Mol Gen Genet (1992) 234:337–345

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Activation of the *Rhizobium leguminosarum glnII* gene by NtrC is dependent on upstream DNA sequences

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Received December 19, 1991

Summary. The cloning and sequence determination is reported of the DNA region of *Rhizobium leguminosarum* coding for glutamine synthetase II (GSII). An open reading frame (ORF) encoding 326 amino acids was defined as the *glnII* gene on the basis of its similarity to other *glnII* genes and the ability of a DNA fragment carrying this ORF to complement the glutamine auxotrophy of a *Klebsiella pneumoniae glnA* mutant. We find that the *glnII* gene in *R. leguminosarum* is transcribed as a monocistronic unit from a single promoter, which shows structural features characteristic of *rpoN(ntrA)*-dependent promoters. In *K. pneumoniae*, such promoters require the *ntrC* and *rpoN(ntrA)* gene products for transcription. The intracellular level of *glnII* mRNA changes when *R. leguminosarum* is grown on different nitrogen sources, as expected for regulation by the nitrogen regulatory system. Promoter deletion analysis has shown that an extensive upstream DNA sequence (316 bp) is essential for in vivo activation of the *glnII* promoter in different biovars of *R. leguminosarum*. This DNA region requires a wild-type *ntrC* gene for activity and includes two conserved putative NtrC-binding site sequences. The results conclusively show that transcription from the *R. leguminosarum glnII* promoter is fully dependent on positive control by NtrC protein and on an upstream activator sequence (UAS).

Key words: Nitrogen assimilation – Gene regulation – Promoter – Deletion analysis

Introduction

In enteric bacteria, nitrogen is assimilated in the form of ammonia by the co-ordinated activities of glutamine synthetase (GS) and glutamate synthase. When the nitrogen source becomes limiting the ammonia assimilation capacity is enhanced by increased synthesis and activity of GS. Indeed, transcriptional and post-translational (adenylylation) regulation takes place in response to available nitrogen. Transcription is controlled by the nitrogen regulatory system (*ntr* system; Gussin et al. 1986), which regulates the synthesis of various enzymes involved in nitrogen metabolism and uptake of nitrogen-containing compounds. Two of the genes involved in the *ntr* system are *ntrC*, coding for the NtrC protein, a transcriptional activator that binds DNA at an upstream activating sequence (UAS), and *rpoN (ntrA)*, coding for the alternative sigma factor σ^{54}, which allows RNA polymerase to recognise specific promoters (Thoeny and Hennecke 1989). *Rhizobium meliloti* or *R. leguminosarum rpoN(ntrA)* and *ntrC* mutant strains have been isolated (Ronson et al. 1987; Shatters and Kahn 1989; Szeto et al. 1987; Moreno et al. 1992).

The isolation of the GSII structural gene *glnII* has been reported for *Agrobacterium tumefaciens, R. meliloti* and *Bradyrhizobium japonicum* (Rossbach et al. 1988; Shatters et al. 1989; Carlson and Chelm 1986). In *B. japonicum* the rate of transcription of the *glnII* gene was demonstrated to change markedly in response to the nitrogen source (Carlson et al. 1987). Furthermore, under conditions of nitrogen-starved aerobic growth, the *ntrA* and *ntrC* gene products are essential for *glnII* expression in *R. meliloti* and *B. japonicum*, respectively (Shatters et al. 1989; Martin et al. 1988). The *R. meliloti* and *B. japonicum glnII* promoters contain putative NtrC-binding sites (Shatters et al. 1989; Carlson et al. 1987), as suggested by comparison with the consensus sequence 5′-TGCACC-N_4-TGGTGCA-3′ (Thoeny and Hennecke 1989) but no evidence concerning the function of these sequences has yet been described in the *Rhizobiaceae*. The present work describes the cloning and se-

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quencing of the *R. leguminosarum* biovar *viciae* (hereafter called *R.l. viciae*) *glnII* gene, its organization as a monocistronic unit and presents a comparison of the deduced amino acid sequence with that of other glutamine synthetases. We show that a sequence, upstream of the *glnII* transcription initiation site, containing two putative NtrC-binding sites is essential for the expression of this gene in *R.l. viciae*.

Materials and methods

Strains, media and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *R.l. viciae* was grown on RMM minimal medium (Hooykaas et al. 1977) at 30° C; the nitrogen sources used were glutamate, NH_4Cl, or KNO_3, each at a concentration of 1 g/l. When NH_4Cl was used as a nitrogen source, 100 mM 3-(N-morpholino)propanesulphonic acid (MOPS), pH 7.2, was also added (Rossi et al. 1989). Antibiotics used were: tetracycline (Tc), 5 μg/ml; nalidixic acid (Nal) 20 μg/ml; kanamycin (Km), 30 μg/ml; chloramphenicol (Cm), 50 μg/ml; rifampicin (Rf) 100 μg/ml. *Klebsiella pneumoniae* was grown on NFDM minimal medium (Dixon et al. 1977) at 30° C with glutamate (1 mg/ml) or glutamine (200 μg/ml) as a nitrogen source. Antibiotics used were: Tc, 15 μg/ml; ampicillin (Ap), 50 μg/ml; carbenicillin (Cb), 250 μg/ml.

R.l. viciae *genomic library.* Total chromosomal DNA was partially digested with *Eco*RI and ligated to the cosmid cloning vector pLAFR1 (Friedman et al. 1982) digested with *Eco*RI.

