Transcription of the *glnB* and *glnA* genes in the photosynthetic bacterium *Rhodospirillum rubrum*

Magnus Johansson and Stefan Nordlund

Author for correspondence: Stefan Nordlund. Tel: +46 8 16 29 32. Fax: +46 8 15 77 94.
email: stefan@biokemi.su.se

Department of
Biochemistry, Arrhenius
Laboratories for Natural
Sciences, Stockholm
University, S-10691
Stockholm, Sweden

The P_{II} protein, encoded by *glnB*, has a central role in the control of nitrogen metabolism in nitrogen-fixing prokaryotes. The *glnB* gene of *Rhodospirillum rubrum* was isolated and sequenced. The deduced amino acid sequence had very high sequence identity to other P_{II} proteins. The *glnA* gene, encoding glutamine synthetase, was located 135 bp downstream of *glnB* and was partially sequenced. *glnB* is cotranscribed with *glnA* from a promoter with high similarity to the σ^{54} -dependent promoter consensus sequence. A putative σ^{70} promoter was also identified further upstream of *glnB*. Northern blotting analyses showed that in addition *glnA* is either transcribed from an unidentified promoter or, more likely, that the *glnBA* transcript is processed to give the *glnA* mRNA. The total level of the two transcripts was much higher in nitrogen-fixing cells than in ammonia-grown cells.

Keywords: P_{II} protein, *glnB*, *glnA*, *Rhodospirillum rubrum*, regulation of nitrogen metabolism

INTRODUCTION

The photosynthetic purple bacterium *Rhodospirillum rubrum* is a free-living nitrogen-fixing organism. The fixed nitrogen is assimilated via the glutamine synthetase–glutamate synthase pathway (Nordlund *et al.*, 1985; Soliman & Nordlund, 1989; Carlberg & Nordlund, 1991). In enteric bacteria, glutamine synthetase, encoded by *glnA*, is regulated both by feedback control and by covalent modification by adenylation controlled through a regulatory cascade in which a number of enzymes participate (Rhee *et al.*, 1989).

The uridylyltransferase/uridyl-removing enzyme (UTase), encoded by *glnD*, senses the intracellular ratio of glutamine/2-oxoglutarate and catalyses uridylylation/deuridylylation of the P_{II} protein (Rhee *et al.*, 1989). P_{II} is a trimer (Vasudevan *et al.*, 1994) of identical subunits, encoded by *glnB*. Each subunit can be uridylylated on a conserved tyrosine, residue 51 (Son & Rhee, 1987). Unmodified P_{II} stimulates the enzyme adenylyltransferase (ATase), encoded by *glnE*, to catalyse inactivation of glutamine synthetase by adenylation, whereas P_{II} -UMP promotes the deadenylylation activity of the enzyme,

leading to activation of glutamine synthetase (Rhee *et al.*, 1989).

In addition to regulating glutamine synthetase activity, P_{II} also has an important function in controlling transcription from NtrC-activated σ^{54} -dependent promoters (Holtel & Merrick, 1988; Magasanik, 1989), which are of particular interest in nitrogen-fixing organisms (for a review see Kennedy *et al.*, 1994). These promoters regulate transcription from genes encoding proteins involved in nitrogen metabolism, e.g. *glnA*. Transcription from these promoters is activated by the phosphorylated form of NtrC, NtrC-P, which binds upstream of the promoter and interacts, by a loop on the DNA, with the σ^{54} -dependent RNA polymerase, leading to initiation of transcription (Minchin *et al.*, 1989). Binding of NtrC-P to DNA in some cases also prevents transcription from σ^{70} -dependent promoters. Phosphorylation/dephosphorylation of NtrC is regulated by the status of the P_{II} protein. Unmodified P_{II} interacts with NtrB, thereby favouring the phosphatase activity of this bifunctional enzyme, whereas P_{II} -UMP cannot interact with NtrB, which then works as a kinase, phosphorylating NtrC. In enteric bacteria *glnA* is localized in the same operon as *ntrB* and *ntrC* (Miranda-Rios *et al.*, 1987). *glnA* is transcribed from a σ^{70} promoter during nitrogen excess, but from a σ^{54} promoter during nitrogen limitation. *glnB*

The EMBL accession number for the sequence reported in this paper is X84158.

is localized elsewhere on the chromosome in these organisms (Rhee *et al.*, 1989).

The metabolic regulation of glutamine synthetase in some phototrophic bacteria is similar to that of the *Escherichia coli* enzyme. Adenylation of glutamine synthetase has been shown in *Rhodospseudomonas palustris* (Alef & Zumft, 1981), *Rhodobacter sphaeroides* (Engelhardt & Klemme, 1982) and *Rhodobacter capsulatus* (Johansson & Gest, 1977; Michalski & Nicholas, 1984). However, in *Rsp. rubrum* the situation seems to be more complex, although the enzyme can be adenylylated (Nordlund *et al.*, 1985; Woehle *et al.*, 1990). Upon addition of ammonium ions to a nitrogen-fixing culture of *Rsp. rubrum* both the transferase and the biosynthetic activity of glutamine synthetase are substantially decreased and cannot be reactivated by treating the enzyme with snake venom phosphodiesterase (Nordlund *et al.*, 1985; Soliman & Nordlund, 1989). Glutamine synthetase in *Rsp. rubrum* can also be ADP-ribosylated (Woehle *et al.*, 1990).

The *glnB* gene in *Rb. capsulatus* has been identified upstream of *glnA*. In this organism *glnB* is cotranscribed with *glnA* either from a σ^{70} promoter or from a promoter, σ^{54} , which requires NtrC-P but not the *rpoN* gene product (Foster-Hartnett & Kranz, 1994). The *Rb. sphaeroides glnB* gene is also localized upstream of *glnA* but it is transcribed from a single σ^{70} promoter (Zinchenko *et al.*, 1994). Other organisms in which *glnB* is localized upstream of *glnA* are *Azospirillum brasilense* (de Zamaroczy *et al.*, 1990) *Rhizobium leguminosarum* (Chiurazzi & Iaccarino, 1990) and *Bradyrhizobium japonicum* (Martin *et al.*, 1989).

