (−500 to +1215) was cloned into the same restriction enzyme site of pOXL6803-4-6. Natural transformation (21–23) was carried out with pOXL6803-COMP plasmids, and transformants were selected on BG 11 plates containing spectinomycin (40 μg/ml) and kanamycin (15 μg/ml).

Polyclonal Antibody for NtcA and Western Blot Analysis—Overexpression and purification of PCC 6803 NtcA were achieved as described previously (17). The purified NtcA was subjected to SDS-PAGE and recovered from the gel. The gel was splinted off and mixed with avidin, and this mixture was injected into a rabbit whose serum (1:500 dilution) did not cross-react to PCC 6803 total protein (50 μg) during Western blotting. The Western blotting was performed as described previously (4). The dilution for rabbit sera of the antibody was 1:1,000, 1:500, 1:1,000, 1:100, 1:500, and 1:1,000 for PCC 6803 SigA, SigB, SigC, SigD, SigE, RpoB, and NtcA, respectively.

**RNA Polymerase Core Enzyme and σ Factors—Purification of the reconstituted PCC 6803 core enzyme with each recombiant subunit was performed as reported previously (6) but with some improvements to the renaturation and reconstitution steps. Previously, the renaturation and reconstitution of RNAP core enzyme were done at the same time in one tube. During these steps, θ (RpoC1) subunits in particular tended to aggregate and consequently the RNAP sometimes lacked the θ subunit. Therefore, for the renaturation of RNAP subunits, purified RpoA-His (His tag attached at the C-terminal domain) or crude RpoB, RpoC1, and RpoC2 dissolved in Buffer G were separately dialyzed against the renaturation buffer at 4 °C for 16 h (6). After clearance of the debris by centrifugation, each supernatant was mixed in the following molar ratio, αβθ:γ = 1:1:2:4, and incubated at 30 °C for 14 h to reconstitute the PCC 6803 core enzyme. After incubation, the reconstituted core enzyme was purified as described previously (6), and the purified fractions were concentrated by centrifugation using an Amicon Ultra-4 filter unit (100-kDa molecular mass cut-off; Millipore Corp.). PCC 6803 σ factors were also prepared by methods reported previously (4, 6).

**In Vitro Transcription Analysis—**Multiple-round run-off assays were performed as described previously (6, 24). The assay mixture (40 μl) comprised 50 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 0.05 mM EDTA-2Na, 0.5 mM dithiothreitol, 0.2 mM each ATP/CTP/GTP/UTP, 25 nM RNAP subunit. Therefore, for the renaturation of RNAP core enzyme, purified RpoA-His (His tag attached at the C-terminal domain) or crude RpoB, RpoC1, and RpoC2 dissolved in Buffer G were separately dialyzed against the renaturation buffer at 4 °C for 16 h (6). After clearance of the debris by centrifugation, each supernatant was mixed in the following molar ratio, αβθ:γ = 1:1:2:4, and incubated at 30 °C for 14 h to reconstitute the PCC 6803 core enzyme. After incubation, the reconstituted core enzyme was purified as described previously (6), and the purified fractions were concentrated by centrifugation using an Amicon Ultra-4 filter unit (100-kDa molecular mass cut-off; Millipore Corp.). PCC 6803 σ factors were also prepared by methods reported previously (4, 6).

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RESULTS

Growth Phase-dependent Regulation of the glnB Transcript by SigB and SigC—Our previous study has indicated that SigC controls synthesis of the glnB transcript from PglN-B-54,-53 induced by nitrogen deprivation in the stationary phase. However, which group 2 σ factors recognize and regulate the glnB promoter during the log phase? To answer this question, we examined the glnB transcript using primer extension in all other knock-out strains missing the group 2 factors SigB, SigD, and SigE (Fig. 1). PCC 6803 glnB possesses two transcription start points: PglN-B-33, an E. coli RpoD-type promoter, transcription from which is constitutive, and PglN-B-54,-53, an NtcA-dependent promoter, transcription from which is induced under nitrogen-deprived conditions (Table 1). Primer extension analyses showed that the amount of transcript synthesized from PglN-B-54,-53 in the sigB knock-out strain (ΔsigB) was significantly reduced during the log phase (Log) regardless of nitrogen levels. A slight reduction was observed in the stationary phase (Sta) under nitrogen-deprived conditions (−N). These results indicate that SigB mainly contributes to the expression of glnB in the log phase. In contrast, similar amounts of the glnB transcript were observed in the ΔsigD and ΔsigE strains, compared with wild-type cells during both phases, indicating that SigD and SigE do not contribute to glnB expression. In this situation, SigA drives the transcription from PglN-B-33, because the transcripts were almost constitutively expressed even in the knock-out strains. This supports previous results (17).

