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NOTES

Expression of P_{II} and Glutamine Synthetase Is Regulated by P_{II}, the *ntrBC* Products, and Processing of the *glnBA* mRNA in *Rhodospirillum rubrum*

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We have studied the transcription of the *glnB* and *glnA* genes in *Rhodospirillum rubrum* with firefly luciferase as a reporter enzyme. Under NH_4^+ and N_2 conditions, *glnBA* was cotranscribed from a weak and a strong promoter. In nitrogen-fixing cultures, activity of the latter was highly enhanced by NtrC, but transcription from both promoters occurred under both conditions. There is no promoter controlling transcription of *glnA* alone, supporting our proposal that the *glnA* mRNA is produced by processing.

Rhodospirillum rubrum, a photosynthetic purple free-living bacterium, is capable of fixing nitrogen anaerobically in N-free medium. As in other diazotrophs, the ammonium produced by nitrogenase is assimilated via the glutamine synthetase-glutamate synthase pathway in *R. rubrum* (2).

The P_{II} protein, a homotrimer encoded by *glnB*, plays a key role in controlling nitrogen metabolism in enteric bacteria. P_{II} can be either unmodified or uridylylated, with different regulatory properties in the control of glutamine synthetase activity and transcriptional regulation involving NtrC, which in the phosphorylated form acts as a transcriptional activator. Under nitrogen-limited conditions, P_{II} is uridylylated by a bifunctional enzyme, the uridylyltransferase, encoded by *glnD*. Conversely, under conditions of nitrogen excess, the uridyl-removing activity of GlnD dominates and the unmodified form of P_{II} is produced. This form of P_{II} stimulates the adenylylation activity of another bifunctional enzyme, adenylyltransferase, the product of *glnE*, which leads to the adenylylation of glutamine synthetase. P_{II} also binds to NtrB, yet another bifunctional enzyme, which then acts as a phosphatase, catalyzing the hydrolysis of phosphate from NtrC-P and thereby inactivating transcription from NtrC-P-dependent promoters. The uridylylated form of P_{II}-UMP is not able to bind to NtrB, which then acts as a kinase catalyzing the phosphorylation of NtrC, leading to the activation of transcription from NtrC-P-dependent promoters. P_{II}-UMP also stimulates the deadenylylating activity of adenylyltransferase, causing deadenylylation (activation) of glutamine synthetase (8, 14, 19, 21). In *R. rubrum*, glutamine synthetase is not only adenylylated but also ADP-ribosylated (29), although the effect of this modification on activity has not been demonstrated.

As in *R. rubrum*, the *glnB* gene has been identified upstream of *glnA* in *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Azospirillum brasilense*, *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, and *Azorhizobium caulinodans*, but the regulation of the *glnBA* operon varies among these bacteria (3–6, 12, 15, 20, 25, 31). The *glnB*-like gene of *Herbaspirillum seropedicae* is not clustered with *glnA* and its expression is constitutive and independent of NtrC, as it is in *Escherichia coli* and *Klebsiella pneumoniae* (1).

In *R. rubrum*, the *glnB* gene is cotranscribed with *glnA* from a putative σ^{54}-dependent promoter (glnBp2) and from a proposed σ^{70}-dependent promoter (glnBp1), overlapping a possible NtrC binding site (9). However, in addition to the *glnBA* transcript we have demonstrated a *glnA* mRNA and proposed that this is due to specific mRNA processing. We have used firefly luciferase as a reporter enzyme to further analyze the transcriptional regulation of the *glnB* and *glnA* genes of *R. rubrum* and to provide additional evidence for processing of the *glnBA* mRNA.