P_{II} in the cyanobacterium *Synechococcus* sp. PCC 7942 has not been shown to be uridylylated, but phosphorylation on a serine residue has been demonstrated (Forchhammer & Tandeau de Marsac, 1994) and serine 49 has been suggested as the phosphorylation site (Cheah *et al.*, 1994). Regulation of this phosphorylation process seems to be coupled to the redox state in the cell (Tsinoremas *et al.*, 1991).

In this investigation we have sequenced the *glnB* gene from *Rsp. rubrum* and shown that it is localized upstream of *glnA*. We have also studied the transcription of these two genes under different conditions with respect to the nitrogen source supplied.

METHODS

Bacterial strains, plasmids, media and growth conditions.

Strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria Broth (LB) supplemented, when needed, with ampicillin at a final concentration of 50 µg ml⁻¹. *Rsp. rubrum* strain S1 was grown in the minimal medium described by Ormerod *et al.* (1961), with the omission of glutamate. *Rsp. rubrum* strain UR 381 (Zhang *et al.*, 1995) was grown in the presence of kanamycin (10 µg ml⁻¹) and streptomycin (100 µg ml⁻¹). Cultures were incubated at 30 °C in the light. For growth under nitrogen-fixing conditions, referred to as N⁻, cultures were grown in sterile serum bottles and bubbled with a gas mixture of 95% N₂/5% CO₂. Ammonia-grown cells, N⁺, were supplemented with 28 mM NH₄Cl.

Cloning and sequencing of *glnB*. The *Rsp. rubrum* λ -DNA library constructed by Falk *et al.* (1985) was screened by using the *glnB* gene from *Rhizobium leguminosarum* as a probe. A 6·7 *SacI*–*SacI* fragment (Fig. 1) was isolated and cloned into a pGEM3zf(+) vector (Promega), to construct the plasmid pJOM. A 0·8 kb *Bam*HI–*Bam*HI fragment (Fig. 1) containing the *Rsp. rubrum glnB* gene was subcloned, to produce plasmid pJOB. Both strands of the 0·8 kb fragment were sequenced by using T7 Sequencing Mixes (Pharmacia). Compressed GC-rich areas were resolved by using the Deaza G/A T7 Sequencing mixes (Pharmacia). M13 universal primers (forward and reverse) and designed oligonucleotides were used as primers. Both strands of the upstream region of *glnB* were sequenced from pJOM with designed oligonucleotides as primers.

RNA isolation. All solutions used were treated with diethyl pyrocarbonate (1 ml l⁻¹) over night and autoclaved to remove RNase activity. *Rsp. rubrum* cultures were grown either with ammonia (N⁺) or under nitrogen-fixing conditions (N⁻) to an OD₆₀₀ of 0·6. Then 50 ml of each culture was transferred to a 250 ml tube filled with ice and centrifuged at 6000 *g* for 10 min. The pellets were washed with resuspension buffer (0·3 M sucrose, 10 mM sodium acetate, pH 4·5), transferred to micro-centrifuge tubes and centrifuged. Each pellet was resuspended in 0·25 ml resuspension buffer, followed by addition of 75 µl 250 mM EDTA to each tube and incubation on ice for 5 min. Then 375 µl lysis buffer (2%, w/v, SDS, 10 mM sodium acetate, pH 4·5) was added to each tube and the mixture incubated at 65 °C for 3 min. Hot phenol (700 µl at 65 °C) was added and after incubation at 65 °C for 3 min, the tubes were placed in an ethanol/dry ice bath for 15 s. The samples were centrifuged at 12000 *g* for 5 min. The phenol extraction was repeated twice followed by a phenol/chloroform extraction. RNA was precipitated with 0·3 M sodium acetate and 2·5 vols ethanol. The pellets were washed with 70% (v/v) ethanol, dried and dissolved in RNA storage buffer (20 mM Na₂HPO₄, 1 mM EDTA, pH 6·5) and stored at –70 °C.

Northern blotting, primer extension and S1 nuclease mapping analyses. For Northern blotting, 20 µg total RNA, from N⁺ and N⁻ cultures, was analysed on a gel containing 2% (v/v) formaldehyde and transferred to a Hybond N filter. The filter was baked under vacuum at 80 °C, hybridized with part of the *Rsp. rubrum glnB* gene, and reprobated with part of *glnA* (Fig. 1). The ECL direct nucleic acid labelling detection system (Amersham) was used for detection. The amount of transcript was estimated by laser densitometry using a Molecular Dynamics Personal Densitometer.

To identify the start of the mRNA transcripts for *glnB* and *glnA* two methods were used: primer extension and S1 nuclease mapping. Primer extension analyses were carried out according to Ausubel *et al.* (1991) with the modification that the primers (Fig. 2) were incubated with total RNA for 10 min at 65 °C and then slowly cooled to 30 °C, followed by hybridization overnight at this temperature. Twenty-five micrograms of total RNA purified from N⁺ and N⁻ cells was used.