Complementation of SigB Function in the Knock-out Strain—To confirm that SigB and SigC actually function in cells, a complementation test was conducted for glnB expression. Plasmids pOXL6803-COMP-B and pOXL6803-COMP-C (see “Experimental Procedures”), carrying sigB and sigC, respectively, were constructed and introduced into the knock-out strains by a method of natural transformation (21–23). After homologous recombination between the plasmids and PCC 6803 genomes (Fig. 2A), we obtained a transformant called “Comp-ΔsigB,” which is resistant to kanamycin and spectinomycin. Unfortunately, we could not obtain a transformant of “Comp-ΔsigC,” suggesting that some factor would not allow the transformation. To verify the recombination in Comp-ΔsigB, we conducted PCR and Western blot analyses. The results are shown in Fig. 2B and C. When a set of primers, 0306II-F and 0306II-R (4), which can amplify a region of sigB (1.0 kb) were used, bands of 1.0 and 2.5 kb were detected with Comp-ΔsigB genomic DNA (Fig. 2B, left). However, when a set of primers, IS-F (5′-TTGGAGGGCATCGAGTTTTG-3′) and IS-R (5′-GAAGACCGTATGGAGTTT-3′), which amplify a region around the integration site (IS) of ss0410, were used, a band of 3.6 kb was detected with Comp-ΔsigB genomic DNA (Fig. 2B, right), confirming that this recombination involved a double-crossover reaction. We further confirmed the complementation of glnB expression at the protein level in Comp-ΔsigB (Fig. 2C). The RpoB antibody was used as a loading control for all proteins in Western blotting. Finally, we verified that SigB compensated for the transcription from PglN-B-54,-53 under Log/−N, in which SigB significantly influences the expression of glnB as shown in Fig. 1 (Fig. 2D). Although the amount of transcript from PglN-B-33 was the same in the three strains, the amount from PglN-B-54,-53 was restored in Comp-ΔsigB to the level observed in the wild type. Thus, we concluded that SigB contributes to the expression of glnB under nitrogen-deprived conditions.

### Table 1

| Gene     | Promoter sequence |
|----------|-------------------|
| glnB     | 5′-GAGAGCATTTTCGCTTTCGTTTGC-3′ |
| glnB     | 5′-GAGAGCATTTTCGCTTTCGTTTGC-3′ |
| sigC     | 5′-GAGAGCATTTTCGCTTTCGTTTGC-3′ |
| sigC     | 5′-GAGAGCATTTTCGCTTTCGTTTGC-3′ |
| sigD     | 5′-GAGAGCATTTTCGCTTTCGTTTGC-3′ |
| sigE     | 5′-GAGAGCATTTTCGCTTTCGTTTGC-3′ |
| ntcA     | 5′-GAGAGCATTTTCGCTTTCGTTTGC-3′ |

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**FIGURE 1. Growth phase-dependent regulation of glnB expression by SigB and SigC**. 5′-End mapping of the glnB transcript is shown. The PCC 6803 cells were grown in BG11 medium until the Log or Sta phase. The cells (50 ml) were harvested by centrifugation, washed with BG11 (+N) or BG11 (−N) medium (2 ml), given fresh BG11 (+N) or BG11 (−N) medium (50 ml), and then continuously cultivated in a triangular flask (100 ml) for 6 h. After that, total RNA was prepared from the PCC 6803 wild type (ΔB, ΔC, ΔD, or ΔE). The RNA (7 μg) was subjected to extension with the primer glnB-R2. The ladders were prepared from the primer glnB-R2 and the glnB-R1 primer. The RNA products were precipitated with 2-propanol and dissolved in 10 μl of RNase-free water. The RNA was precipitated with 2-propanol and dissolved in 10 μl of RNase-free water. The RNA samples were subjected to primer extension as mentioned above.
SigB and SigC Contribute to Nitrogen-related Gene Expression—The glnB transcript was further analyzed by QRT-PCR. Amounts of the transcript were reduced ∼30% relative to levels in wild-type cells in the ΔsigB strain during the log and stationary phases (Fig. 3A). On the other hand, there was also a 45% reduction in the ΔsigC strain in the stationary phase (Fig. 3A). These results well support the data shown in Fig. 1 and again indicate that SigB and SigC mainly contribute to transcription from PglnB-53,-54 under conditions of nitrogen deprivation in a growth phase-dependent manner. In this case, the QRT-PCR analysis was useful for measuring the amount of glnB transcript although the gene possesses multiple promoters (Fig. 3A, top), PglnB-54,-53 (an NtcA-dependent nitrogen deprivation-responsive promoter recognized by SigB and SigC) and PglnB-33 (a constitutive promoter recognized by SigA), and could practically resolve the decrease in transcription from the NtcA-dependent promoter in the knock-out strains.

