Tandem promoters determine regulation of the *Klebsiella pneumoniae* glutamine synthetase (glnA) gene

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**ABSTRACT**

Transcription of the structural gene for glutamine synthetase (glnA) in *Klebsiella pneumoniae* is controlled by the nitrogen regulatory genes ntrA, ntrB and ntrC. The nucleotide sequence of the regulatory region upstream of the glnA gene is reported here. High resolution S1 mapping of in vivo transcripts indicates that the regulatory region contains tandem promoters separated by 100 nucleotides. Measurements of β-galactosidase activities determined in vivo from glnA-lac fusions suggest that the upstream promoter (for RNA2) is negatively regulated by the ntrBC gene products whereas transcription from the downstream promoter (for RNA1) is positively activated by the ntrA gene product in the presence of either the ntrBC or the nifA genes. The nucleotide sequence of the upstream promoter resembles the consensus sequence for *E. coli* promoters, whereas the downstream promoter shows homology with the nitrogen fixation (nif) promoters of *K. pneumoniae*.

**INTRODUCTION**

Many operons in enteric bacteria which are regulated in response to nitrogen status are subject to transcriptional control by the nitrogen regulation (ntr) system, which comprises three genes, ntrA (glnF), ntrB (glnL) and ntrC (glnG) (1). The ntrBC (glnLG) genes are located in a complex operon, downstream from glnA, the structural gene for glutamine synthetase (2,3,4), whereas the ntrA (glnF) gene is unlinked (5, 6,7). Both the ntrA (glnF) and ntrC (glnG) genes are required for transcriptional activation of a number of nitrogen regulated promoters. In addition the ntrC (glnG) gene product has been implicated in negative control of the glnA ntrBC regulon and is therefore a bifunctional regulatory protein (2,4, 8,9). Three classes of promoters can be distinguished among ntr-controlled operons: (a) those subject solely to negative
control, e.g. the ntrBC promoter, (b) promoters subject to positive control, e.g. the nif promoters of Klebsiella pneumoniae and (c) promoters subject to both positive and negative control (4). The glnA promoter is an example of the last class, it is activated under nitrogen-limiting conditions, repressed under conditions of nitrogen excess and shows an intermediate level of activity in strains lacking a functional ntr system. This complex mode of regulation is perhaps not surprising since the glnA gene product, glutamine synthetase, is not only required for synthesis of an essential amino acid, it is also an important component of the ammonia assimilation pathway under nitrogen-limiting conditions.

Genetic studies have shown that the ntrB and ntrC genes are involved in negative control of glnA expression. Whereas the products of the K. pneumoniae ntrA and ntrC genes, but not the ntrB gene, are required for activation of other ntr promoters such as nifL, three ntr genes, ntrA, ntrB and ntrC are required for activation of glnA expression in vivo (4). The additional requirement for ntrB for activation of glnA transcription suggests a role for ntrB product in modulating the activator or repressor function of the ntrC product. Studies with cis-dominant regulatory mutations suggest that functionally distinct sequences determine positive and negative control at the glnA promoter (10,11).

The glnA gene from the cyanobacterium Anabaena has recently been shown to be transcribed from two different promoters, one of these resembles the consensus sequence for E. coli promoters and is utilised during growth on excess ammonia, whereas the second promoter sequence resembles that of the Anabaena nitrogen fixation (nif) promoters and is utilised under nitrogen-fixing conditions (12). Several lines of evidence suggest that tandem promoters may also regulate glnA transcription in enteric bacteria (4,13). It was therefore of interest to determine the sequence of the glnA regulatory region in K. pneumoniae and to locate the 5' end of glnA transcripts by S1 nuclease mapping. These experiments confirm the existence of two separate promoters for glnA transcription in this organism and show that positive and negative control of glnA transcription occurs at distinct sites within the regulatory region. The upstream
promoter shows homology with the consensus sequence for *E. coli* promoters and is solely subject to repression whereas the sequence of the downstream promoter shows homology with other *nif* promoters and is subject to activation.