The 5'-end-labelled probes (Fig. 1) used for S1 nuclease mapping were obtained by digesting pJOD and pJOB (Table 1) with *Pst*I and *Bam*HI, respectively. The fragments generated were isolated and purified, treated with alkaline phosphatase and labelled with [γ -³²P]ATP (Amersham) by polynucleotide kinase according to the manufacturer's instructions (Pharmacia). The fragments were digested with *Sph*I and *Bgl*II respectively and the probes (Fig. 1) were separated and extracted from low-melting-point agarose and purified by Wizard DNA clean up (Promega). S1 nuclease mapping was done according to Berk & Sharp (1977).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Comments
Strains		
<i>E. coli</i> Q358	<i>hsdR_k⁻ hsdM_k⁺ supE</i> ϕ 80 ^r	An su host used for growth of phage λ (Karn <i>et al.</i> , 1980)
<i>E. coli</i> JM109	<i>endA1 recA1 gyrA96 thi hsdR17</i> (<i>r_k⁻</i> , <i>m_k⁺</i>) <i>relA1 supE44</i> Δ (<i>lac-proAB</i>) [F' <i>traD36 proAB lac^a</i> Z Δ M15]	Promega
<i>Rsp. rubrum</i> S1	Wild-type	
<i>Rsp. rubrum</i> UR 381	Δ <i>nutrBC1::kan</i> Sm ^r	Zhang <i>et al.</i> (1995)
Plasmids		
pAH3	pUC19 derivative containing 0.4 kb <i>Rhiz. leguminosarum</i> DNA fragment with part of <i>glnB</i> and flanking region	Constructed by A. Holtel, University of Sussex, UK
pJOM	pGEM3zf(+) derivative containing 6.7 kb <i>Rsp. rubrum</i> DNA fragment with <i>glnB</i> , <i>glnA</i> and flanking regions	This work
pJOB	pGEM3zf(+) derivative containing 0.8 kb <i>Rsp. rubrum</i> DNA fragment with <i>glnB</i> and flanking regions	This work
pJOD	pGEM3zf(+) derivative containing 2.6 kb <i>Bgl</i> II– <i>Bgl</i> II <i>Rsp. rubrum</i> DNA fragment with upstream region and part of <i>glnB</i>	This work

Fig. 1. Map of the 6.7 kb fragment containing *glnB* and *glnA*. The location and size of the *glnB* and *glnA* probes used for Northern blotting analyses and for S1 nuclease mapping are also shown.

as modified by Weaver & Weissman (1979). Total RNA (50 μ g) and probe (0.5 μ g) were mixed, precipitated, dried and dissolved in 30 μ l hybridization buffer (40 mM PIPES pH 6.4, 80%, v/v, formamide, 0.4 M NaCl and 1 mM EDTA). The samples were denatured for 10 min at 85 $^{\circ}$ C and hybridized overnight at 42 $^{\circ}$ C. Then 300 μ l S1 nuclease buffer (280 mM NaCl, 5 mM sodium acetate pH 4.6 and 4.5 mM ZnSO₄) and 300 U S1 nuclease were added and the samples were incubated for 1 h at 30 $^{\circ}$ C. The samples were extracted once with phenol/chloroform, ethanol-precipitated with carrier yeast tRNA, resuspended in formamide/dye mixture and analysed on a 4%

(w/v) polyacrylamide gel. Sequenced M13 DNA was used as size markers for the trimmed RNA–DNA hybrids.

Computer analysis. Sequence analyses were done using the GCG subprogram FASTA (Genetics Computer Group, Madison, WI, USA) to identify similar sequences. The amino acid alignment of the P_{II} proteins from *Rsp. rubrum*, *A. brasilense*, *Rb. capsulatus*, *Rhiz. leguminosarum*, *B. japonicum*, *Klebsiella pneumoniae*, *E. coli*, *Rb. sphaeroides* and the cyanobacterium *Synechococcus* sp. PCC 7942 was carried out with the program Gene Works (IntelliGenetics).

RESULTS

Nucleotide sequence of the *glnB* locus

The 0.8 kb *Bam*HI–*Bam*HI fragment cloned into pJOB was sequenced. To facilitate investigation of the transcriptional regulation of *glnB* the sequence was extended by sequencing the pJOM plasmid with two designed oligonucleotides as primers. This generated an additional 202 bp upstream of the *Bam*HI site. The total sequence is presented in Fig. 2.

The nucleotide sequence of the ORF shows up to 83% identity to *glnB* genes from other organisms (Son & Rhee, 1987; Holtel & Merrick, 1988; Martin *et al.*, 1989; Chiurazzi & Iaccarino, 1990; de Zamaroczy *et al.*, 1990; Kranz *et al.*, 1990; Tsinoremas *et al.*, 1991; Zinchenko *et al.*, 1994). A possible ribosome-binding site was identified 12 bp upstream of *glnB*, and a sequence similar to the σ^{54} -dependent promoter consensus was identified 63 bp upstream of the gene. In this sequence the C normally

```

AAAGATCCGCCATCCGAGTCCAAGCGGGGAACACGAACCCAGACTAGCGGCCCATGCG
-260          -240
*****
CTTGGCGCATGCCATATATTTTCGGGCAGACTGCCTTCCTTCAGGCAACCCCCAAGGGGA
-200          -180          -160
AACGACTGTCCGTCGCCAGGGAAGAAAGATATTTTTCAGCCACTTGGGTGATCATTGAA
-140          -120          -100
CCAGTGGCACGAGGCGGTATAGGGATCCCCATCGTCCCGCAAGGACGGGTGCCGACCA
-80  Primer1      -60          -40

          M K K I E A I I K P F K L
ACCAGCCGAGCGACCGGATTTCTGAAGATCGAAGCGATCATCAAGCCCTTCAAGC
-20          1          20  Primer2

D E V K E A L H E I G L Q G I T V T E A
TCGATGAGGTGAAGGAAGCCCTTCACGAAATCGGCCCTGACGGGATCACCGTCACCGAGG
40          60          80

K G F G R Q K G H T E L Y R G A E Y V V
CGAAGGCTTTGGCCGCCAAAGGGCCACACGGAACCTGACCGCGTCCGGAGTACGTTG
100        120        140

D F L P K V K I E L V I E D A L V E R A
TGGATTTCTGCCCAAGGTCAGATCGAGCTGGTGATCGCGCTTGTGGAACCGG
160        180        200

I E A I Q Q A A Q T G R I G D G K I F V
CGATCGAGGCCATCCAGGAGCGGCCCAACGGGCGCATTTGGCGACGGCAAGATCTTG
220        240        260

Y A I E E A I R I R T G E R G G D A I *
TCTACGCCATCGAAGAGGCCATCCGCATCCGTACAGGGAGCGGGCGCGACCGGATCT
280          300          320

AGCGCCACCGCCCGTCAATTCGGGGGGCTTGGCCCGCGGCTCCGSCCTTCGGGCGCGT
340          360          380  Primer3

TCGCCGGGGTTCGGAGCGAATACGAACCGGATCGCCGCTTTCAGTCAGGCAGCACGACA
400          420          440

          M S D V K T I L E M I K E N D
AATGGGAATTAAGCTCATGTGTCGACGTTAAGACCATTCCTCGAATGATTAAAGAAAACG
460          480          500

V K F V D F R F T D P K G K W Q H T A Q
ACGTTAAGTTCGTCGATTTCCGCTTCACCGATCCCAAGGGCAAGTGGCAGCATACCGCC
520  Primer4      540          560

Y V T T I D E E S L V E G I M F D G S S
AGTACGTGACCACCATCGACGAGGAAAGCTGGTGAAGGCATGATGTCAGCGTTCTGT
580          600          620

I A G W K D I N E S D M I L K P D L K S
CGATCGCCGGTGAAGGACATCAACAGTCCGACATGATCTGAAGCCGACCTGAAGT
640          660          680

A F M D
CGGCCTTCATGGATCC
700