To clarify whether SigB and SigC contribute to the expression of other nitrogen-related genes, we characterized another four NtcA-dependent promoters (Table 1), transcription from which is induced under nitrogen-deprived conditions in PCC 6803: glnA, a type-1 glutamine synthase gene; sigE, a group 2 σ factor gene; amtl, an ammonium permease gene; and glnN, a type-3 glutamine synthase gene, in respective knock-out strains. QRT-PCR analyses also revealed that the transcription of glnA, sigE, and amtl decreased ∼30–40% in the sigB knock-out strain in the log phase and ∼34–50% in the sigB or sigC knock-out strain in the stationary phase under conditions of nitrogen deprivation (Fig. 3, B–D). We used rrr165Sa as a control and observed an almost constant level of expression in the knock-out strains (Fig. 3F). These results were similar to those found in the case of glnB, indicating that the contribution of SigB and SigC to the NtcA-dependent promoters might be conserved in PCC 6803.

The profile of transcription of glnN in the sigB or sigC knock-out strain was different from that of glnB, glnA, sigE, or amtl (Fig. 3E). The level of the glnN transcript was increased about 1.8- and 1.5-fold in the sigB and sigC knock-out strains, respectively, in the log phase. In contrast, the level was reduced about 25% in the sigB and sigE knock-out strains during the stationary phase and both phases, respectively. Muro-Pastor et al. (15) reported that SigE contributes to glnN expression under nitrogen-deprived conditions. Our results support theirs. The distinct roles of SigB, SigC, and SigE will be discussed below. Of note, none of the transcripts analyzed in this study disappeared completely in the group 2 σ factor knock-out strains, suggesting that the basal transcription is driven by the group 1 σ factor SigA.

Expression Levels of SigB, SigC, and NtcA under Nitrogen Deprivation—Amounts of σ factors change in response to environmental or internal physiological stress to coordinate cellular processes. Therefore, the protein levels of SigB and SigC were examined by Western blotting (Fig. 4). The amount of SigB increased ∼2-fold under nitrogen-deprived conditions in both growth phases (Fig. 4A). In addition, the relative amount of SigB increased slightly from the log to stationary phase. These increases do not contradict the timing of the expression of the nitrogen-related genes (Figs. 1 and 3, A–D), indicating that the relative amount of SigC decreased in the stationary phase, but the nitrogen-related gene activated by SigC was expressed under nitrogen-deprived conditions in the stationary phase (Figs. 1 and 3, A–D). This means that the activation of nitrogen-related genes by SigC might not depend on protein levels but on other factors (NtcA and/or 2-OG) or a change in the level of enzyme activity itself in a specific manner in the stationary phase.

NtcA plays a key role in regulating the assimilation of nitrogen in cyanobacteria, and the amount of NtcA rose about 2-fold under conditions of nitrogen deprivation in the PCC 6803 cells (26). In fact, García-Domínguez et al. (27) demonstrated that the transcription from NtcA-dependent promoters significantly decreased along with a reduction in ntcA expression. The ntcA gene itself also has an NtcA-binding motif upstream of the promoter (Table 1). This raises another question, whether the amount of NtcA changes in the sigB or sigC knock-out

![FIGURE 2. Complementation test in the sigB knock-out strain](image)
strain. To clarify this point, we performed Western blotting with the NtcA polyclonal antibody. The results are shown in Fig. 4B. Almost the same protein levels of NtcA (25 kDa) were observed in the wild-type and the knock-out strains even with nitrogen deprivation in both growth phases. Under the same conditions, we confirmed the constant expression of RpoB (Fig. 4A) and a 2-fold increase in NtcA in the wild-type strain the same as reported previously (data not shown) (26). These results implied that the reduced transcription of NtcA-dependent genes strain the same as reported previously (data not shown) (26). These results implied that the reduced transcription of NtcA-dependent genes

**FIGURE 3.** QRT-PCR analysis for NtcA-dependent transcripts. Total RNA (1 μg) was prepared from wild-type (W), ΔB, ΔC, and/or ΔE cells under the same conditions as in Fig. 1 and subjected to QRT-PCR. The levels of transcripts of glnB (A), glnA (B), sigE (C), amtl (D), glnN (E), and rrn16Sa (F) were calculated with the standard curve (see “Experimental Procedures”) and are presented in n = 3, means ± S.D. Relative values (value for wild type/Log/−N as 100%). The positions of primers (arrowheads) for QRT-PCR and transcription start sites (arrows) from the NtcA-dependent promoters (small black circles) or the others (small white circles) are shown at the top.
noncoding RNA (5). It is also noted that sigB expression is autoregulated (Fig. 6B, left) (4).

