Interplay between CRP-cAMP and PII-Ntr systems forms novel regulatory network between carbon metabolism and nitrogen assimilation in Escherichia coli

Xian-Jun Mao¹, Yi-Xin Huo¹,³, Martin Buck², Annie Kolb³ and Yi-Ping Wang¹,*

¹National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, P. R. China, ²Department of Biological Science, Imperial College of Science, Technology and Medicine, London SW72AZ, UK and ³Unité des Régulations Transcriptionnelles, URA-CNRS 2172, Institut Pasteur, 75724 Paris, France

Received November 21, 2006; Revised December 13, 2006; Accepted December 14, 2006

ABSTRACT

In Escherichia coli, utilization of carbon sources is regulated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which modulates the intracellular levels of cAMP. The cAMP receptor protein (CRP) controls the transcription of many catabolic genes. The availability of nitrogen is sensed by the PII protein at the level of intracellular glutamine. Glutamine is transported mainly by GlnHPQ, and synthesized by glutamine synthetase (GS) encoded by glnA. Previous studies suggest that CRP affects nitrogen assimilation. Here we showed that at least two mechanisms are involved. First, CRP activates glnHp1 via synergistic binding with sigma 70 RNA polymerase (Eσ70) and represses glnHp2. As a consequence, in the presence of glutamine, the overall enhancement of glnHPQ expression alters GlnB signalling and de-activates glnAp2. Second, in vitro studies show that CRP can be recruited by sigma 54 holoenzyme (Eσ54) to a site centred at −51.5 upstream of glnAp2. CRP-induced DNA-bending prevents the nitrogen regulation protein C (NtrC) activator from approaching the activator-accessible face of the promoter-bound Eσ54 closed complex, and inhibits glnAp2. Therefore, as the major transcriptional effector of the ‘glucose effect’, CRP affects both the signal transduction pathway and the overall geometry of the transcriptional machinery of components of the nitrogen regulon.

In Escherichia coli and related bacteria, the presence of glucose in the growth medium prevents the utilization of other carbohydrates, the so-called classic ‘glucose effect’ (1). Preferential utilization of different carbon sources is regulated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) which modulates the intracellular levels of cAMP (2). cAMP levels are high when cells are grown on non-PTS carbon sources such as glycerol. When grown on carbohydrates such as glucose, cAMP levels are low (2,3). The activity of CRP can only be triggered by binding to cAMP (3), and therefore, in this article in order to simplify the text, the liganded CRP will be further designated as CRP, not CRP-cAMP. CRP contains three transcription activation regions and is considered as a proximal activator interacting over short distances with the major form of σ70-RNA polymerase (Eσ70), often as the promoters of sugar catabolic genes. As a CRP point mutation defective in its activation region I (ARI), CRP H159L is unable to interact with the aCTD of Eσ70, but is still able to bind and bend DNA in a manner similar to its wild-type counterpart (4). Location of CRP-binding sites upstream of CRP-dependent promoters, their affinity for CRP and the intrinsic promoter strength determine the extent of activation (3). Detailed three-dimensional models of CRP–Eσ70–promoter complexes constructed by Lawson et al. (5) have yielded insights into how CRP binds DNA and activates transcription.

Assimilation of ammonia and acquisition of various nitrogen sources is tightly controlled in E. coli. A key operon, glnALG, encodes a key enzyme of nitrogen assimilation, glutamine synthetase (GS), and two regulatory proteins that control expression of the Ntr regulon,
Table 1. Bacterial strains and plasmids used in this work