The bacterial strains and plasmids used in this study are listed in Table 1. *R. rubrum* S1MJ is a spontaneous streptomycin-resistant strain, identical to the wild-type S1 in all respects studied (8a). *E. coli* DH5α was used for plasmid transformation and *E. coli* S17-1 was used for plasmid mobilization by conjugation into *R. rubrum*. *E. coli* strains were grown in Luria-Bertani medium (26). *R. rubrum* strains were grown anaerobically either with ammonium as a N source (N+) or with N_2 (N−) at 30°C (24). A red filter (cutoff at 610 nm) was used to minimize tetracycline phototoxicity (16). Where required, antibiotics were added to the growth medium (final concentrations are in micrograms per milliliter): tetracycline, 15 for *E. coli* and 3 for *R. rubrum*; streptomycin, 200 for S1MJ and 100 for the *ntrBC* mutant UR381 of *R. rubrum* (30); kanamycin, 20 for UR381; and ampicillin, 50 for *E. coli*.

Plasmid purification, cloning, and transformation and restriction enzyme digestion were performed according to standard methods (26). Six PCR primers (28- to 33-mers) were synthesized, based on the nucleotide sequence of the *glnBA* operon of *R. rubrum* (9), including a *Kpn*I, *Bgl*II, or *Bam*HI restriction site near the 5′ end. The same ribosome binding site

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5α	F^- Δ Φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17*(r_m^- m_K^+) *supE44 thi-1 gyrA96* Δ(*lacZYA-argF*)*U169*	7
S17-1	Sm^r Sp^r Tra^+; *pro thi hsdR recA*; chromosomal integration of RP4-2-Tc::Mu-*Kan*::Tn*7*	27
R. rubrum		
S1MJ	Sm^r; a spontaneous strain of S1	This laboratory
UR381	Km^r Sm^r; Δ *ntrBC1*::*Kan*	30
Plasmids		
pRKD418	Tc^r Tp^r; cloning vector	17
pGEM-*luc*	Ap^r; source for *luc*	Promega
pJJ303	Ap^r; pGEM-*luc* derivative containing *tac* promoter upstream of *luc*	22
pJOM	Ap^r; pGEM3zf(+) derivative containing *glnB, glnA*, and flanking regions	9
pJCN100	Ap^r; pJJ303 derivative containing *glnB* and *glnA*	This work
pSNC208	Tc^r Tp^r;pRKD418 digested by *Bam*HI-*Xho*I and ligated with *Bam*HI-*Xho*I *luc* from pGEM-*luc*	This work
pRKD419	Tc^r Tp^r; pRKD418 with a complete *Bg*III-*Bam*HI digestion and religation	This work
pSNC109	Tc^r Tp^r; pRKD419 digested by *Nsi*I-*Xho*I and ligated with *Nsi*I-*Xho*I *luc* from pGEM-*luc*	This work
pSNCBP1	Tc^r Tp^r; pSNC109 derivative containing glnBp1	This work
pSNCB1	Tc^r Tp^r; pSNC109 derivative containing glnBp1 and glnBp2	This work
pSNCB2	Tc^r Tp^r; pSNC109 derivative containing glnBp2	This work
pSNCB1A	Tc^r Tp^r; pSNC109 derivative containing glnBp1 and glnBp2, *glnB* and upstream of *glnA*	This work
pSNCB2A	Tc^r Tp^r; pSNC109 derivative containing glnBp2, *glnB* and upstream of *glnA*,	This work
pSNCA1+	Tc^r Tp^r; pSNC208 derivative containing *glnB* and upstream of *glnA*	This work
pSNCA1−	Tc^r Tp^r; same as pSNCA1+ but with insertion in opposite direction	This work
pSNCglnA	Tc^r Tp^r; pSNC109 derivative containing 189-bp 3′-end of *glnB* and upstream of *glnA*	This work
pSNCref	Tc^r Tp^r; pSNC109 derivative containing 53 bp upstream of *glnA*	This work

and similar numbers of nucleotides between the ribosome binding site and the coding start site of *luc* in the amplified DNA as those in chromosomal *glnB* and *glnA* were designed. With pJOM (Fig. 1A) as a template, DNA fragments containing the possible promoter region and/or *glnB* were amplified by PCR. After each amplification, DNA was digested by *Kpn*I and *Bam*HI or *Bgl*II and inserted upstream of *luc* (encoding firefly luciferase) in a broad-host-range promoterless plasmid, pSNC109 or pSNC208 (Table 1; Fig. 1B). The constructs were verified by restriction enzyme digestion analysis.