```

Fig. 2. Nucleotide sequence of promoter regions, *glnB* and the 5'-end of *glnA*. Possible ribosome-binding sites are underlined. The mRNA start sites identified are indicated by bold and underlining. Putative promoter sequences are bold and a putative NtrC-P binding site is marked with *. The translated P_{II} protein shows the conserved tyrosine residue 51 (bold). Also shown are the primers used for primer extension.

located at -12 from the mRNA start is replaced with an A. We also identified a possible σ^{70} promoter sequence 180 bp upstream of *glnB*. Interestingly, the sequence 177-194 bp upstream of *glnB* shows high similarity to the consensus sequence for a NtrC-P binding site. This site overlaps the possible σ^{70} promoter.

Another ORF that starts 135 bp downstream of *glnB* shows high similarity to *glnA* from other organisms. Only 239 bp (approximately 17% of *glnA*) have so far been

sequenced. We have not yet been able to identify any obvious promoter sequences upstream of *glnA*.

Features of the predicted gene products

The polypeptide deduced from *glnB* shows very high similarity to P_{II} proteins from other organisms (Fig. 3). The identity with *Rb. capsulatus* and *Rb. sphaeroides* is 77 and 73% respectively, but the highest identity is to *A. brasilense*, 88%. The *Rsp. rubrum* sequence also has a tyrosine at residue 51, which is the uridylylation site of the *E. coli* protein. Residue 49, which in *Synechococcus* sp. PCC 7942 is the serine that has been suggested to become phosphorylated (Cheah *et al.*, 1994; Forchhammer & Tandeau de Marsac, 1994), is replaced with an alanine in the *Rsp. rubrum* sequence and in all other known P_{II} proteins.

The product of the other ORF shows high similarity to the N-terminal part of glutamine synthetase from other organisms. The highest similarity is with glutamine synthetase from *A. brasilense*, with which the identity is 66% (Bozouklian & Elmerich, 1986). The ORF and a sequence of 36 amino acids from the N-terminus of glutamine synthetase from *Rsp. rubrum*, which has previously been sequenced (Woehle, 1992), differ by one amino acid in the region now sequenced; a lysine at position 21 is a histidine in the sequence now reported. We believe these findings are convincing evidence for the gene downstream of *glnB* being the structural gene for glutamine synthetase.

Expression of *glnB* and *glnA* in *Rsp. rubrum*

Northern blotting analyses showed that *glnB* is expressed as a single mRNA transcript (Fig. 4). The size of the transcript is approximately 2.0 kb, as estimated by comparison with rRNA standards. *glnB* is expressed under high-ammonia conditions, but the level of transcript is 4-8 times higher under nitrogen-fixing conditions.

When *glnA* was used as a probe for Northern blotting analysis, two transcripts, one of 2.0 kb and one about 400 bp shorter, were detected (Fig. 4). This indicates that *glnA* is cotranscribed with *glnB* from the *glnB* promoter(s). The presence of the shorter transcript could be explained either by transcription from another promoter or by specific processing of the *glnBA* mRNA. The expression of this shorter transcript was also much higher during nitrogen-fixing conditions.

Identification of mRNA start sites for *glnBA* and *glnA*

To identify the transcription start sites of the mRNAs demonstrated by Northern blotting, primer extension and S1 nuclease mapping were used with the primers and probes shown in Fig. 2 and Fig. 1, respectively. Both methods clearly identified two start sites upstream of *glnB* (Fig. 5). The one localized 52 bp from *glnB* is in agreement with a putative σ^{54} -dependent promoter (*glnBp2* in Fig. 5) at a location similar to that normally found for such