On the other hand, the amount of SigC was significantly reduced during both phases in the sigB knock-out strain irrespective of nitrogen status (Fig. 6A, right), whereas the amount of sigC transcript produced from PsigC-141, -140, -38, and -31 was almost the same in both phases among the three strains (Fig. 6B, right). These results indicate that SigB positively controls the expression of SigC at the post-transcriptional level. Thus, we concluded that there is growth phase-dependent cross-talk between SigB and SigC.

**DISCUSSION**

We present a possible model in Fig. 7. This study shows that multiple group 2 σ factors, SigB and SigC (or SigE), take part in the control of NtcA-dependent nitrogen-related gene expression in cooperation with a group 1 σ factor, SigA. The replacement of σ factors and growth phase-dependent activation for transcription by the group 2 σ factors were also implied. Therefore, the coexistence and cooperation of group 1 and group 2 σ factors are required for nitrogen-related gene activation in cyanobacteria. The cyanobacterial σ factors are quite different from the *E. coli* system in which RpoN recognizes promoters of nitrogen-related genes. We discuss the expression and contributions of NtcA/2-OG, SigB, and SigC below.

The coupling of NtcA with 2-OG improves its DNA binding affinity, and NtcA is a key positive regulator at the first step under conditions of nitrogen deprivation in cyanobacteria (13, 14). The ntcA gene is autoregulated by the binding of NtcA to its own promoter (Table 1) (26). Here, we could confirm the constitutive expression and no differences...
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FIGURE 6. Growth phase-dependent cross-talk between SigB and SigC. A, SigB and SigC protein levels. Western blotting was performed for SigB (left) and SigC (right). Others are the same as in Fig. 4, B, levels of sigB and sigC transcripts. Primer extension with sigB-R2 or 0184V-R3 was done for the 5′-end mapping of the sigB and sigC transcripts, respectively. Transcription start points are presented at the right, as reported previously (4, 5).

FIGURE 7. A possible model for transcription from the NtcA-dependent promoters in a cyanobacterium. Positive (+) or negative (−) effects are indicated with the arrows. See “Results and Discussion” for details.

in the amount of ntcA transcript (data not shown) and NtcA protein (Fig. 4) even in the sigB and sigC knock-out strains, suggesting that SigA mainly recognizes the ntcA promoter. However, we unfortunately could not detect in vitro transcripts synthesized by RNAP-SigA from the ntcA promoter (data not shown). The unique structure of the ntcA promoter with a long spacer between the NtcA-binding motif and the −10 hexamer might be required under some conditions (Table 1). After the induction of NtcA, the NtcA-dependent gene transcription might be sequentially accelerated by SigB and SigC (or SigE).

SigB contributes to the gene expression for nitrogen assimilation (Figs. 1–4). Our previous results showed that SigB was also required for transcription of heat-shock and dark-responsive genes (4, 5). Therefore, SigB is a multifunctional σ factor for manifold environmental stress. Concerning the functional diversity of the PCC 6803 SigB-type σ factor in cyanobacteria, the transcript of Synechococcus sp. strain PCC 7002 sigB, corresponding to PCC 6803 sigB, is specifically expressed in response to nitrogen deprivation (28). The PCC 7120 strain possesses four sigB-type σ factors in its genome (sigB2) and plasmids (sigB, sigB3, and sigB4) in the cell. Among them, sigB and sigB2 respond to nitrogen deprivation (29, 30). These findings suggest an evolutionary conservation of the SigB-type σ factors in cyanobacteria for nitrogen metabolism. Signal transduction with SigB should also be discussed. SigB is an autoregulated heat-shock σ factor that can specifically recognize the hspA promoter (4, 6). PCC 7942 HspA plays a central role in ameliorating harmful effects of light during heat stress through stabilization of the photosystem II complex and light-harvesting phycobilisomes (31). PCC 6803 sigB and hspA transcripts are remarkably accumulated (about a 5-fold increase) upon the exposure of cells to salt stress, which also leads to inactivation of photosystem II activity (32, 33). Furthermore, under conditions of nitrogen deprivation, phycocyanin, the major constituent of phycobilisome, acts as a source of nitrogen released from degraded phycobilisomes for the synthesis of polypeptides required for acclimation to a new nitrogen status (34). Therefore, SigB may be a σ factor sensing the status of the photosystem II complex and phycobilisomes. One possibility for sensing is the redox state of the electron transport chain in photosynthesis, because the degradation of phycobilisomes is also considered to be useful for minimizing the absorption of excess excitation energy under stressful conditions (35). Recent results also support the idea that SigB expression is coupled with the redox state of the electron transport chain (5). Reportedly, most of the promoters recognized by SigB bear only the −10 hexamer (4–6), and the binding of NtcA/2-OG contributed to the marked ability to recognize the NtcA-dependent promoters (Fig. 5).