Plasmid transfer from *E. coli* S17-1 into *R. rubrum* was essentially carried out according to the mating procedure of Liang et al. (13), but tetracycline and streptomycin or tetracycline, streptomycin, and kanamycin were used to select transconjugated *R. rubrum*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of P_{II}, glutamine synthetase, and luciferase were essentially performed as described previously (10). An affinity-purified luciferase antibody (Promega) was used to probe the luciferase. The amount of protein was estimated by laser densitometry with a Molecular Dynamics Personal Densitometer. Luciferase activity was determined in cell extracts by a luciferase assay system (Promega). Glutamine synthetase activity was measured by the transferase assay (28), except that 0.1 M Tris-HCl buffer (pH 7.6) was used. Protein concentration was measured by the Bio-Rad protein assay.

In a previous report, evidence was provided for the transcription of the *glnBA* operon from two promoters, glnBp1 and glnBp2, with similarity to the consensus sequences of σ^{70}- and σ^{54}-dependent promoters, respectively, whose activity was dependent on N status (9). However, unlike what was observed for other N-regulated promoters (18), transcription from glnBp2 under nitrogen-sufficient conditions could also be demonstrated and, in an *ntrBC* mutant of *R. rubrum* (30), a glnBp2 transcript was still produced (9). Another central issue is the origin of a *glnA* mRNA, as we were not able to identify a third promoter controlling the transcription of *glnA* alone. To address these questions, a reporter system was constructed with firefly luciferase as the reporter enzyme. In *R. rubrum* strains containing the plasmids (Table 1; Fig. 1B), P_{II} and luciferase were expressed separately from plasmids carrying *luc* and/or *glnB*; no truncated forms of P_{II} or luciferase were present, as demonstrated by SDS-PAGE and Western blotting (data not shown).

TABLE 2. Luciferase activity in *R. rubrum* cultures carrying plasmids with inserts upstream of *luc*[a]

Plasmid	Features upstream of *luc*	Activity of strain in medium			
		S1MJ		UR381	
		N−	N+	N−	N+
pSNCBP1	glnBp1	769	509	947	741
pSNCB1	glnBp1 and glnBp2	21,007	1,231	2,291	1,950
pSNCB1A	glnBp1, glnBp2, and *glnB*	9,302	1,508	2,543	2,170
pSNCB2	glnBp2	4,070	1,042	1,418	1,140
pSNCB2A	glnBp2 and *glnB*	3,494	1,169	1,662	1,302
pSNCA1+	*glnB*	194	168	171	159
pSNCA1−	*glnB*, reversed direction	150	138	140	110
pSNCglnA	3′ of Bg in *glnB*	165	141	150	135
pSNCref	53 bp upstream of *glnA*	179	163	123	110
pSNC109	No promoter	140	128	127	125
pSNC208	No promoter	135	141	124	131

[a] *R. rubrum* S1MJ and UR381 (*ntrBC*$^-$) were anaerobically grown in NH_4^+-containing (N+) or N-free (N−) medium. Luciferase activity was measured in the cell extract as quanta $seconds^{-1}$ $nanograms\ of\ protein^{-1}$. The data shown are the averages of three independent assays. The standard deviation was less than 15% of the average.