**MATERIALS AND METHODS**

**Strains and Plasmids**

The strain of *K. pneumoniae* used was KP5022 (hisD2 hsdR1) a derivative of M5al (14). All *E. coli* strains are derivatives of ET8000 (*rbs lacZ::IS1 gyrA hutC*) (9) and their genotypes are listed in the figure legends.

The 'high copy' lac translational fusion vector pMC1403 has been described previously (15). The 'low copy' translational (pJEL126) lac vectors (16) are derivatives of plasmid R1 carrying an insertion of the lambda P* R* promoter and the lambda cI*857* gene upstream of the R1 cop region. At low temperature (30°-35°) the plasmid copy number is one per genome equivalent whereas 'runaway' replication of these plasmids occurs following thermal inactivation of the lambda repressor (45).

**Cloning and DNA Sequencing**

Restriction enzymes and DNA-modifying enzymes were obtained from commercial sources and used according to the manufacturer's instructions. Dideoxy sequencing reactions were carried out using clones prepared in M13 mp8 and mp9 vectors (17) with \([\alpha^{35}S]\)-dATP as the labelled nucleotide (18).

**\(\beta\)-galactosidase Assays**

Strains were grown anaerobically in NFDM medium (19) containing glutamine at 200 \(\mu g/ml\) (-N) or glutamine at 2 mg/ml plus ammonium sulphate at 2 mg/ml (+N) in the presence of the appropriate antibiotics to maintain plasmid selection. \(\beta\)-galactosidase assays were performed as described previously (20).

**RNA Extraction and S1 Nuclease Mapping**

Strains were grown anaerobically overnight in NFDM medium containing ammonium sulphate at 2 mg/ml plus appropriate supplements. Cells were centrifuged then resuspended in fresh medium either with (+N) or without (-N) ammonia as nitrogen...
source and were grown for 4-5 hrs. at 28°C. RNA was extracted by the method of Aiba et al. (21).

Strand-specific DNA probes for S1 nuclease mapping were prepared from various M13 mp8 clones using single-stranded phage DNA as a template for primed synthesis by the Klenow fragment of DNA polymerase in the presence of [α³²P] dCTP (22), essentially as described above for sequencing reactions, except that the dideoxynucleotides were omitted. After 30 to 45 min. incubation at 23°C, the reaction products were digested with BamH1 in order to introduce a cut at the downstream insert-vector junction. Unincorporated nucleotides were removed by passage through a micro-column of Sephadex G50.

Probes were mixed with 25-50 μg of the appropriate RNA, lyophilised and resuspended in 30 μl of hybridisation buffer (20 mM HEPES pH 6.5, 0.4 M sodium chloride, 80% formamide) heated at 80°C for 10 min., then incubated at 49°C for 3 hrs. 450 μl of ice-cold S1 buffer (30 mM sodium acetate pH 4.5, 3 mM zinc chloride, 300 mM sodium chloride) was then added and nucleic acids were digested with 1000-2000 (Boehringer) units of S1 nuclease for 1 hr. at 28°C. The digestion products were ethanol precipitated, resuspended in formamide dyes and analysed on 7% polyacrylamide gels containing 8 M urea. Size markers were [α³²P] d-CTP labelled dideoxy sequencing products which were also digested at the insert-vector junction with BamH1.