| Strain/plasmid | Relevant characteristics | Source/reference |
|---------------|--------------------------|------------------|
| **E. coli strains** | | |
| TP2339-1 | F' , xyl, cya, crp-39, lacΔX74, argH1, gln | Tian et al. (9) |
| BD4000 | F' , xyl, cya, crp-39, lacΔX74, argH1, gln | This article |
| **Plasmids** | | |
| pKU101 | glnHAp2::CRP::lacZY fusion in pGD926 | Tian et al. (9) |
| pKU101H | entire glnHp1 operon inserted into pKU101 | This article |
| pKU550 | glnHp1P2::lacZYA fusion in pGD926 | This article |
| pLG339CRP | plG339 carrying crp under the control of the crp promoter | Kolb. A (23) |
| pLG339CRP<sup>H159L</sup> | H159L mutant in pLG339CRP | Busby. S (28) |
| pLG339ΔRS | plG339 with E.coli R1 SalI internal deletion | Busby. S (28) |

mCRP, mutated CRP-binding site; ... novel joint; pLG339ΔRS is the crp deleted version of plasmid pLG339CRP, as the negative control for CRP and its ARI mutant CRP<sup>H159L</sup>. which in turn causes a decrease in glnAp2 expression. Second, in vitro CRP is recruited to a sequence upstream of the core glnAp2 promoter through direct interaction between the ARI of CRP and the σCTD of E<sup>54</sup> resulting in a reduced activity of glnAp2. The latter direct down-regulatory effect is evident in vitro when a limiting amount of ammonium is used as the nitrogen source.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Table 1. The entire glnHPQ operon in TP2339-1 was replaced by bla gene using pKO3 system (24). This results in strain BD4000.

For construction of plasmid pKU550, the entire glnHp1P2 region together with the first 7 codons of the glnHPQ open reading frame (ORF) (from −289 to +63 of glnHPQ) was amplified as a 364-bp DNA fragment by PCR using E. coli strain MG1655 chromosome DNA as template and 5′-CCCAGGTTCTACAAGCGGATTTTCTCAACGTCTG-3′ (p1) and 5′-CGGAGCTTATTTTATACAGAATCTAG-3′ (p2) as primers. The 364-bp DNA fragment was digested with HindIII and BamHI, cloned into pUC18 and verified by DNA sequencing. The 364-bp HindIII–BamHI DNA fragment was cloned into pGD926, and the glnA ORF was fused in-frame with the eighth codon of the lacZ reporter gene. This results in plasmid pKU550.

The entire glnHPQ operon was amplified by PCR using p1 and 5′-CCCCAGGTTCTATAAGCGGATTTTCTCAACGTCTG-3′ (p3) as primers and E. coli strain MG1655 chromosome DNA as template. The PCR product was restricted with HindIII, cloned into pUC18 and verified by DNA sequencing. The HindIII DNA fragment was cloned...
into the glnAp2 reporter plasmid pKU101. This results in plasmid pKU101H, which complements strain BD4100 for glnHPQ.

A CRP consensus-binding site was introduced into position −50.5 nucleotides upstream of the glnAp2 by Takara Biotechnology (Dalian) and the promoter was designed as CC-50.5 according to the location of the centre of the CRP consensus-binding site (22).

**Growth media and enzyme assays**

M63 minimal medium was prepared as previously described (25) with glycerol (0.4% w/v) as the sole carbon source, and either glutamine (0.2% w/v) or 0.2 mM ammonium as nitrogen source, in the absence/presence of exogenous cAMP (2 mM), as indicated. Cells were grown at 30°C under aerobic conditions. The growth of the cells was measured spectrophotometrically, by following the optical density of the culture (OD600). The cultures that were used for measurements were initiated by diluting a cell suspension from an overnight-grown culture in the appropriate medium to an initial OD600 of 0.04–0.05. When the day-culture reached an OD600 between 0.8 and 1.0, β-galactosidase assays were performed as described before (25).

**Primer extension and in vivo KMsO4 footprinting experiments**

As described before (9), two primers (5'-CCCGATCCACGACGTTGAAAACG-3' (p#4) and 5'-CAACCGTTCCGCCAGTTTGCATG-3' (p#5)) hybridize with the structural gene of glnH (p#1, for probing transcripts p1p2) and tetR (p#5, for the control) (p#5) hybridize with the structural gene of lacZ (p#4, for probing transcripts from glnHp1p2) and tetR (p#5, for the control) on pGD926-derived plasmids, respectively, in primer extension experiments.