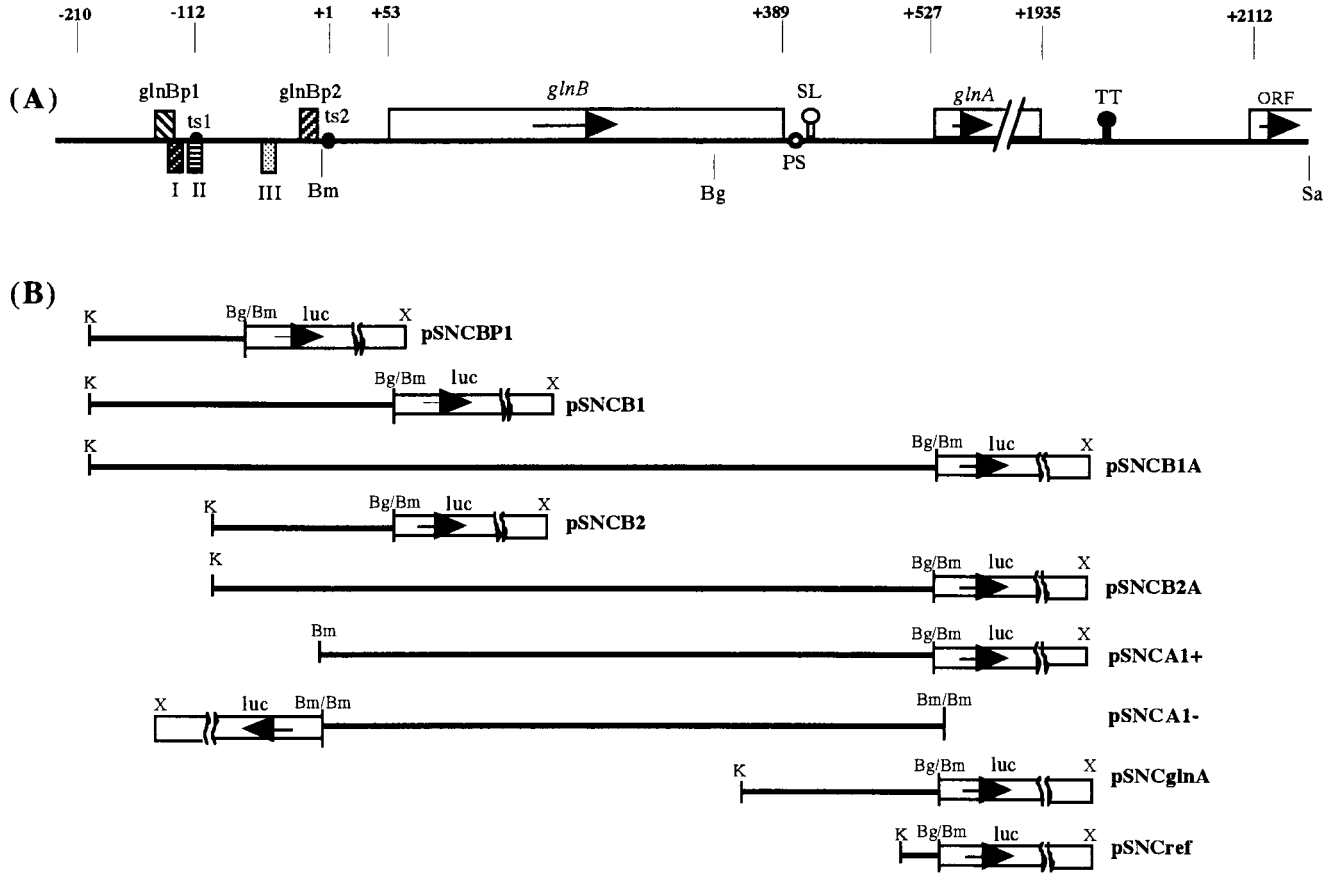

FIG. 1. Genetic mapping of the *glnBA* region of *R. rubrum* and construction of plasmids carrying promoters *glnB* and/or *glnA*. (A) The putative σ^{70}-dependent (glnBp1) and σ^{54}-dependent (glnBp2) promoters and NtrC binding sites (I, II, and III) are shown by rectangles. The arrows indicate the direction of transcription. Transcription start sites of *glnB* and *glnA* are marked ts1 and ts2. The point of origin +1 in the numbering above the map corresponds to ts2. A stem-loop structure (SL) in the intergenic region of *glnB* and *glnA* and a potential transcriptional terminator (TT) downstream of *glnA* are shown as open- and closed-circle pins, respectively. A putative processing site of the *glnBA* transcript is designated PS. (B) Relevant DNA fragments amplified by PCR and inserted upstream of the firefly *luc* gene in the broad-host-range promoterless plasmid pSNC109 or pSNC208. Restriction enzymes sites: Bg, *Bgl*II; Bm, *Bam*HI; K, *Kpn*I; X, *Xho*I; Sa, *Sac*I.