RESULTS
Functional Analysis of the glnA Regulatory Region

The starting material for this work was the plasmid pAM127 (4) which carries the K. pneumoniae glnA regulatory region and the N-terminal end of the glnA structural gene cloned in-frame with the lacZ gene on the translational fusion vector, pMC1403. In order to delineate the promoter sequences more precisely, a number of glnA-lac fusion plasmids were constructed containing various portions of the 5' non-coding region of the glnA gene (Fig. 1). Fragments were cloned into both 'high copy' and 'low copy' lac fusion vectors so that any titration of regulatory molecules could be examined. All translational fusions contain the first eighteen codons of the glnA structural gene adjacent
Fig. 1. Construction of fusions between the glnA regulatory region and the E. coli lac operon. (a): The horizontal black line indicates the 1.8 kb fragment which was cloned into the vector pMC1403 to form the glnA-lac translational fusion pAM127 (4). The wider portion of the line indicates the glnA coding sequence and the wide open arrow represents lac DNA (not drawn to scale). (b): Enlarged map of the 510 bp AluI- BamHI fragment derived from pAM127. This fragment and its derivatives were cloned into the vectors pMC1403 (15) and pJEL122 (16) to form 'high copy' and 'low copy' translational lac fusions respectively. These plasmids (with the exception of pJAC20) contain a unique BamHI site at the glnA-lac junction and a unique EcoRI site (not shown) flanking the opposite end of the insert. The wide open arrows indicate lacZ DNA and the direction of its transcription relative to the inserted fragment. Plasmid pJAC20 contains a transcriptional fusion on the 'low copy' lac vector pJEL126 (16). The 340 bp AlyI-AhaIII fragment was first cloned into the Smal site of pEMBL8 (44) was excised with EcoRI and BamHI and then inserted into the unique EcoRI and BamHI sites of pJEL126. The wide arrow schematically represents the trp (shaded) and lac (open) DNA on this vector and the direction of its transcription relative to the insert.

to a unique BamHI site at the lacZ junction (see Fig. 3).

The fusion plasmids were introduced into a glnA ntrBC deletion strain of E. coli and β-galactosidase activities were measured. Previous studies have shown that transcription from the glnA promoter is repressed by the presence of an NtrC+ plasmid (which produces NtrC product constitutively from a vector promoter) and is activated by the presence of a plasmid which carries both the ntrB and ntrC genes (4). In addition it has been shown that the nitrogen fixation gene nifA can substitute for the ntrC gene as a transcriptional activator of several ntr-controlled operons (23,24). NifA can activate
Fig. 2. Effect of ntr and nif regulatory genes on β-galactosidase activity of glnA-lac fusions. All strains were grown anaerobically on NFDM medium containing 20 μg glutamine⁻¹. The strain background in all cases was ET8894 (Δ(rha-ntrC)1703 rbs_gyrA hutC lacZ::IS1 Mu cts62). The symbols on the horizontal axes indicate the presence of an additional plasmid providing ntr or nif gene products in trans: Δ, none; C, pMM14(ntrCC); BC, pRD565(ntrBCc); A, pMC71A(nifAc). Promoter lac fusion plasmids were: (a) pRD572; (b), pRD576; (c), pRD573; (d), pRD577; (e) pJAC20.

transcription at the glnA promoter in the absence of ntrB and unlike ntrC it does not repress transcription (4). Analogous experiments were therefore carried out by measuring the β-galactosidase activity of glnA-lac fusions in strains containing the relevant K. pneumoniae ntr or nifA gene in trans (Fig. 2).

Plasmid pAM127 and plasmids carrying the 510 bp Alul-BamHl fragment or the 330 bp Sau3A-BamHl fragment (Fig. 1) gave similar patterns of regulation and equivalent levels of β-galactosidase activity, indicating that the latter fragment contains the entire regulatory region. Therefore, for the sake of brevity, data obtained with plasmids carrying large fragments (pAM127, pRD567 and pRD574) are not included. Both the 'high' and 'low' copy number plasmids carrying the 330 bp Sau3A-BamHl fragment gave similar patterns of regulation (Fig. 2a and b). A basal level of β-galactosidase expression was observed in the absence of the glnA, ntrB ntrC operon, which was not significantly altered by the presence of a NtrCC plasmid. This result contradicts previous data obtained with derivatives of
Fig. 3. Nucleotide sequence of the glnA regulatory region. The thick lines between the DNA strands indicate homology with the E. coli glnA sequence (26). The junction between the glnA sequence and vector DNA on pAM127 is shown by the dotted line at position 323. SD denotes the presumed Shine and Dalgarno sequence. The amino acid sequence of glutamine synthetase predicted from the DNA sequence is also shown. The location of the 5' ends of transcripts RNA1, RNA2 and RNA3 are shown by large arrows. The thin horizontal arrows indicate putative repressor binding sites shown in Fig. 5. The boxed sequences indicate potential features of promoters.

*K. pneumoniae*, in which repression by ntrC was observed (4). β-galactosidase expression was enhanced by the presence of an NtrBC<sup>C</sup> or NifA<sup>C</sup> plasmid in trans, in agreement with previous results.