Northern blot analysis. RNA (10 μg) was extracted from bacteria grown under various conditions and purified as described previously (Rossi et al. 1989). The RNA was precipitated with ethanol and resuspended in running buffer (20 mM MOPS, 10 mM sodium acetate, pH 5.3, and 1 mM EDTA) containing 50% formamide and 2.2 M formaldehyde, heated for 10 min at 65° C and cooled on ice. Samples were separated by electrophoresis on 1.5% denaturing agarose gel in running buffer. The RNA was then transferred onto a nitrocellulose filter in 20 × SSC (1 × SSC is 0.15 M NaCl and 0.15 M sodium citrate, pH 7.0) and the filters were hybridized in 5 × SSC, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll and 0.02% bovine serum albumin) and 50% formamide at 42° C overnight. Filters were washed twice with 0.1% SDS, 1 × SSC at 50° C and then autoradiographed.

Preparation of radioactive probes. Single-stranded, uniformly labelled RNA probes were prepared with T7 or SP6 RNA polymerase by run-off transcription of appropriate fragments cloned into vector pGEM3 (Promega)

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Source or reference
Rhizobium leguminosarum biovar *viciae:*		
LPR1105	*Rif*^{r} derivative of RCR1001	Hooykaas et al. 1977
R.l. phaseoli:		
CE3	Wild type	Noel et al. 1984
CFN2012	Tn*5* derivative of CE3	Moreno et al. 1992
Klebsiella pneumoniae:		
UNF122	*hisD2, lac2002*	Holtel and Merrick 1988
UNF1827	*glnA201, lac2001, recA56, sbl300*::Tn*10*	M. Merrick
UNF2792	*ntrA*::Tn*5*	M. Merrick
UNF1828	*ntrC209, hisD2, recA56, sbl300*::Tn*10, hsdR1*	M. Merrick
Escherichia coli:		
HB101	F$^-$, *hsd520, recA, proA2, lacY1, galK2, rpsL20, xyl5, mtl1, supE44*	Boyer and Roulland-Dussoix 1969
Plasmids:		
pBJ196A	*Bradyrhizobium japonicum glnII* in pBR322	Carlson and Chelm 1986
pLAFR1	Vector for genomic library	Friedman et al. 1982
pMP220	Vector for promoter selection	Spaink et al. 1987
pAR35	*glnII* upstream sequence up to −494 in pMP220	This work
pAR36A	*glnII* upstream sequence up to −316 in pMP220	This work
pAR36B	As pAR36A but with inverted insert	This work
pAR38	*glnII* upstream sequence up to −219 in pMP220	This work
pAD7A	4.2 kb *Pvu*II fragment in pBR329	This work
pAD7B	As pAD7A, but with inverted insert	This work
pAR21	1.9 kb *Xho*I fragment in pGEM-7Zf(+)	This work
pAR26	0.7 kb *Eco*RI-*Pvu*II fragment in pGEM-7Zf(+)	This work
pGEM3	Vector for in vitro run-off	Promega
pGEM-7Zf(+)	Sequencing vector	Promega

as described by Melton et al. (1984). Oligodeoxynucleotides were labelled with ^{32}P at the 5′ end the T4 polynucleotide kinase as described in Maniatis et al. (1982).

DNA sequencing and computer analysis. Sequencing was performed according to the method of Sanger et al. (1977) using the Sequence kit (US Biochemicals). Clones were sequenced on both strands. For sequence analysis the multiple alignment was obtained with the program MALI (Vingron and Argos 1989). The release 25.0 (December 1990) of the NBRF sequence database (George et al. 1986) was screened by the method of Pearson and Lipman (1988) using the FASTA program distributed by the Genetics Computer Group Inc.

Preparation of crude extracts and GS assay. Cells were harvested by centrifugation, resuspended in 10 mM imidazole-HCl buffer, pH 7.2, containing 10 mM $MgCl_2$ and 1 mM β-mercaptoethanol and disrupted by sonic oscillation at 0° C. Determinations of protein concentration and GS activity (γ-glutamyl transferase assay) were as described by Rossi et al. (1989).

Immunoblots. Bacteria were harvested while in the exponential phase by centrifugation at 4° C, washed with 0.9% NaCl and resuspended in 10 mM imidazole, pH 7.2, containing 2.5 mM $MgCl_2$. The cells were disrupted by sonication at 0° C and the cell debris was removed by centrifugation at $27000 \times g$ for 15 min. The samples (100 µg) were boiled for 5 min and electrophoresed in 15% SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblots were made according to Rossi et al. (1989). Molecular weight protein standards, obtained from Sigma Chemical Co., included bovine albumin (67000 Da), egg albumin (43000 Da), carbonic anhydrase (30000 Da) and trypsin inhibitor (20100 Da).

β-Galactosidase assay. Bacteria were assayed for β-galactosidase according to the method of Miller (1972), as modified by Rossen et al. (1985).

Primer extension. An appropriate primer (0.7 pmoles, labelled with ^{32}P) eluted from a sequencing gel (which also verified its size) was mixed with 4 or 8 µg of RNA in 12 µl of a solution containing 50 mM TRIS-HCl, pH 8.3, 60 mM NaCl and 30 mM magnesium acetate. The mixture was heated for 3 min at 63° C, frozen in a dry ice/ethanol bath and then slowly thawed on ice. Two microlitres of this mixture were added to 3 µl containing dNTPs (0.4 mM, final concentration) and 1 unit of AMV reverse transcriptase. After 30 min incubation at 48° C the reaction was stopped by adding the loading dye. The reaction products were separated on a 6% polyacrylamide-8 M urea sequencing gel. After electrophoresis, gels were transferred to 3 MM paper (Whatman), dried and autoradiographed at −70° C. The signal due to the unreacted oligonucleotide was in excess (>100-fold) compared to the bands reported in Fig. 3.