The results shown in Table 2 indicate that in wild-type (S1MJ) strains containing plasmids with the glnBp2 promoter included, alone or together with glnBp1, there was a 3- to 17-fold increase in luciferase activity when cells were grown under N− conditions, compared to N+ conditions. The highest activity was obtained with plasmids containing both promoters (pSNCB1 and pSNCB1A), indicating that the entire glnBp1 and glnBp2 regions are required for maximal transcription. In the absence of either one or the other, expression was significantly lower than with both. Expression from glnBp2 alone was higher than from glnBp1 alone, and the former was required for N regulation (compare pSNCB2 with pSNCBP1). There are three potential NtrC binding sequences upstream of glnBp2 (Fig. 1) which could account for the observed N regulation, confirmed by the absence of N regulation in the *ntrBC* mutant strain (9, 30). The observation that significant NtrC-dependent transcription occurs from glnBp2 even without two of the potential NtrC binding sequences is interesting and might indicate that NtrC can activate transcription from solution, as was recently shown (23), or that NtrC can bind to another sequence less like the consensus NtrC binding sequence. Other possibilities could be that there are alternate activators in *R. rubrum* or that transcription by another RNA polymerase can occur from glnBp2 or from a sequence overlapping this region. In this context, it is interesting that the C normally found at position −12 in σ^{54}-dependent promoters is an A in glnBp2 in *R. rubrum* (19).

When the *glnB* gene was included in the insert upstream of *luc* (pSNCB1A and pSNCB2A), a lower level of transcription than that with the corresponding plasmid without *glnB* (pSNCB1 and pSNCB2) was obtained. We suggest that the reason for this effect is an accumulation of P_{II} and thereby a higher level of the unmodified form. This would lead to an increase in the phosphatase activity of NtrB and thus a lower level of phosphorylated NtrC, i.e., decreased activation of transcription. This proposal is supported by the results shown in Fig. 2, where an increase in the total amount of both P_{II} (compare B1 and B1A; Fig. 2, right) and the unmodified form (compare − lanes in B1 and B1A; Fig. 2, right) is demonstrated in the strain containing pSNCB1A (the separation of two bands in the B1A + lane is clearly seen with a shorter exposure of the film, but then the B1 bands are barely detectable). Furthermore, the amount of glutamine synthetase was significantly lower in that strain (compare B1A and BA; Fig. 2, left), indicating that transcription from the glnBp1-glnBp2 region of the chromosomal copy of the *glnBA* operon is regulated in the same way as the one in the plasmid. These results are supported by measurements of glutamine synthetase activity (Table 3). The strains carrying plasmids with the *glnB* gene show about 50% activity compared to those without *glnB*.

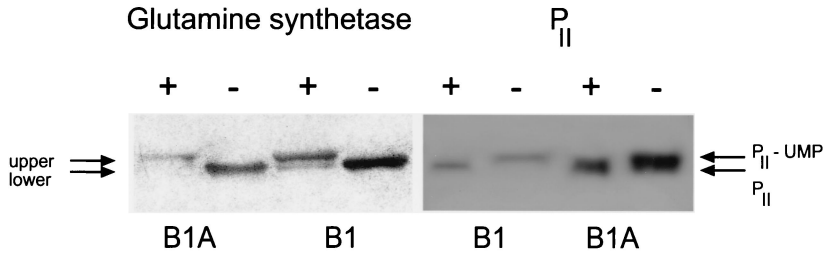

FIG. 2. Accumulation and modification of P_{II} and glutamine synthetase in *R. rubrum*. Extracts from cells grown under N+ or N− conditions were subjected to SDS-PAGE and Western blotting. B1 denotes strain S1MJ, carrying pSNCB1, and B1A denotes strain S1MJ, carrying pSNCB1A. The left panel shows glutamine synthetase and the right panel shows PII. Upper, adenylylated form of glutamine synthetase, lower, unmodified form of glutamine synthetase.