An entirely different regulatory pattern was observed with plasmids carrying the 170 bp AhaIII-BamH1 fragment (Fig. 2c and d). The basal level of activity in the glnA-ntrBC deletion decreased considerably, particularly in the low copy plasmid pRD577, as did the level of expression observed in the presence of the ntrBC genes, whereas the activity in the presence of the nifA gene remained virtually unchanged. With the 'low' copy-number vector, promoter function was activated 10-fold by NtrC, 8-fold by NtrBC and 27-fold by NifA. This regulatory pattern is similar to that shown by the nifLA promoter in *K. pneumoniae* (25).

Figure 2 also shows the regulatory pattern given by the plasmid pJAC20 which carries a 340 bp AluI-AhaIII fragment cloned into a 'low copy' lac transcriptional vector (Fig. 1). Promoter activity on this plasmid was not repressed by ntrC.
Fig. 4. Sl nuclease mapping of the 5' ends of glnA transcripts using uniformly labelled DNA probes and m-RNA's extracted from various strains. A. Schematic interpretation of the experiments: (i): Probes prepared from an mp8 clone carrying the 330 bp Sau3A-BamHI fragment allow the detection of DNA-RNA hybrids of various sizes. (ii): Probes prepared from an mp8 clone carrying the AhaIII-BamHI fragment allows the detection of short (>170 bp) DNA-RNA hybrids. Transcripts initiated upstream of the AhaIII site will be truncated to the length of the homologous region of the probe (170 bp). The dashed lines indicate that the probes
contained labelled M13 DNA at their 3' ends.

B. Autoradiographs of protected fragments obtained by S1 nuclease digestion of RNA-DNA hybrids. Probes were derived from the Sau3A-BamHI fragment (lanes 1-4, 9 and 10) or the AhaIII-BamHI fragment (lanes 5-8 and 11). For lanes 1-8, RNA was extracted from the same E. coli strains used for the β-galactosidase assays of pRD576 in Fig. 2b; for lanes 1 and 5, the strain contained no additional plasmid (equivalent to Δ in Fig. 2b); for lanes 2 and 6; the strain contained pMM14 (equivalent to C in Fig. 2b); for lanes 3 and 7, the strain contained pRD565 (BC in Fig. 2b); for lanes 7 and 8, the strain contained pMC71A (equivalent to A in Fig. 2b). For lanes 9-11, RNA was extracted from K. pneumoniae KP5022 which contains a normal chromosomal copy of the glnA gene. This strain was grown anaerobically in either low nitrogen medium (-N) (lanes 9 and 11) or high nitrogen medium (+N) (lane 10). The S1 digestion products were analysed on 7% polyacrylamide sequencing gels. Lanes marked G, A, T and C are dideoxy sequencing products cut with BamHI to generate 5' termini equivalent to those of the probe.

or nifA but was strongly repressed by the presence of both the ntrB and ntrC genes. The results in Fig. 2 provide tentative evidence for the existence of two promoters in the glnA regulatory region, one located on the AhaIII-BamHI fragment and one located upstream of the AhaIII site (see Fig. 1).