**Protein purification, in vitro transcription assays and DNA I footprinting**

As described before (22).

**Real time RT-PCR assays**

Total RNA was isolated from 5 ml E. coli cells (TP2339-1 with pKU101 and pLG339CRP/pLG339CRPHi594) at OD600 0.8 using RNase Mini Kit (QIAGEN). About 1.5 μg of total DNase I-treated RNA were reverse-transcribed using SuperScriptII™ RT (Invitrogen) according to the manufacturer’s instructions. A real-time PCR was performed with each pair of primers (5'-CGGATCCGCCTTTCCGG-3' (p#6) and 5'-ACC GCCCTTACGCAACAACC-3' (p#7)) that were designed for quantifying transcripts from glnHp1p2; 5'-CCAGATGGTCATCTTCAGG-3' (p#8) and 5'-TCAGCTTCTTTAGCGATGGCAG-3' (p#9) were designed for quantifying transcripts from glnHp1; 5'-ACGGCCCAAGCTGAGATGA-3' (p#10) and 5'-ACGGGTCTGACGACACGCAA-3' (p#11) were designed for quantifying transcripts of tetR from pKU101 as internal control) on a MJ OPTICON® 2 using SYBR Green PCR Master Mix (Applied Biosystems). Data analyses for a relative quantification of gene expression were performed by the comparative Ct (threshold cycle) method according to the manufacturer’s instructions. The parameter Ct is defined as the cycle number at which intensity of fluorescence (which is proportional to the quantity of DNA present in the tube during the exponential phase of the PCR) passes a fixed threshold value. The relative amount of target in two preparations is AE(Ct condition A internal control—Ct condition B sample) = (Ct condition B internal control—Ct condition B sample), where conditions A and B were in the presence/absence of exogenous cAMP (2 mM), respectively, sample is glnHp1 or glnHp1p2 product, internal control is tetR, AE = 1.8. Experiments were performed three times.

**Determination of the modification status of GlnB**

The modification status of GlnB was determined by non-denaturing polyacrylamide gel electrophoresis followed by immunodetection of GlnB using GlnB-specific antibodies as described before (26). The cell pellet was resuspended in 50 μl of ice-cold buffer (50 mM Tris-HCl pH 7.4; 5 mM EDTA, 1 mM DTT and 1 mM benzamidin), added with glass beads (0.11 mm) to about the same volume as the cell pellet, and broken in a FastPrep (Thermo). Cell debris and glass beads were removed by centrifugation and the protein concentration in the supernatant was estimated as described before (27). A total of 2.5 μg protein was loaded per lane for non-denaturing PAGE. The proteins were blotted onto nitrocellulose membranes (Hybond-C Extra, Amersham) and the blots were probed with specific anti-GlnB (provided by K. Forchhammer), which were subsequently visualized using peroxidase-conjugated secondary antibodies (Promega) and DAB (Pierce) diluted 1:10 in stable peroxide substrate buffer (Pierce) as substrate.

**RESULTS**

**CRP activates the transcription of glnHp1 in vitro through its synergistic binding with Eσ70**

Previous studies suggested that glutamine uptake is increased by the presence of CRP and increased extra-cellular glutamine elicits a nitrogen-excess signal (16). The glnHPQ operon encodes the high-affinity glutamine uptake system, and is under the control of the cAMP-dependent glnHp1 and σ^54-and NtrC-P-dependent glnHp2 (12,13). Under in vivo conditions, glnHp2 is repressed by CRP (Li et al., unpublished data, see also Figure 1). However, in a macroarray assay, we have found that the overall transcription of glnHPQ is activated by the presence of exogenous cAMP (2 mM, data not shown). Therefore whether glnHp1 transcription and the overall transcription of the glnHPQ operon are activated by CRP in ARI-dependent manner was studied.