Results

Isolation and sequencing analysis of the glnII *gene*

The insert of plasmid pBJ196A carrying the *B. japonicum glnII* gene (Carlson et al. 1987) revealed by hybridization a high degree of homology to specific restriction fragments of *R.l. viciae* DNA (data not shown). The insert was used to screen, by colony hybridization, a genomic DNA library in pLAFR1 (see Materials and methods) and to isolate a clone containing the *glnII* gene of *R.l. viciae*. The DNA of this clone was prepared and analysed by hybridization with the insert of pBJ196A after restriction with several endonucleases. The results (not shown) demonstrate colinearity of restriction sites between the cloned DNA and the chromosomal DNA. A 4.2 kb *Pvu*II fragment was subcloned into plasmid pBR329 to obtain plasmids pAD7A and pAD7B (containing the same insert in either orientation). A partial restriction map of the hybridizing region was determined and the region of homology to the insert of pBJ196A was narrowed down to a 2.0 kb *Xho*I fragment (data not shown). For sequence analysis, the 2.0 kb *Xho*I fragment and a 0.6 kb *Pvu*II-*Eco*RI fragment were isolated from pAD7A (Fig. 1 A) and subcloned into the sequencing vector pGEM-7Zf(+), generating plasmids pAR21 and pAR26, respectively (Fig. 1 B). The sequencing strategy is shown in Fig. 1 B.

The nucleotide sequence of 1617 bp of *R.l. viciae* DNA is shown in Fig. 2. The sequence analysis revealed only one significant open reading frame (ORF). The first 27 codons of this ORF match the previously determined (G. Manco et al. submitted) amino acid sequence of *R.l. viciae* GSII and thus we conclude that this ORF codes for GSII. The predicted molecular mass of the coded protein is 36900 Da, which corresponds fairly well to the estimate (M_r 39000) obtained by SDS-polyacrylamide gel electrophoresis of GSII (Fig. 3). This ORF is 981 nucleotides long and shows extensive homology to glutamine synthetases of other organisms. The five highly conserved regions found in virtually all GS pro-

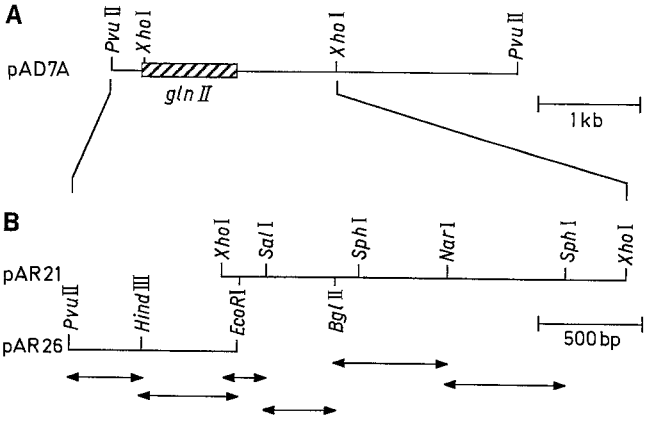

Fig. 1. Physical map of the *Rhizobium leguminosarum* biovar *viciae* DNA cloned into plasmid pAD7A, showing partial restriction map and localization of the *glnII* gene (*striped box*). **B** Sequencing strategy