Mapping the 5' Ends of glnA Transcripts in vivo

The nucleotide sequence of the 330 bp Sau3A-BamHI fragment which contains the glnA regulatory region was determined and is presented in Fig. 3. This sequence shares considerable homology with published data for the corresponding E. coli sequence (26) and the regions of complete homology are indicated in the figure. The first 18 amino acids of K. pneumoniae glutamine synthetase are identical to those in E. coli and Salmonella typhimurium (37). The in vivo expression studies presented above suggest that the regulatory region contains tandem promoters which are differentially regulated. In order to test this possibility RNA was extracted from bacteria carrying the 'low copy' plasmid pRD576 using the same strains used for the β-galactosidase assays as in Fig. 2b. Transcripts were identified by S1 nuclease mapping using strand-specific M13 hybridisation probes. When an mp8 clone carrying the 330 bp Sau3A-BamHI fragment was used for probe preparation, two protected fragments were detected in RNA extracted from the glnA ntrBC deletion carrying pRD576; a major band of 240 nucleotides designated RNA2 and a less abundant transcript RNA3, of
approximately 285 nucleotides (Fig. 4, lane 1). These bands were also detected in RNA extracted from strains carrying a NtrC<sup>C</sup> plasmid in trans but an additional band, RNA1, of 140 nucleotides was also detected (Fig. 4, lanes 2 and 4). In contrast, only RNA1 could be detected in RNA prepared from strains carrying a NtrBC<sup>C</sup> plasmid (lane 3). When the probe was prepared from an mp8 clone carrying the 170 bp AhaIII-BamHI fragment, RNA1 was also detected in RNA prepared from NtrC<sup>C</sup>, NtrBC<sup>C</sup> and NifA<sup>C</sup> strains (Fig. 4, lanes 6, 7, 8). A protected fragment of 170 nucleotides which corresponds to the homologous region of this probe was also detected in all RNA preparations except those from the NtrBC<sup>C</sup> strain (lanes 5-8). This band presumably represents RNA2 and RNA3 transcripts which have been truncated by nuclease S1. RNA1, but not RNA2 or RNA3, was also detected in RNA extracted from K. pneumoniae cells containing a normal chromosomal copy of the glnA ntrBC regulon; this transcript was detected in nitrogen-fixing cultures (Fig. 4, lanes 9 and 11) but was repressed in ammonia-grown cells (Fig. 4, lane 10).

The position of the 5' ends of these transcripts (determined by the corresponding sequencing ladders in Fig. 4) is indicated in Fig. 3. The sequence upstream of the transcription start for RNA1 shows considerable homology to the nif promoters of K. pneumoniae (27) and is unlike the consensus sequence for prokaryotic promoters, whereas the promoter for RNA2 resembles the canonical sequence for E. coli promoters. The significance of the less abundant transcript, RNA3, is unclear since the sequence upstream of the 5' end of this transcript shows poor homology with prokaryotic promoters. Analogous transcripts of unknown function have also been detected in the upstream region of the Anabaena glnA promoter (12).

The Role of the ntrA Gene in glnA Regulation

The combined results of the β-galactosidase measurements and the transcript mapping studies suggest that transcription from the downstream promoter (for RNA1) is activated in the presence of the ntrBC genes whereas the expression from the upstream promoter (for RNA2) is repressed in these conditions. Strains carrying ntrA mutations show a very low level of glnA
expression resulting in a Gln^- phenotype (5,6,7). However, the Gln^- phenotype of ntrA mutants can be suppressed by a secondary mutation in either ntrB or ntrC (28), which suggests that the ntrBC products strongly repress glnA transcription in ntrA^- strains. de Bruijn and Ausubel (29) showed that the Gln^- phenotype of ntrA mutants could be suppressed by a multi-copy plasmid carrying the K. pneumoniae glnA promoter and attributed this suppression to titration of repressor(s) by multiple copies of the glnA regulatory region. High copy plasmids carrying the 330 bp Sau3A-BamHI fragment, but not the 170 bp AhaIII-BamHI fragment, suppressed the Gln^- phenotype of an ntrA^- strain whereas low copy number plasmids had no effect (Table 1). A high copy plasmid carrying the 340 bp AluI-AhaIII fragment also suppressed the Gln^- phenotype (data not shown). These results suggest that binding sites for repression by regulatory molecules are located on the Sau3A-AhaIII fragment (between positions 1 and 158 in Fig. 3).

β-galactosidase expression from the glnA promoter(s) on these plasmids was also monitored in ntr mutant backgrounds (Table 1). Expression from the upstream promoter(s) (RNA2 and RNA3) on pJAC20 was repressed in an Ntr^+ as well as an NtrA^- strain but was not repressed in an ntrC mutant or in an ntrA, ntrC double mutant (Table 1). This result again demonstrates that the upstream promoter is repressed in the presence of the NtrBC products; repression was observed in both nitrogen starved (-N) and ammonia-grown (+N) cultures (data not shown).
Plasmids carrying the 170 bp AhaIII-BamHI fragment (pRD573, pRD577) gave a low level of β-galactosidase activity in strains carrying ntrC or ntrA mutations, reflecting a requirement for the ntrC and ntrA gene products in activation of the downstream promoter (RNAl). As expected, plasmids carrying both promoters on the Sau3A-BamHI fragment (pRD572, pRD576) gave a composite expression phenotype. The lower level of β-galactosidase activity observed in the ntrC mutant presumably results from a lack of activation at the downstream promoter although repression of upstream transcription is presumably relieved.