Sequence analysis indicates that no obvious CRP-binding site can be found upstream of glnHp1, and DNA fragment containing glnHp1 cannot be retarded by CRP alone in gel shift assays (data not shown). In order to investigate the biochemical behaviour of CRP and Eσ70 on glnHp1, DNase I footprinting assays were carried out in vitro. The results show that in the absence of Eσ70, CRP cannot occupy the upstream regulatory sequences.
of glnH1 even at high concentrations (lanes 1–5 of Figure 2A). Also, in the absence of CRP, only weak occupancy of E70 at the core promoter of glnH1 is evident (lanes 6 and 10 of Figure 2A; lanes 2 and 9 of Figure 2B). In contrast, when wild-type CRP is present together with E70, the binding of E70 is strongly increased (Figure 2A and B). Also, wild-type CRP is recruited by E70 to a site centred at /C061.5 by E70-RNA polymerase, which suggests a higher affinity for DNA binding of E70 than that of CRP.

In order to investigate whether CRP can activate the transcription of glnH1 in an ARI-dependent manner in vitro, run-off and multiple-round transcriptional assays were carried out with different concentrations of CRP or its ARI mutant CRP H159L (Figure 2B). In contrast, when wild-type CRP is present together with E70, the binding of E70 is strongly increased (Figure 2A and B). Also, wild-type CRP is recruited by E70 to a site centred at /C061.5, organized as Class I σ70-promoters (28). Moreover, the above synergistic binding is abolished when wild-type CRP is replaced by its ARI mutant CRP H159L (Figure 2B).

In order to investigate whether CRP can activate the transcription of glnH1 in an ARI-dependent manner in vitro, run-off and multiple-round transcriptional assays were carried out with different concentrations of CRP or its ARI mutant CRP H159L. Results showed that, regardless of the concentration of E70, wild-type CRP, but not its ARI mutant CRP H159L, can activate glnH1 by a factor up to 6- and 14-fold in single- and multiple-round transcriptional assays, respectively. The activation factor increased when the concentration of CRP was increased (Figure 3).

Taken together, we have identified a naturally occurring case, where E70 RNA polymerase binds very weakly to glnH1. Binding of E70 to glnH1 recruits CRP to a degenerate site at −61.5 (“ttgTATCCacatcaTCACA”, which has a score of 1.44 bits, according to methods described before ((29), in which 17 bits was used as the cutoff score for strong CRP-binding sites; and 10 bits as the cutoff score for weak CRP-binding sites)), and the synergistic binding between CRP and E70 clearly helps both partners in a reciprocal manner, which results in transcriptional activation of glnH1.

The overall transcription of the glnHPQ operon is activated by CRP in vivo

To investigate the effect of CRP on the expression of glnHPQ in vivo, pKU550 carrying glnH1p2 fused with the lacZ reporter gene was transformed into cya crp double mutant TP2339-1 containing either pLG339CRP or pLG339CRP H159L. The cells were grown in

Figure 1. Effect of CRP and its ARI mutant CRP H159L on the glnH1p2 expression in the E. coli cya crp double mutant TP2339-1 detected by primer extension analysis. Lane 5 is G marker ladder. Cells were grown in M63 minimal medium with glycerol (0.4% w/v) as the sole carbon source, and glutamine (0.2% w/v) as nitrogen source, in the absence/presence of exogenous cAMP (2 mM).

Figure 2. DNase I footprints of E70 in the presence or absence of CRP and CRP H159L on glnH1 (non template strand). (A) Titration with increasing concentrations of CRP (lanes 2 and 7, 30 nM; lanes 3 and 8, 100 nM; lanes 4 and 9, 300 nM) were performed in the absence (2–4) or presence of 50 nM E70 (lanes 7–9). Lane 11 is A + G marker ladder. The protected regions were monitored by adding increasing concentrations of CRP in the presence or absence of E70. (B) Titration with increasing concentrations of CRP (lane 3, 33 nM; lane 4, 100 nM; lane 5, 300 nM) and CRP H159L (lane 10, 33 nM; lane 11, 100 nM; lane 12, 300 nM) were performed in the presence of 25 nM E70 (lanes 3–5 and 10–12). Lane 7 is A + G marker ladder. The limits of protected regions are indicated. Note that wild-type CRP is recruited to a site centred at −61.5 by E70-RNA polymerase, which suggests a higher affinity for DNA binding of E70 than that of CRP.