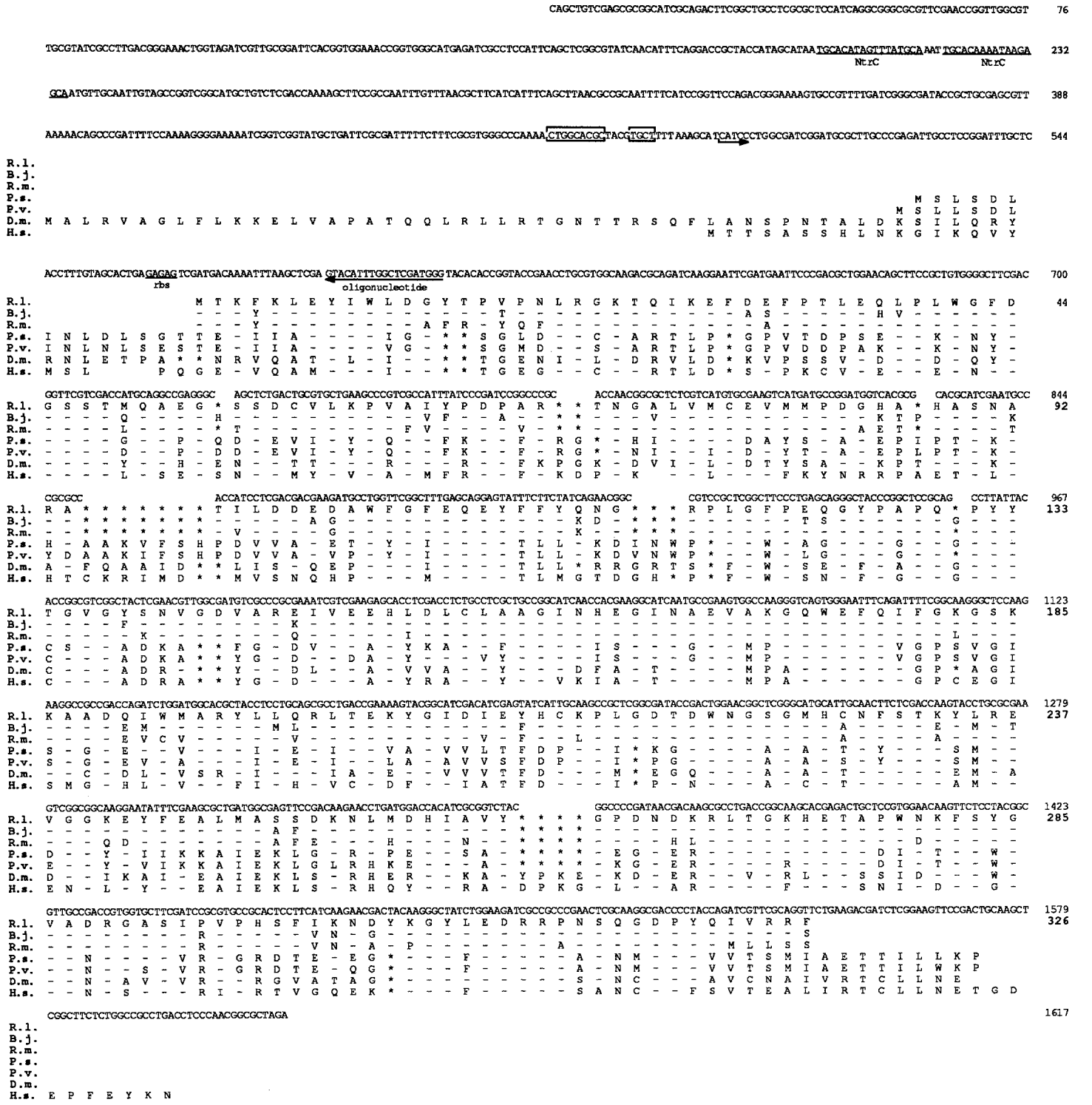

Fig. 2. DNA sequence of the *glnII* region and comparison of the deduced amino acid sequence of *R.l. viciae* GSII to that of other glutamine synthetases. *R.l., R.l. viciae; B.j., Bradyrhizobium japonicum; R.m., R. meliloti; P.s., Pisum sativum; P.v., Phaseolus vulgaris; D.m., Drosophila melanogaster; H.s., Homo sapiens. Dashes* indicate the presence of a match with the GSII amino acid sequence; *asterisks* represent a gap introduced to optimize the sequence alignment. The 5′ end of *glnII* mRNA is indicated by an *arrow;* a promoter sequence similar to a σ^{54}-dependent promoter is *boxed*; sequences for putative NtrC$^-$ and ribosome-binding sites (rbs) are *underlined;* an oligonucleotide synthesized for primer extension is indicated by an *arrow*. The nucleotide sequence has been deposited in the EMBL Data Library, accession number X67296

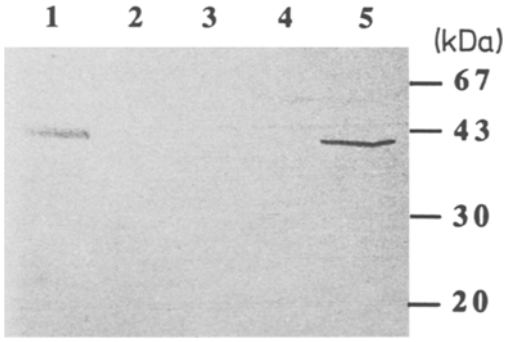

Fig. 3. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot with affinity-purified antibodies againt GSII of crude extracts of: lane 1, UNF122(pAD7A); lane 2, UNF122; lane 3, UNF1828(pAD7A); lane 4, UNF2792(pAD7A); lane 5, *R.l. viciae* LPR1105. Relevant genotypes of *Klebsiella pneumoniae* strains are wild-type (UNF122), *ntrC*$^-$ (UNF1828) and *ntrA*$^-$ (UNF2792). Bacteria were grown in NFDM (*Klebsiella*) or RMM (*Rhizobium*) medium containing glutamate as the nitrogen source (see Materials and methods). Extracts (100 µg) were separated on SDS-PAGE (15% acrylamide) transferred to a nitrocellulose sheet and treated with the immunostaining procedure

Table 2. Comparison of *glnII* promoter sequences

Source	Sequence[a]	Reference
NtrA binding site		
	-24 -12	
B. japonicum	t TGG CACGtTtga TGC TT	Carlson et al. 1987
R. meliloti	t TGG CACGtTtga TGC TT	Shatters et al. 1989
R.l. viciae	c TGG CACGcTacg TGC TT	This work
NtrC binding site		
	-119 -103	
B. japonicum	tGCgCc gctat gacGCA	Carlson et al. 1987
	-298 -282	
B. japonicum	gGCaCc gctat gacGCA	Martin et al. 1988
	-114 -98	
R. meliloti	aGCgCc agctt ggtGac	Shatters et al. 1989
	-276 -260	
R.l. viciae	tGCaCa aaata agaGCA	This work
	-296 -280	
R.l. viciae	tGCaCa tagtt tatGCA	This work