R.r.	MKKIEAIIKP	FKLDEVKEAL	HEIGLQGITV	TEAKSFGROK	GHTELYRGAE	YVVDLFPKVK	60
A.b.	MKKIEAIIKP	FKLDEVKEAL	HEVGIGITV	TEAKSFGROK	GHTELYRGAE	YVVDLFPKVK	60
E.c.	MKKIDAIIPK	FKLDDVREAL	AEVGITGMTV	TEVKQFGROK	GHTELYRGAE	YVVDLFPKVK	60
K.p.	MKKIDAIIPK	FKLDDVREAL	AEVGITGMTV	TEVKQFGROK	GHTELYRGAE	YVVDLFPKVK	60
R.c.	MKKVEAIIKP	FKLDEVKEAL	QEAGIQGLSV	IEVKSFGROK	GHTELYRGAE	YVVDLFPKVK	60
R.s.	VKKIEAIIKP	FKLDEVKEAL	QAAGVQGLSV	TEVKQFGROK	GHTELYRGAE	YVVDLFPKVK	60
R.l.	MKKIEAIIKP	FKLDEV-SL	SGVGLQGITV	TEAKSFGROK	GHTELYRGAE	YVVDLFPKVK	59
B.j.	MKKIEAIIKP	FKLDEV-SL	SGVGLQGITV	TEAKSFGROK	GHTELYRGAE	YVVDLFPKVK	59
S.sp.PC.7942	MKKIEAIIKP	FKLDEVKIAL	VNAGIVGMTV	SEVRSFGROK	GOTERYRGSE	YVVEFLQKLN	60
Consensus	MKKIEAIIKP	FKLDEVKEAL	.EVGIQG.TV	TEVKQFGROK	GHTELYRGAE	YVVDLFPKVK	60

R.r.	IELVIEDALV	ERAIEAEQQA	AQTGRIGDGK	IFVYAEAEI	RIRTERGGD	AI	112
A.b.	IEVVMEDSLV	ERAIEAEQQA	AHTGRIGDGK	IFVTPVEEVV	RIRTERGGD	AI	112
E.c.	IEIVVPDDIV	DTCVDTIIRT	AQTGRIGDGK	IFVFDVARVI	RIRTEGEDDA	AI	112
K.p.	IEIVVTDDIV	DTCVDTIIRT	AQTGRIGDGK	IFVFDVARVI	RIRTEGEDDA	AI	112
R.c.	IEVVLPEEMV	DIAIEAIVGA	ARTEKIGDGK	IFVSSIEQAI	RIRTERGGED	AV	112
R.s.	IEVVLADMV	EAAREAVISA	SRTEKIGDGK	IFISPEVQAI	RIRTERGGED	AV	112
R.l.	VEVVLADENA	EAVIEATKKA	AQTGRIGDGK	IFVSNVEEVI	RIRTERGGID	AI	111
B.j.	IEIVIGDDLV	ERAIDAIRRA	AQTGRIGDGK	IFVSNIEEAI	RIRTERGGID	AI	111
S.sp.PC.7942	LEIVVEDAQV	DTVIDKIVAA	ARTGEIGDGK	IFVSPVQITI	RIRTERGNAD	AI	112
Consensus	IE.V..D..V	E.AIEAI..A	AQTG.IGDGK	IFVS.VE..I	RIRTERG.G.D	AI	112

Fig. 3. Amino acid sequence comparison of the P_{II} proteins from *Rsp. rubrum*, *Azospirillum brasilense* (de Zamaroczy et al., 1990), *Escherichia coli* (Son & Rhee, 1987), *Klebsiella pneumoniae* (Holtel & Merrick, 1988), *Rhodobacter capsulatus* (Kranz et al., 1990), *Rhodobacter sphaeroides* (Zinchenko et al., 1994), *Rhizobium leguminosarum* (Chiurazzi & Iaccarino, 1990), *Bradyrhizobium japonicum* (Martin et al., 1989) and *Synechococcus* sp. PCC7942 (Tsinoremas et al., 1991). Conserved amino acids (46%) are shaded.

Fig. 4. Northern blotting analyses of *glnB* and *glnA* expression from nitrogen-fixing (N-) and ammonia-grown (N+) cells. The total RNA shown (used as size markers) was stained with ethidium bromide and visualized under UV light.

promoters. As shown in Fig. 5, an mRNA starting 164 bp upstream of *glnB* can also clearly be identified. This would be in good agreement with glnBp1 (Fig. 5), which we propose to be a σ^{70} -dependent promoter. Fig. 5 also shows the results of S1 nuclease mapping of mRNAs in the *ntrBC* mutant of *Rsp. rubrum* (Zhang et al., 1995). Also in this strain two start sites can be identified corresponding to glnBp1 and glnBp2, although the latter is much weaker than in the wild-type. These results verify that NtrC enhances transcription from glnBp2, but also indicate that transcription from this promoter is not strictly dependent on NtrC in *Rsp. rubrum*.

Both methods were also used to determine the start of the *glnA* mRNA. Primer extension identified a site within

Fig. 5. Localization of the transcription start sites upstream of *glnB*. (a) S1 nuclease mapping of mRNA from *Rsp. rubrum* wild-type and the *ntrBC* mutant using probe A shown in Fig. 1. (b) Primer extension of mRNA from *Rsp. rubrum* wild-type using primer 1 (Fig. 2). (c) Primer extension of mRNA from *Rsp. rubrum* wild-type using primer 2 (Fig. 2). (d) Schematic representation of the location of the promoters deduced from (a-c).

glnB; however, this could not be verified by S1 nuclease mapping. By the latter method two 5' ends in the region between *glnB* and *glnA* could be clearly demonstrated

Fig. 6. Localization of the mRNA start site upstream of *glnA*. (a) S1 nuclease mapping of mRNA from *Rsp. rubrum* wild-type using probe B shown in Fig. 1. (b) Schematic representation of the location of the *glnA* mRNA start site. (c) Sequence of the region around the site deduced from (a). The suggested processing site is similar to that proposed by Fritsch et al. (1995).

(Fig. 6), one of which gave a stronger signal. This site was also supported by primer extension experiments (data not shown).