With the 'low copy' plasmid, pRD576, the level of expression in the ntrA mutant was much lower than in the ntrA ntrC double mutant or in the ntrC mutant, again implicating the involvement of ntrC in repression at the upstream promoter. The absence of ntrA product therefore has two consequences; the downstream promoter is not activated and transcription from the upstream promoter is repressed.

DISCUSSION

Extensive nucleotide sequence homology in the regulatory regions of the glnA gene from K. pneumoniae and E. coli suggests that both organisms contain tandem promoters for glnA transcription. The arrangement of these promoters is analogous to that in the regulatory region of the glnA gene from the cyanobacterium Anabaena, where transcripts in ammonia-grown cells are initiated from two promoters: an upstream 'E. coli-like' promoter and a downstream promoter which shows sequence homology with Anabaena nif promoters (12). In nitrogen-limiting conditions the Anabaena glnA transcripts are initiated exclusively from the 'nif-like' promoter. It should be noted however that Anabaena nif-promoter sequences are very different from K. pneumoniae (Table 2). So it is unlikely that the Anabaena 'nif-like' promoter could be regulated by the enteric ntr system; indeed the downstream Anabaena 'nif-like' promoter is not expressed in an Ntr+ E. coli strain (12). Moreover, whereas the upstream promoter from Anabaena is expressed in ammonia-grown cells both in its original host and in E. coli, transcription from the analogous upstream promoter (for RNA2)
Table 2. Homologous sequences in nitrogen-regulated promoters

| (1) ntrA-independent promoters                                      | -35 region | -10 region | reference |
|----------------------------------------------------------------------|------------|------------|-----------|
| **E. coli consensus promoter**                                       | T T G A C A| T A T A A T | (31)      |
| **Anabaena glnA ('E. coli-like') promoter**                         | T T G T G C| T A A T A T | (12)      |
| **K. pneumoniae glnA (RNA2)**                                       | G T G C C C| T A T A T T | this paper|

| (2) ntrA-dependent promoters                                        | -24        | -12        |           |
|----------------------------------------------------------------------|------------|------------|-----------|
| **K. pneumoniae**                                                    |            |            |           |
| glnA (RNA1)                                                          | T T G G C A C A G A T T T C G C T | this paper|
| nifP                                                                 | C T G G C A C A G C C T T C G C A | (27)      |
| nifJ (nifC) (a)                                                      | C T G G C A C A G G C T G T G C T | (34)      |
| nifL                                                                 | A G G G C G C A C G G T T T G C A | (25)      |
| nifB                                                                 | C T G G C C G G A A A T T T G C A | (27)      |
| nifM                                                                 | C T G G T A T C G C A A A T T G C T | (27)      |
| nifU                                                                 | C T G G T A T C G C A A A T T G C T | (27)      |
| nifE                                                                 | C T G G A G C G C G A A A T T G C A | (27)      |
| nifH                                                                 | C T G G T A T G T T C C C C T G C A | (35)      |
| **S. typhimurium**                                                   |            |            |           |
| dhuA (a)                                                             | A T G G C A C G A T A G T C G C A | (36)      |
| argTr (a)                                                            | A T G G C A T A A G A C C T G C A | (36)      |
| consensus                                                            | C T G G C A C N N N N N T T G C A |           |

| (3) Anabaena nif-promoters                                           | -35        | -14        |
|----------------------------------------------------------------------|------------|------------|
| nifH                                                                 | C A T A A C | T C T A C  | (12)      |
| glnA ('nif-like')                                                    | C A A A A C | T C T A C  | (12)      |

(a): indicates that the transcriptional start-site of these promoters has not yet been reported and the promoter location is assumed on the basis of sequence homology only.
in *K. pneumoniae* is repressed in these conditions, provided that both the *ntrB* and *ntrC* genes are present. Therefore in *K. pneumoniae*, unlike *Anabaena*, the two different promoters do not appear to be utilised in response to the nitrogen source although they both respond to *ntr*-mediated control.