[a] Sequences are numbered starting at the site of initiation of transcription

tein sequences analysed (Hill et al. 1989) are also conserved in the putative *R.l. viciae* protein (region I, from amino acids 42 to 70; region II, 168–178; region III, 210–231; region IV, 287–297; region V, 308–321). A comparison of the predicted amino acid sequence of *R.l. viciae* GSII with published sequences of GSs from other *Rhizobiaceae* shows that *R.l. viciae* GSII displays 87.8% identity in an overlap of 328 amino acids with GSII from *B. japonicum* (Carlson and Chelm 1986) and 82.2% identity in an overlap of 326 amino acids from *R. meliloti* (Shatters and Kahn 1989). Furthermore, comparison of the deduced amino acid sequence with those of various plant (*Pisum sativum, Phaseolus vulgaris, Hordeum vulgare*) and mammalian (*Homo sapiens, Rattus norvegicus, Mus musculus, Cricetulus griseus*) glutamine synthetases shows an average of 44.9% and 38.1% identity, respectively (Fig. 2 and data not shown). Thus we confirm that the sequence of GS proteins is highly conserved and that the divergence of these proteins is proportional to the degree of phylogenetic divergence of the organisms concerned (Shatters and Kahn 1989). The sequence analysis reveals a putative ribosome-binding site in the 5′ flanking region of the ORF. Furthermore, potential transcriptional regulatory signals are present in this region: a sequence highly homologous to a −24/−12 promoter and two putative NtrC-binding sites (Table 2).

Expression of the R.l. viciae glnII *gene in* K. pneumoniae

In order to confirm that the cloned sequence encodes GSII of *R.l. viciae*, we tested it for the ability to suppress the glutamine auxotrophy phenotype caused in *K. pneumoniae* by a *glnA* mutation. Using the *glnA* mutant strain UNF1827 complementation was observed when either pAD7A or pAD7B (containing the same insert in either orientation) was introduced, suggesting that the *glnII* gene was expressed and that the expression initiated from a promoter of the insert. The growth rate of strain UNF1827 carrying pAD7A or pAD7B was the same as that of the *glnA*$^+$ strain UNF122 when incubated at 30° C. However at 37° C we observed no growth in the absence of glutamine either in liquid cultures or on plates; this phenomenon was not studied further.

Attempts to clone the *glnII* gene from other *Rhizobiaceae* by direct complementation of *glnA*$^-$ strains of *Escherichia coli* have previously failed and for this reason it has been suggested that the *glnII* promoter is not expressed in *E. coli* (Carlson et al. 1987; de Bruijn et al. 1989; Somerville et al. 1989; Rossbach et al. 1988). The genetic background of the strains used by the above authors was *glnA*$^-$ *ntrC*$^-$, whereas UNF1827 is *glnA*$^-$ *ntrC*$^+$; therefore expression of the *R.l. viciae glnII* promoter is likely to be dependent upon the host *ntr* system. Enzyme (γ-glutamyl transferase) assays showed that the introduction of either pAD7A or pAD7B induced the appearance of substantial GS activity which, like wild-type GSII activity from *R.l. viciae* (Rossi et al. 1989), was heat labile. An immunoblot with affinity-purified antibodies raised against GSII purified from *R.l. viciae* (G. Manco et al. submitted) is presented in Fig. 3. A specific protein is recognized in a crude extract of UNF122 (pAD7A), and is absent in both the *ntrC* mutant UNF1828 (pAD7A) and the *rpoN(ntrA)* mutant UNF2792 (pAD7A) and in the wild-type strain UNF122. This experiment confirms that pAD7A codes for GSII and shows that NtrC and NtrA are required for expression of *R.l. viciae glnII* in *K. pneumoniae*.

Transcriptional regulation of the R.l. viciae glnII *gene*

The in vivo start point of transcription was mapped precisely by reverse transcription, extending a [^{32}P]end-labelled synthetic oligonucleotide 5′-CCCATCGAGC-