DISCUSSION

In a number of diazotrophs, transcription of *nif* genes is regulated through the *ntr* system, in which the P_{II} protein plays a key role as a signal of the nitrogen status in the cell. However, in photosynthetic diazotrophs the transcriptional regulation has not been established in detail, although *ntr* genes have been identified. In fact not even a general consensus promoter for housekeeping genes has been determined.

The high similarity of the amino acid sequence deduced from the *glnB* gene of *Rsp. rubrum* to those of other

organisms indicates that the P_{II} protein probably plays an essential role in the regulation of nitrogen fixation and ammonium assimilation in this phototroph. This is also supported by the demonstration that glutamine synthetase from *Rsp. rubrum* can be adenylylated and the identification of an ATase (Woehle et al., 1990; Woehle, 1992). However, whether P_{II} is also involved in the ADP-ribosylation of glutamine synthetase (Woehle et al., 1990) remains to be established.

The sequence of *glnBp2*, the putative σ^{54} -dependent promoter, TGGCACGN₄GTGAT, is 75% similar to the consensus TGGCACGN₄TTGCA (Merrick, 1992) but it is localized at -13/-25 bp from the transcription start site instead of the usual -12/-24. Interestingly, the conserved C normally located at -12 is replaced with an A. This has to our knowledge only previously been found in the promoter sequences of *nifH* in *Rhiz. leguminosarum* (Roelvink et al., 1990), *Rhiz. trifolii* (Watson & Schofield, 1985) and *Rhiz. phaseoli* (Quinto et al., 1985) and of *nifN* in *Rhiz. meliloti* (Aguilar et al., 1987). The possible NtrC-P binding site TGCCTATATTTCGGGCA contains all the conserved nucleotides (bold) compared with the extended consensus for the NtrC recognition site TGCACCA(A/T)₃TGGTGCA (Foster-Hartnett & Kranz, 1994). These similarities with the Ntr system and the fact that the level of the *glnBA* transcript seems to be regulated according to the nitrogen status, make it likely that a similar system regulating *glnBA* transcription is present in *Rsp. rubrum*. Furthermore, Zhang et al. (1995) have recently identified the *ntrBC* genes in *Rsp. rubrum* and shown that in a mutant in which part of *ntrBC* was deleted, glutamine synthetase activity and protein level were both approximately 50% of wild-type. Our studies with this mutant are in good agreement with that report. Taken together these results suggest the operation of a *ntr* system in *Rsp. rubrum* and the transcription of *glnBA* from a NtrC-activated promoter but also from one that is NtrC-independent.

In many organisms, e.g. *Azospirillum brasilense*, a σ^{70} -dependent promoter has been identified upstream of the σ^{54} -dependent *glnB* promoter (de Zamaroczy et al., 1990). The sequence GCGCCAN₁₇TATATT, *glnBp1*, found upstream of *glnB* in *Rsp. rubrum*, resembles the consensus sequence for σ^{70} binding, especially in the -10 region. Repeated primer extension and S1 nuclease mapping experiments indicate that a transcription start site is located 164 bp upstream of *glnB*, giving a promoter and an mRNA start more distal than usually found for σ^{70} dependent transcription. The transcript was more abundant relative to the *glnBp2* transcript in ammonia-grown wild-type cells than in cells grown under nitrogen-fixing conditions, and under both conditions in the *ntrBC* mutant. This could be due to blocking of the promoter by NtrC-P in diazotrophically grown cultures, as the possible NtrC-P binding site overlaps the proposed σ^{70} promoter sequence.

The Northern blotting experiments showed that the expression of the other *glnA* transcript in *Rsp. rubrum* also varies according to the nitrogen status. In *Rb. capsulatus* (Foster-Hartnett & Kranz, 1994), *A. brasilense* (de

Zamaroczy *et al.*, 1993) and *Rhiz. leguminosarum* (Amar *et al.*, 1994), studies using reporter genes have shown the existence of a specific *glnA* promoter. Transcription from the promoter in *A. brasilense* is weaker under N— compared to N+ conditions. In *Rhiz. leguminosarum* the promoter is weak but constantly expressed. The situation is different in *Rsp. rubrum*, where the *glnA* mRNA is 70 % more abundant than the *glnBA* transcript during N— conditions, indicating a nitrogen-regulated promoter, upstream of the transcription start site of *glnA*. S1 nuclease mapping and primer extension experiments identify mRNAs starting between *glnB* and *glnA*, as well as within the 3'-terminal part of the *glnB* gene. In the light of these results, and as we have not been able to identify any sequence similar to a known promoter sequence in this region, we suggest that the *glnA* mRNA is the product of specific processing of the *glnBA* transcript. A similar proposal was recently made for the *glnBA* transcript in *Rb. capsulatus* (Borghese & Wall, 1995). Fig. 6(c) shows a possible RNase E cleavage site in the region between *glnB* and *glnA*. This enzyme has been suggested by Fritsch *et al.* (1995) to be involved in the specific cleavage of the polycistronic *puf* mRNA in *Rb. capsulatus*; these authors also propose a cleavage site in the *puf* sequence similar to that in the region between *glnB* and *glnA* in *Rsp. rubrum*.

The results presented in this investigation demonstrate for the first time that *glnB*, encoding the regulatory P_{II} protein, is present in *Rsp. rubrum*. The similarity of the sequences identified with established promoter and NtrC-binding sites indicates that N-regulated gene expression in *Rsp. rubrum* has features in common with that in other diazotrophs. To investigate the physiological role(s) of P_{II} in *Rsp. rubrum*, we have tried to generate *glnB* mutants, but have so far been unsuccessful. This could be due to such mutations being lethal, or alternatively that it is difficult to get a high recombination frequency in the *glnB* region, as has been reported for some *Rhizobium* species (Martin *et al.*, 1989; Amar *et al.*, 1994). Other investigations have however been initiated to obtain information about the involvement of P_{II} not only in the transcriptional regulation of nitrogen fixation and ammonia assimilation, but possibly also in the metabolic regulation operating in *Rsp. rubrum* (Ludden & Roberts, 1989), which has recently been suggested on the basis of studies of the *ntrBC* mutant (Zhang *et al.*, 1995).