Although maximal transcription of the *glnBA* operon occurs under N− conditions, requiring NtrC and the region containing the putative NtrC binding sequence(s) close to glnBp1, there was still significant transcription in UR381 (the *ntrBC* mutant), containing the same plasmids as the S1MJ strains (Table 2), which again shows that transcription from glnBp2 is not absolutely dependent on NtrC. It should be emphasized that NtrC is not required for *nif* transcription in *R. rubrum*, as shown by Zhang et al. (30).

Transcription from the glnBp1 promoter was also investigated by inserting this promoter directly upstream of the *luc* gene (pSCNBP1). As shown in Table 2, transcription was rather low, indicating that even under N+ conditions transcription from glnBp2 plays a more important role. It should be noted that transcription from glnBp1 was above background, as defined by the luciferase activity in strains containing plasmids with *luc* but no promoter (pSNC109 and pSNC208). The possible physiological role of glnBp1 is to provide a basic (low) level of *glnBA* products, whereas activation from glnBp2 occurs when an increase in glutamine synthetase is required. In the light of the results reported here, the mechanism(s) of such activation may involve more than the Ntr system.

An important goal of this investigation was to establish the origin of the *glnA* mRNA. Four plasmids (pSNCA1+, SNCA1−, pSNCglnA, and pSNCref) containing different parts of the *glnBA* operon between glnBp2 and the start of *glnA* (Fig. 1B) were constructed to address this issue. As shown in Table 2, neither of the strains containing these plasmids showed luciferase activity that was significantly higher than background. We believe that this clearly shows the absence of a complete promoter 3′ of the *Bam*HI site in *glnB* and therefore that the *glnA* mRNA results from processing of the *glnBA* transcript.

In conclusion, we have shown that the transcriptional regulation of the *glnBA* operon in *R. rubrum* is, in some central aspects, different from that in *R. capsulatus*, the phototrophic bacterium for which the genetics of nitrogen fixation and ammonium assimilation have so far been best characterized. Transcription from the glnBp2 promoter region is dominant under both N+ and N− conditions, and although significantly enhanced by NtrC under N− conditions, it is not strictly dependent on this activator. This could indicate that either the σ^{54}-dependent RNA polymerase is activated by other factors in addition to NtrC or there is additional polymerase(s) catalyzing transcription from the glnBp2 region. We have never been able to detect significant changes in the amount of P_{II} in *R. rubrum* under conditions where glutamine synthetase increases, and we believe that this is due to the processing of the *glnBA* transcript, which results in an increased level of *glnA* mRNA and a rapid degradation of the *glnB* mRNA, which we never detected. It is reasonable to assume that the P_{II} level remains unchanged, as there is no evidence for an increased level in its target proteins, NtrB and GlnE, in *R. rubrum*. It is also possible that specific mRNA processing is more common in phototrophs than has been reported so far. However, whether the processing is regulated in *R. rubrum* (as it is in the best-studied processing system in phototrophs, the *puf* genes in *R. capsulatus* [11]) remains to be established.

TABLE 3. Glutamine synthetase activity of *R. rubrum* S1MJ and the *ntrBC* mutant UR381 harboring plasmids with different inserts upstream of *luc*[a]

Plasmid	Activity of strain or mutant in medium			
	S1MJ		UR381	
	N−	N+	N−	N+
None	4.4	1.0	0.7	0.6
pSNCB1	4.5	0.9	0.9	0.7
pSNCB1A	2.7	0.8	1.0	0.9
pSNCB2	4.6	1.1	1.0	1.0
pSNCB2A	2.8	1.0	0.9	0.8
pSNCA1+	4.6	1.1	0.9	0.8
pSNCA1−	4.3	1.0	1.1	0.9
pSNCglnA	4.7	1.1	0.9	0.7
pSNCref	4.8	1.3	1.1	0.9

[a] *R. rubrum* cultures were grown in N-free (N−) medium or NH_4^+-containing (N+) medium, and glutamine synthetase activity was measured in cell extracts as γ-glutamyltransferase activity (in micromoles minutes^{-1} milligrams of protein^{-1}). The results shown are the average of two different experiments.

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