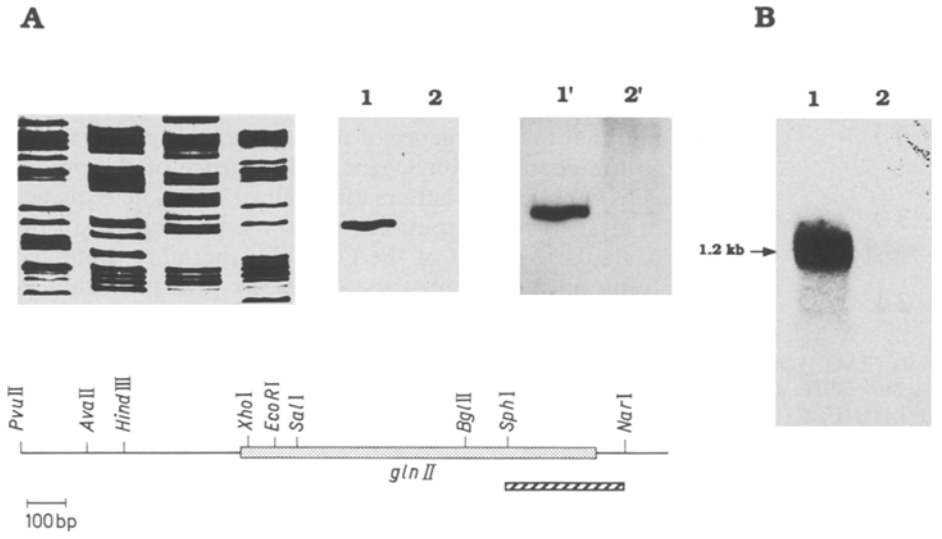

Fig. 4. A Primer extension analysis of the *glnII* transcript. Lanes 1 and 2, primer extension with 5 µg of RNA extracted from *R.l. viciae* grown with KNO_3 and NH_4Cl as nitrogen sources, respectively. Sequencing lanes were dideoxy sequencing reactions specific for the G, A, T, C residues, respectively, which were primed with the same end-labelled oligonucleotide used for the RNA analysis. A longer exposure time (lanes 1′ and 2′) reveals a band in lane 2, coincident with that of lane 1. **B** Northern blot of *glnII*. RNA was extracted from *R.l. viciae* grown on minimal medium containing KNO_3 (lane 1) or NH_4Cl (lane 2) as the sole nitrogen source. The *arrow* indicates the size of the transcript, measured using RNA molecular weight markers (Boehringer) including 1.6, 1.0, 0.6, 0.4 and 0.3 kb RNA fragments (not shown). A longer exposure time reveals a clear-cut band in lane 2, coincident with that of lane 1. In the lower part of the figure a partial restriction map of the *glnII* region is shown: the *black bar* indicates the *glnII* coding region; the *cross-hatched* bar indicates the fragment used in the hybridization (an appropriate riboprobe was synthesized)

CAAATGTAC-3′ complementary to the 5′ end of the *glnII* ORF, as indicated in Fig. 2. The size of the product obtained after primer extension was determined by electrophoresis, using as marker a sequencing ladder obtained from dideoxy reactions in which the same oligonucleotide was used as a primer. Only one 5′ end, located 72 nucleotides (nt) upstream of the *glnII* initiation codon (Fig. 4A), was found. Primer extension analysis was performed with RNA extracted from *R.l. viciae* grown on different nitrogen sources and was carried out under conditions of probe excess (see Materials and methods) to allow estimation of the relative levels of these transcripts under these conditions. Using a film scanner and films exposed for different times, the intensity level, given in arbitrary units, of the positive band representing the *glnII* transcript in *R.l. viciae* grown on KNO_3 was found to be tenfold higher than that found in bacteria grown on NH_4Cl. Northern blot analysis was performed in order to determine the size of this transcript. RNA extracted from *R.l. viciae* grown on KNO_3 or NH_4Cl as nitrogen sources was hybridized with a riboprobe internal to the *glnII* gene, as shown in Fig. 4B. This experiment reveals a single band of 1.2 kb indicating that the *glnII* gene is transcribed as a monocistronic unit terminating about 100 nt downstream of the TGA stop codon.

Analysis of the glnII *promoter*

In order to identify the region upstream of the *glnII* gene, which is essential for its expression in *R.l. viciae*, we recloned defined fragments from the inferred *glnII* promoter region of pAD7A into the pMP220 promoter-probe vector (Spaink et al. 1987) carrying the *E. coli lacZ* reporter gene. Data from Fig. 5 show levels of β-galactosidase activity obtained in *R.l. viciae* wild-type strain and in *R. leguminosarum* bivolar *phaseoli* (*R.l. phaseoli*) wild-type and *ntrC* (Moreno et al. 1992) strains. The control plasmid pMP220 had a basal level of β-galactosidase activity (52 ± 11), which was not affected by the nitrogen source. It can be seen that the introduction of a 407 bp *Ava*II-*Xho*I fragment (pAR36A) containing a region of 316 bp located upstream of the 5′ end of the *glnII* transcript promotes activity in the wild-type strain. The activity was fourfold higher when the strain was grown on KNO_3 as compared to NH_4Cl. When introduced in the opposite orientation (pAR36B) the fragment shows no β-galactosidase activity. Plasmid pAR36A introduced in a *R.l. phaseoli ntrC* mutant showed a background level of β-galactosidase activity indicating that *glnII* expression requires the presence of an intact *ntrC* gene. We conclude that the DNA fragment cloned in pAR36A contains a promoter which is regulated by the nitrogen source and requires NtrC for expression. This interpretation is consistent with the presence of an 18 bp sequence, at positions $-27/-10$ (boxed in Fig. 2), which is very similar to the $-24/-12$ promoter consensus sequence (Thoeny and Hennecke 1989) and in particular to the version of this sequence found in the *B. japonicum* (Carlson et al. 1987) and *R. meliloti* (Shatters et al. 1989) *glnII* promoters (Table 2). The conserved GC doublet of this sequence lies 12 nt upstream of the mapped transcriptional start site. Another feature present in many $-24/-12$ promoters is observed upstream of the *glnII* gene, where two sequence elements are present (at positions -296 to -280 and