ACKNOWLEDGEMENTS

We are indebted to Dr Ray A. Dixon for helpful discussions and for providing *glnB* from *Rhizobium leguminosarum*, to Dr Gary P. Roberts for kindly providing the *ntrBC* *Rsp. rubrum* mutant and to Dr Janet Jansson for valuable discussions and advice. This work was supported by grants to S.N. from the Swedish Natural Science Research Council.

REFERENCES

- Aguilar, O. M., Reiländer, H., Arnold, W. & Pühler, A. (1987). *Rhizobium meliloti* *nifN* (*fixF*) gene is part of an operon regulated by a *nifA*-dependent promoter and codes for a polypeptide homologous to the *nifK* gene product. *J Bacteriol* **169**, 5393–5400.
- Alef, K. & Zumft, W. G. (1981). Regulatory properties of glutamine synthetase from the nitrogen-fixing phototrophic bacterium *Rhodospseudomonas palustris*. *Z Naturforsch* **36c**, 784–789.
- Amar, M., Patriarca, E. J., Manco, G., Bernard, P., Riccio, A., Lamberti, A., Defez, R. & Iaccarino, M. (1994). Regulation of nitrogen metabolism is altered in a *glnB* mutant strain of *Rhizobium leguminosarum*. *Mol Microbiol* **11**, 685–693.
- Ausubel, M. F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1991). *Current Protocols in Molecular Biology*. New York: John Wiley.
- Berk, A. J. & Sharp, P. A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**, 721–732.
- Borghese, R. & Wall, J. D. (1995). Regulation of the *glnBA* operon of *Rhodobacter capsulatus*. *J Bacteriol* **177**, 4549–4552.
- Bozouklian, H. & Elmerich, C. (1986). Nucleotide sequence of the *Azospirillum brasilense* Sp7 glutamine synthetase structural gene. *Biochemie* **68**, 1181–1187.
- Carlberg, I. & Nordlund, S. (1991). Purification and partial characterization of glutamate synthase from *Rhodospirillum rubrum* grown under nitrogen-fixing conditions. *Biochem J* **279**, 151–154.
- Cheah, E., Carr, P. D., Suffolk, P. M., Vasudevan, S. G., Dixon, N. E. & Ollis, D. L. (1994). Structure of the *Escherichia coli* signal transducing protein PII. *Curr Biol* **2**, 981–990.
- Chiuarazzi, M. & Iaccarino, M. (1990). Transcriptional analysis of the *glnB-glnA* region of *Rhizobium leguminosarum* biovar *viciae*. *Mol Microbiol* **4**, 1727–1735.
- Engelhardt, H. & Klemme, J.-H. (1982). Purification and structural properties of adenylylated and deadenylylated glutamine synthetase from *Rhodospseudomonas sphaeroides*. *Arch Microbiol* **133**, 202–205.
- Falk, G., Hampe, A. & Walker, J. E. (1985). Nucleotide sequence of the *Rhodospirillum rubrum* *atp* operon. *Biochem J* **228**, 391–407.
- Forchhammer, K. & Tandeau de Marsac, N. (1994). The PII protein in the cyanobacterium *Synechococcus* sp. strain PCC 7942 is modified by serine phosphorylation and signals the cellular N-status. *J Bacteriol* **176**, 84–91.
- Foster-Hartnett, D. & Kranz, R. G. (1994). The *Rhodobacter capsulatus* *glnB* gene is regulated by NtrC at tandem *rpoN*-independent promoters. *J Bacteriol* **176**, 5171–5176.
- Fritsch, J., Rothfuchs, R., Rauhut, R. & Klug, G. (1995). Identification of an mRNA element promoting rate-limiting cleavage of the polycistronic *puf* mRNA in *Rhodobacter capsulatus* by an enzyme similar to RNase E. *Mol Microbiol* **15**, 1017–1029.
- Holtel, A. & Merrick, M. (1988). Identification of the *Klebsiella pneumoniae* *glnB* gene: nucleotide sequence of wild-type and mutant alleles. *Mol Gen Genet* **215**, 134–138.
- Johansson, B. C. & Gest, H. (1977). Adenylylation/deadenylylation control of the glutamine synthetase of *Rhodospseudomonas capsulata*. *Eur J Biochem* **81**, 365–371.
- Karn, J., Brenner, S., Barnett, L. & Cesareni, G. (1980). Novel bacteriophage λ cloning vector. *Proc Natl Acad Sci USA* **77**, 5172–5176.
- Kennedy, C., Doetsch, N., Meletzus, D., Patriarca, E., Amar, M. & Iaccarino, M. (1994). Ammonium sensing in nitrogen fixing bacteria: functions of the *glnB* and *glnD* products. *Plant Soil* **161**, 43–57.
- Kranz, R. G., Pace, V. M. & Caldicott, I. M. (1990). Inactivation, sequence, and *lacZ* fusion analysis of a regulatory locus required for repression of nitrogen fixation genes in *Rhodobacter capsulatus*. *J Bacteriol* **172**, 53–62.
- Ludden, P. W. & Roberts, G. P. (1989). Regulation of nitrogenase