SigC specifically contributes to the NtcA-dependent transcription of glnB, glnA, sigE, amtl, and sigB in the stationary phase (Figs. 1, 3, and 6). Interestingly, the amount of SigC protein was almost constant even when the nitrogen source was depleted, and the amount present in the stationary phase was almost half that in the log phase (Fig. 4) (4). How is SigC specifically activated in the stationary phase? Possible explanations include (i) a post-translational regulatory effect, (ii) a phosphorylation effect, and (iii) a ppGpp effect, reported previously in some bacteria (36–38). It has also been reported that PCC 7002 sigE, corresponding to PCC 6803 sigC, is required for gene expression during the postexponential growth phase, but its mRNA decreased after the midexponential phase (39). In addition, the cell viability of the sigC knock-out strain was apparently reduced in the stationary phase, suggesting that SigC contributes to long-term survival (17). Therefore, the PCC 6803 SigC-type σ factor is evolutionarily conserved in cyanobacteria and may function as a key protein for stationary-specific gene expression to acclimate and coordinate cellular processes involved in the assimilation of nitrogen. How does SigC effectively recognize NtcA-dependent nitrogen-related promoters? Previous in vitro assays showed that SigC preferentially recognizes typical E. coli consensus-type promoters carrying −10 and −35 hexamers, similar to SigA (4, 6, 17). In this study, the addition of NtcA/2-OG enhanced transcription not only from PglB−54, −53 but also from PglA−48, −47 and Paml−142, strongly indicating that they support effective recognition of the nitrogen-related promoters lacking the −35 hexamer (Fig. 5). Of note, SigC probably does not contribute to recognition of the glnN promoter (Figs. 3 and 5). Reyes et al. (40) suggested that the control mechanisms for PCC 6803 glnA and glnN are different. An additional modification of NtcA or an additional factor is required for the activation of glnN (40). They pointed out possible regulation with a palindromic inverted repeat sequence in the proximal upstream region of the NtcA-binding motif of glnN (−130 to −88, +1 as the initiation codon). The lack of recognition by SigC may be caused by the unique structure in addition to the low similarity of NtcA-binding motifs of glnN among the NtcA-dependent promoters (40) (Table 1). In
addition, recent studies have indicated that competition between σ factors for the limited core enzyme is regulated by the rate at which σ factors are degraded, an anti-σ factor, or ppGpp (38, 41–45). Regarding the stoichiometry of σ factors and the core enzyme in PCC 6803 cells, the intracellular core enzymes (~35 fmol/total protein) exist in excess of the sum of all group 1 and group 2 σ factors (~25 fmol/total protein) in a log phase under normal physiological conditions (4). On the other hand, the sum of σ factors was estimated as ~40 fmol/total protein (from data presented here) under conditions of nitrogen deprivation in the log phase, suggesting a limited amount of core enzyme and replacements among the σ factors on the core enzyme.

SigB expression is dependent on SigB and SigC (Figs. 3 and 7), suggesting a hierarchy among group 2 σ factors in nitrogen-related gene expression in the cyanobacterium PCC 6803. Recently, it has been identified that SigE is a σ factor required for positive regulation of sugar catabolic pathways in PCC 6803 (7). It is conceivable that SigB and SigC also contribute to the catabolism of sugar through the control of SigE expression to coordinate the carbon/nitrogen balance. The autoregulation of SigB expression and cross-talk between SigB and SigC found in this study also indicate a functional network among group 2 σ factors. The existence of a regulatory network was inferred from quantification of the sum of all group 1 and group 2 σ factors (from data presented here) under conditions of nitrogen deprivation in PCC 6803 (7).

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