The *ntrA* gene is required for transcriptional activation at the promoter for RNA1 and this promoter shares considerable sequence homology with other *ntrA*-activatable promoters such as the *nif* promoters of *K. pneumoniae* (27). It has been suggested that the *ntrA* gene product may act as a specific sigma-like factor to enhance recognition of *ntr*-activatable promoters by RNA polymerase (29,46). These promoters have a characteristic structure which is very different to the canonical sequence for enteric promoters (30,31) and they lack the conserved '-10' and '-35' regions found in typical *E. coli* promoters. Instead, these promoters contain conserved regions of homology with the invariant purine dinucleotide GG at -24 and an invariant dinucleotide GC at -12 with respect to the transcriptional start-site (Table 2). The conserved region from -15 to -11 TTGCA, has been proposed to substitute for a Pribnow box and in the *nif* promoters the conserved sequence CTGG from -27 to -24 has been suggested to confer activator specificity (27). Indeed, *nif* promoters which are subject to *nifA* control have the CTGG sequence in the -24 region whereas the *nifLA* and *glnA* promoters which are activated by *ntrC* have the sequence AGGG and TTGG respectively (Table 2). However, the heptameric sequence TTTTGCA, which is present in several *ntr*-controlled promoters has also been suggested to act as a recognition sequence for *ntrC* mediated activation (13). This sequence is located around 70 nucleotides upstream of the transcriptional start-site for RNA1 (between positions 115 to 121 in Fig. 3) and although this sequence may form part of a recognition site for regulatory proteins, it is not essential for transcriptional activation since the RNA1 promoter can be activated when this sequence is deleted. Plasmids pRD573 and pRD577 (which carry the 170 bp AhaIII-BamHI fragment) contain only 32 nucleotides upstream of the start-site for RNA1, yet promoter activity on these plasmids was *ntrA*-dependent and was activated by either *ntrC* or *nifA*. 
Likewise, deletions which remove the -35 region of the nifLA promoter decrease promoter expression but do not eliminate transcriptional activation (25). Therefore any interaction between RNA polymerase and transcriptional activators may occur at the conserved -24 and -12 regions in these promoters and the sequence in the -35 region may not be essential for promoter activity.

The -12 region of the RNA1 promoter contains the sequence TTTCGCT and is particularly homologous with the nifF and nifJ promoters; in addition all three promoters contain the sequence CACAG at a corresponding position (Table 2). This homology is of interest since the nifF and nifJ promoters (unlike other nif promoters) show a low level of activator-independent activity (20,25). However it should also be noted that the RNA1 promoter (unlike nifF and nifJ) contains the sequence TATATT in the -10 region which could act as a Pribnow box and perhaps give rise to activator-independent transcription.

The promoter for RNA1 is unlike other ntr-activated promoters such as nifLA in that the ntrB gene product is required in addition to the ntrC product to activate transcription when the regulatory region is intact. However, the ntrB gene product is not required for nifA-mediated activation at this promoter. This may imply that the ntrBC gene products interact in some way with the upstream sequences whereas the nifA gene product does not. The requirement for ntrB may reflect the bifunctional nature of the ntrC gene product which has both positive and negative regulatory functions, whereas no negative function has been ascribed to nifA. Nevertheless the same transcriptional start-site is utilised whether this promoter is activated by nifA or ntrBC.

The upstream promoter (RNA2) shows homology with the consensus sequence for E. coli promoters (30,31) and although the -35 region is somewhat atypical, there is an A-T rich region around -40 which is a common feature of prokaryotic promoters. This promoter is apparently repressed in Ntr+ strains and the data with plasmids in trans indicate that the ntrBC gene products but not the nifA gene product can repress transcription. In contrast to previous results in
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Fig. 5. Putative repressor binding sites in nitrogen-regulated promoters. (a): Homologous sequences in promoters subject to negative control by the ntr genes. (b): Direct and indirect repeats of the motif GTGCA in the K. pneumoniae glnA regulatory region (positions 75 to 134 in Fig. 3).