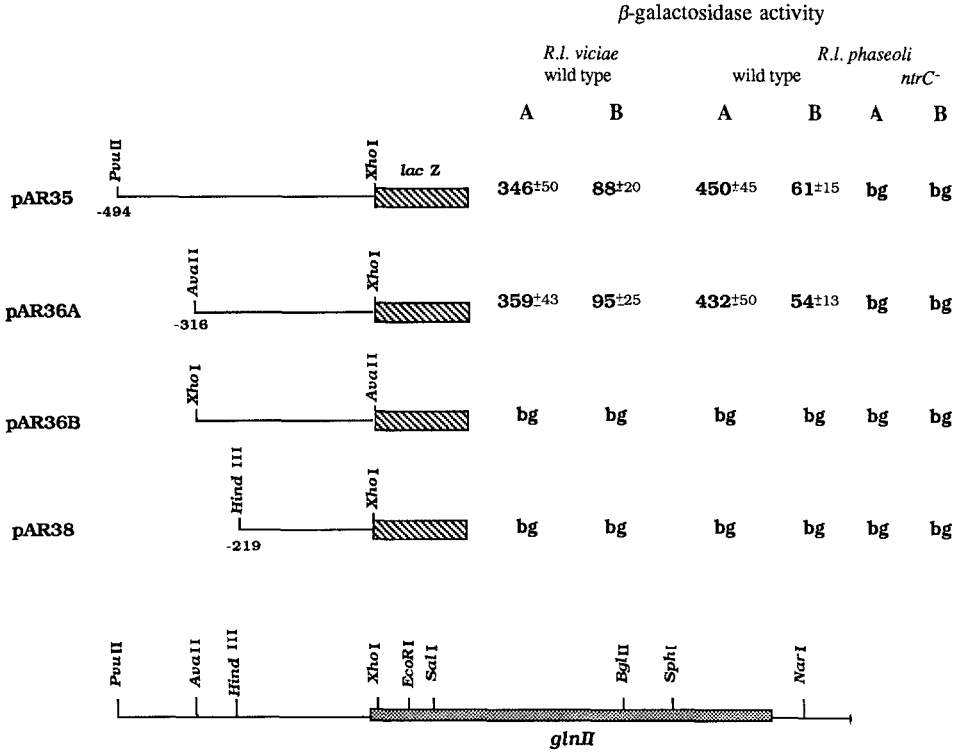

Fig. 5. Functional analysis of the *glnII* upstream region (for details see the Results). Plasmids conjugated in different *R.l. viciae* strains are indicated and for each of them the distance from the transcription initiation site to the left boundary of the cloned fragments is reported. Cultures were grown on **A** KNO_3 or **B** NH_4Cl. *Numbers* indicate β-galactosidase specific activity; the values reported are the average of 4–5 independent assays (±standard deviation) and the β-galactosidase background level of strains containing the vector pMP220 (52 U) was substracted. bg indicates that the experimental value was within the range of the background level (52±11). In the lower part of the figure a partial restriction map of the *glnII* region is shown

−276 to −260) that match the 5′-TGCACC-N_4-TGGTGCA-3′ consensus NtrC-binding site (Table 2).

Plasmid pAR35, containing a longer fragment (*Pvu*II-*Xho*I, 494 bp upstream of the transcription initiation site) gives the same activity level as that shown by pAR36A. This indicates the absence of any further positive regulatory sites between positions −494 and −316 (Fig. 5) that can be revealed under the conditions used. The promoter activity and its regulatory response disappear, in *R.l. viciae* and *R.l. phaseoli* wild-type strains, when the fragment size is reduced to a region of 219 bp upstream of the transcription initiation site (pAR38, *Hin*dIII-*Xho*I fragment). This result is consistent with the requirement for promoter activity of at least one of the two putative NtrC-binding sites identified between positions −296 and −260. We conclude that transcriptional activation of the *glnII* gene by NtrC depends on the presence of an upstream region to which the NtrC protein presumably binds (see Discussion). Putative NtrC-binding sites located upstream of the *glnII* promoter have been previously proposed only by sequence analysis in *B. japonicum* at −103 to −119 bp, and at −282 to −298 bp (Carlson et al. 1987; Martin et al. 1988) and in *R. meliloti* (Shatters et al. 1989) at −98 to −114 bp (Table 2).

Discussion

In this paper we describe the cloning, sequencing and analysis of transcriptional regulation of the *R.l. viciae glnII* gene. Using a *glnII* probe from *B. japonicum*, a DNA region containing the *glnII* gene was identified by screening a genomic library. We determined the entire sequence of the coding and flanking regions and found that the deduced amino acid sequence shows extensive homology with glutamine synthetases of other organisms. In particular it shows 87.8% and 82.2% identity with the deduced sequence of the GSII proteins of *B. japonicum* and *R. meliloti*, resepctively. The multiple alignment of this ORF with GS sequences of eukaryotic origin allows us to compare the percentage identity with mammalian and plant sequences (see Results). These values are similar, confirming that the bacterial sequence diverged from the eukaryotic sequence prior to the separation of plants from animals (Shatters and Kahn 1989).

Plasmids harbouring the *R.l. viciae glnII* gene are able to complement the glutamine auxotrophy of a *K. pneumoniae glnA⁻ ntrC⁺* strain (UNF1827). Expression of the *R.l. viciae* gene from its own promoter is also observed in *K. pneumoniae* wild-type but not in either a *ntrA⁻* or *ntrC⁻* genetic background (Fig. 3). These results are consistent with the presence in the *glnII* upstream region of an 18 bp sequence, which is very similar to the −24/−12 promoter consensus sequence 5′-TGGYRYR-N_4-TTGCA-3′ (Thoeny and Hennecke 1989), and indicate that the enteric bacteria *ntr* system is able to control the expression from the *R.l. viciae glnII* promoter. This explains why previous authors reported failure to express the *glnII* gene from its own promoter in a *glnA⁻ ntrC⁻* strain of *E. coli*. Regulation in a heterologous system was also shown for other *Rhizobium* genes involved in nitrogen fixation and assimilation (Morett and Buck 1989; Chiurazzi and Iaccarino 1990) and is consistent with the high level of homology found between different factors of *Rhizobiaceae* and *Enterobacteriaceae* which are part of the transcriptional apparatus involved in the *ntr* system.