- activity by reversible ADP-ribosylation. *Curr Top Cell Regul* **30**, 23–55.
- Magasanik, B. (1989).** Regulation of transcription of the *glnALG* operon of *Escherichia coli* by protein phosphorylation. *Biochemie* **71**, 1005–1012.
- Martin, G. B., Thomashow, M. F. & Chelm, B. K. (1989).** *Bradyrhizobium japonicum glnB*, a putative nitrogen-regulatory gene, is regulated by NtrC at tandem promoters. *J Bacteriol* **171**, 5638–5645.
- Merrick, M. J. (1992).** Regulation of nitrogen fixation genes in free-living and symbiotic bacteria. In *Biological Nitrogen Fixation*, pp. 835–876. Edited by G. Stacey, R. H. Burris & H. J. Evans. London: Chapman & Hall.
- Michalski, W. P. & Nicholas, D. J. D. (1984).** Regulation of N₂ fixation and ammonia assimilation in *Rhodospseudomonas sphaeroides* f. sp. *denitrificans*. Role of glutamine. *J Gen Microbiol* **130**, 1069–1077.
- Minchin, S. D., Austin, S. & Dixon, R. A. (1989).** Transcriptional activation of the *Klebsiella pneumoniae nifL*A promoter by NtrC is face-of-the-helix dependent and the activator stabilizes the interaction of sigma 54-RNA polymerase with the promoter. *EMBO J* **8**, 3491–3499.
- Miranda-Rios, J., Sanchez-Pescador, R., Ureda, M. & Covarrubias, A. A. (1987).** The complete sequence of the *glnALG* operon of *Escherichia coli* K12. *Nucleic Acids Res* **15**, 2757–2770.
- Nordlund, S., Kanemoto, R. H., Murrell, S. A. & Ludden, P. W. (1985).** Properties and regulation of glutamine synthetase from *Rhodospirillum rubrum*. *J Bacteriol* **161**, 13–17.
- Ormerod, J. G., Ormerod, K. S. & Gest, H. (1961).** Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. *Arch Biochem Biophys* **94**, 449–463.
- Quinto, C., de la Vega, H., Flores, M., Leemans, J., Cevallos, M. A., Pardo, M. A., Azpiroz, R., de Lourdes Girard, M., Calva, E. & Palacios, R. (1985).** Nitrogenase reductase: a functional multigene family in *Rhizobium phaseoli*. *Proc Natl Acad Sci USA* **82**, 1170–1174.
- Rhee, S. G., Chock, P. B. & Stadtman, E. R. (1989).** Regulation of *Escherichia coli* glutamine synthetase. *Adv Enzymol* **62**, 37–92.
- Roelvink, P. W., Harmsen, M., van Kammen, A. & van den Bos, R. C. (1990).** The *nifH* promoter region of *Rhizobium leguminosarum*: nucleotide sequence and promoter elements controlling activation by NifA protein. *Gene* **87**, 31–36.
- Soliman, A. & Nordlund, S. (1989).** Purification and partial characterization of glutamine synthetase from the photosynthetic bacterium *Rhodospirillum rubrum*. *Biochim Biophys Acta* **994**, 138–141.
- Son, H. S. & Rhee, S. G. (1987).** Cascade control of *Escherichia coli* glutamine synthetase. Purification and properties of PII protein and nucleotide sequence of its structural gene. *J Biol Chem* **262**, 8690–8695.
- Tsinoremas, N. F., Castets, A. M., Harrison, M. A., Allen, J. F. & Tandeau de Marsac, N. (1991).** Photosynthetic electron transport controls nitrogen assimilation in cyanobacteria by means of posttranslational modification of the *glnB* gene product. *Proc Natl Acad Sci USA* **88**, 4565–4569.
- Vasudevan, S. G., Gedye, C., Dixon, N. E., Cheah, E., Carr, P. D., Suffolk, P. M., Jeffrey, P. D. & Ollis, D. L. (1994).** *Escherichia coli* PII protein: purification, crystallization and oligomeric structure. *FEBS Lett* **337**, 255–258.
- Watson, J. M. & Schofield, P. R. (1985).** Species-specific, symbiotic plasmid-located repeated DNA sequences in *Rhizobium trifolii*. *Mol Gen Genet* **199**, 279–289.
- Weaver, R. F. & Weissman, C. (1979).** Mapping of RNA by a modification of the Berk–Sharp procedure: the 5' termini of 15S β -globin mRNA precursor and mature β -globin mRNA have identical map coordinates. *Nucleic Acids Res* **7**, 1175–1193.
- Woehle, D. L. (1992).** *ATP-dependent and NAD-dependent modifications of glutamine synthetase from Rhodospirillum rubrum*. PhD thesis, University of Wisconsin-Madison.
- Woehle, D. L., Lueddecke, B. A. & Ludden, P. W. (1990).** ATP-dependent and NAD-dependent modification of glutamine synthetase from *Rhodospirillum rubrum* in vitro. *J Biol Chem* **265**, 13741–13749.
- de Zamaroczy, M., Delorme, F. & Elmerich, C. (1990).** Characterization of three different nitrogen-regulated promoter regions for the expression of *glnB* and *glnA* in *Azospirillum brasilense*. *Mol Gen Genet* **224**, 421–430.
- de Zamaroczy, M., Paquelin, A. & Elmerich, C. (1993).** Functional organization of the *glnB-glnA* cluster of *Azospirillum brasilense*. *J Bacteriol* **175**, 2507–2515.
- Zhang, Y., Cummings, A. D., Burris, R. H., Ludden, P. W. & Roberts, G. P. (1995).** Effect of an *ntrBC* mutation on the posttranslational regulation of nitrogenase activity in *Rhodospirillum rubrum*. *J Bacteriol* **177**, 5322–5326.
- Zinchenko, V., Churin, Y., Shestopalov, V. & Shestakov, S. (1994).** Nucleotide sequence and characterization of the *Rhodobacter sphaeroides glnB* and *glnA* genes. *Microbiology* **140**, 2143–2151.

Received 29 December 1995; accepted 5 January 1996.