K. pneumoniae, repression by the ntrC gene product alone was not observed. Previous data (4) were obtained by measuring glutamine synthetase activities in a strain carrying a glnA ntrB::lac translational fusion which contains approximately 600 nucleotides of the ntrB coding sequence and it is possible that the ntrB::lacZ fusion retained some ntrB function. The role of the ntrB product may be to modulate the repressor properties of ntrC and these two products may form a regulatory protein complex in vivo. However, purified ntrC product represses transcription from the ntrBC promoter in vitro in the absence of the ntrB gene product (32) so the requirement for ntrB for repression may be promoter-specific.

Promoters subject to negative control by the ntrBC gene products might be expected to contain a common repressor-binding site. Comparison of the sequences of the E. coli and K. pneumoniae glnA promoters and the S. typhimurium ntrBC promoter reveals a homologous sequence with the consensus TGCATAANNTGGTGCAA (Fig. 5). This sequence is also found in S. typhimurium dhuA promoter although it is not yet known whether this promoter is negatively regulated by ntrBC (48). These homologous regions have dyad symmetry and conform to the conserved sequences found among known repressor-binding sites (33). The location of this sequence in the glnA regulatory region is of particular interest since the regions of dyad symmetry overlap the Pribnow box for the upstream RNA2 promoter.
Further regions of dyad symmetry characterised by the motif GTGCA, are found in direct and indirect repeat between the tandem promoters (Fig. 5b). These sequences may be indicative of multiple binding sites for regulatory molecules.

What is the function of the upstream glnA promoter if transcription from this site is normally repressed in Ntr+ cells? This promoter may have a physiological role in ensuring synthesis of glutamine under certain metabolic conditions or it may have an important regulatory role in enhancing or hindering transcription initiation at the downstream promoter. Tandem promoters separated by a 120 bp spacer region have been found in the rRNA cistrons of E. coli and it has been suggested that independent transcription from each promoter allows optimal expression under different growth conditions (38). Two promoters P1 and P2, located 72 bp apart regulate transcription of the E. coli ilvGEDA operon. Although the upstream promoter is not transcriptionally active in vivo its presence is required for maximal transcription from the downstream promoter P2; protein-protein interactions between RNA polymerase molecules bound at the tandem promoters may enhance transcription initiation at the downstream promoter (C.W. Adams and G.W. Hatfield, personal communication). At least 90 bp of the sequence upstream from the transcription initiation sites in the nifLA (25) and tyrT promoters (39) are required for optimal activity. In the case of the tyrT and a number of other tRNA genes in E. coli, secondary RNA polymerase-binding sites upstream of the primary RNA polymerase-binding site have been observed and a model has been proposed in which RNA polymerase serves as a positive activator of transcription at the secondary binding sites (40). It seems unlikely that this model could apply to the glnA regulatory region since the two promoters are rather far apart (100 bp) and repression by ntr gene products might preclude interaction of RNA polymerase molecules with the upstream promoter. However there are cases in which binding of RNA polymerase to secondary binding sites can hinder promoter function. One example is the E. coli lac operon where RNA polymerase can form tight binding complexes with a secondary binding site (P2) in the absence of cAMP-CAP,
resulting in poor transcription initiation from P2 and blocking transcription from the normal transcription start. One proposed role for cAMP-CAP is therefore to prevent interaction of RNA polymerase with the inefficient P2 promoter (41-43). In the case of glnA, upstream sequences are important for maximum activation of transcription at the downstream promoter by the ntrBC products and the presence of these products results in repression of the upstream promoter. NtrBC could therefore block interaction of RNA polymerase with the upstream site and prevent inefficient transcription from this sequence. This role cannot be applied to the nifA product which activates transcription at the downstream promoter without repressing upstream transcription. As has been suggested previously (13) the independent activation of glnA transcription by the nifA product may play an important physiological role in optimising glutamine synthetase levels and hence determining efficient ammonia assimilation under nitrogen-fixing conditions.

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