We previously reported (Rossi et al. 1989) that GSII

specific activity varies from a value of more than 1000 when *R.l. viciae* is grown on glutamate or nitrate, to an undetectable level (less than 5) when grown on NH_4Cl. Recently Manco et al. (submitted) reported that this is in part a consequence of a post-translational modification, since NH_4Cl treatment of a *R.l. viciae* culture derepressed for GSII shows a rapid loss of transferase activity, which is not due to changes in the concentration of immuno-reacting material. In this paper an analysis of *glnII* transcription by primer extension is presented, revealing a unique transcriptional initiation site located 12 nt downstream of the conserved GC doublet typical of the *rpoN(ntrA)* promoters. The experiment of Fig. 4 demonstrates that *glnII* is transcribed only as a monocistronic unit. The transcript analysis of the *glnII* gene in *B. japonicum* (Carlson et al. 1987) was only concerned with the determination of the transcription initiation site.

A quantitative analysis indicated that *glnII* transcription is regulated in response to nitrogen source availability. A ratio of 10:1 was found for the amount of the *glnII* transcript detected in *R.l. viciae* grown in the presence of KNO_3 or NH_4Cl, respectively, as the sole nitrogen source. β-Galactosidase activity obtained with plasmids pAR35 and pAR36A (Fig. 5) indicates that promoter activity changes in the presence of KNO_3 or NH_4Cl with a similar ratio and is completely NtrC-dependent. These results indicate that *glnII* expression in *K. pneumoniae* and *R. leguminosarum* is regulated at the transcriptional level in response to the cellular nitrogen status through the *ntr* system. Moreover, the different expression ratios found when analysed at the RNA or protein level suggest a post-transcriptional control.

Our analysis was limited to the role of *ntrC* in aerobiosis. It has been found previously that *glnII* expression in microaerobiosis is NtrC-independent (Martin et al. 1988). Martin and Chelm (1991) have shown that both *nifA* and *ntrC* affect the transcription of *B. japonicum glnII*, the former under low oxygen concentrations, the latter under aerobic conditions similar to those used here.

To date, σ^{54}-dependent promoters have been shown to require a positive control protein for maximal activity. Specific upstream recognition sites play an essential role as target sequences for transcriptional regulatory proteins and enhance gene expression. However, the requirement for an upstream-bound activator to increase transcriptional initiation varies among different promoters (Morett and Buck 1989; Popham et al. 1989). Deletions of these binding sites in *K. pneumoniae* in front of the *glnAp2* or the *nifLA* promoter resulted in a strong decrease of transcriptional activity, but expression was never abolished (Drummond et al. 1983; Austin et al. 1987). Thus, the upstream DNA region is not a prerequisite for expression but does enhance it, depending on the number of NtrC-binding sites present. In fact, higher concentrations of the NtrC protein in the cell could compensate for the down-regulating effect on gene expression caused by upstream deletions in the *glnA* and *nifLA* promoters, thus allowing expression at wild-type levels (Reitzer and Magasanik 1985, 1986; Austin et al. 1987).

The role of NtrC on upstream activator sequences (UAS) has not been clearly defined in *Rhizobiaceae*. The promoters that have been shown to require NtrC in aerobic nitrogen-starved cultures of free-living bacteria are the *nifH* promoter of *R. meliloti* (Sundaresan et al. 1983), the *glnB* promoters of *B. japonicum* and *R.l. viciae* (Martin et al. 1989; Chiurazzi and Iaccarino 1990) and the *glnII* promoters of *R. meliloti* and *B. japonicum* (de Bruijn et al. 1989; Martin et al. 1988). In the latter two promoters, a NtrC-binding site sequence is present upstream of the transcription initiation site, but no evidence for the requirement of these sequences has been reported. In *B. japonicum* it was reported that a truncated NtrC protein lacking its putative DNA-binding region retained its function as an activator of *glnII* expression, raising the possibility that this domain is not required for *glnII* activation at an upstream sequence (Martin et al. 1988). Sequence analysis of the upstream region of the *glnII* gene reveals two putative NtrC-binding sites between positions −261 and −294 (Fig. 2). Results shown in Fig. 5 demonstrate that this upstream sequence is essential to promote β-galactosidase activity in *R.l. viciae* and *R.l. phaseoli* wild-type strains. Moreover, the lack of β-galactosidase activity with plasmid pAR35 and pAR36A in the *R.l. phaseoli ntrC*$^-$ strain is consistent with the conclusion that these sequences are functional in activation of the *R.l. viciae glnII* gene.

Acknowledgements. This study was partially supported by grant "Tecnologie avanzate in agricoltura" of MAF, DPGA, by EEC grants CI1-0408-I and BIOT-0166-C (EDB) and by RAISA-CNR, Subproject 2 (paper no. 335). We thank C. Sole, O. Piedimonte and N. Indaco for technical assistance, B. Chelm for supplying plasmid pBJ196A, G. Espin for suggesting complementation with pAD7, A. Tramontano for help in computer analysis, S. Colonna Romano and R. Driscoll for discussion and R. Campbell for preparing the manuscript.

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Communicated by H. Hennecke