Figure 3. Effect of CRP and its ARI mutant CRP H159L on the glnH1 expression in vitro. (A) multiple-round run-off assay; (B) single-round run-off assay.
In contrast, CRP H159L does not affect the expression of wild-type CRP activates in vivo. First, respectively, two parallel experiments were carried out. Second, a more quantifiable real time RT-PCR was carried out to verify the CRP effect on both promoters from plasmid pKU550 (Figure 1).

Table 2. Effect of CRP and its ARI mutant CRP\^{H159L} on the expression of \(\text{glnHp1p2}\) and \(\text{glnAp2}\) in the \(E.\ coli\ cya\ crp\) double mutant TP2339-1 and \(cya\ crp\) \(\text{glnHPQ}\) triple mutant BD4000

| Strain       | CRP plasmid | Promoter (plasmid) | \(\beta\)-gal |
|--------------|-------------|--------------------|--------------|
|              |             |                    | \(-\text{cAMP}\) | \(+\text{cAMP}\) |
| a            | TP2339-1    | pLG39ARS           | \(\text{glnHp1p2(pKU550)}\) | 6533 ± 350 | 6245 ± 347 |
| TP2339-1    | pLG39CRP    | \(\text{glnHp1p2(pKU550)}\) | 6378 ± 287 | 20190 ± 1851 |
| TP2339-1    | pLG339CRP\^{H159L} | \(\text{glnHp1p2(pKU550)}\) | 6829 ± 393 | 6416 ± 334 |
| b            | TP2339-1    | pLG39ARS           | \(\text{glnAp2(pKU101)}\) | 6650 ± 276 | 6188 ± 265 |
| TP2339-1    | pLG39CRP    | \(\text{glnAp2(pKU101)}\) | 6588 ± 355 | 207 ± 43 |
| TP2339-1    | pLG339CRP\^{H159L} | \(\text{glnAp2(pKU101)}\) | 6423 ± 287 | 5582 ± 255 |
| BD4000      | pLG39CRP    | \(\text{glnAp2(pKU101)}\) | 6478 ± 312 | 6033 ± 283 |
| BD4000      | pLG39CRP    | \(\text{glnAp2(pKU101)}\) | 6431 ± 305 | 3509 ± 167 |
| BD4000      | pLG339CRP\^{H159L} | \(\text{glnAp2(pKU101)}\) | 6249 ± 203 | 5163 ± 179 |
| BD4000      | pLG39CRP    | \(\text{glnAp2(pKU101H)}\) | 912 ± 124 | 712 ± 76 |
| BD4000      | pLG39CRP    | \(\text{glnAp2(pKU101H)}\) | 896 ± 130 | 20 ± 17 |
| BD4000      | pLG339CRP\^{H159L} | \(\text{glnAp2(pKU101H)}\) | 869 ± 117 | 578 ± 50 |
| c            | TP2339-1    | pLG39ARS           | \(\text{glnAp2(pKU101)}\) | 7106 ± 312 | 6779 ± 259 |
| TP2339-1    | pLG39CRP    | \(\text{glnAp2(pKU101)}\) | 6907 ± 296 | 3542 ± 229 |
| TP2339-1    | pLG339CRP\^{H159L} | \(\text{glnAp2(pKU101)}\) | 6891 ± 355 | 6682 ± 203 |
| BD4000      | pLG39CRP    | \(\text{glnAp2(pKU101)}\) | 6485 ± 278 | 6135 ± 264 |
| BD4000      | pLG39CRP    | \(\text{glnAp2(pKU101)}\) | 6431 ± 257 | 3509 ± 305 |
| BD4000      | pLG339CRP\^{H159L} | \(\text{glnAp2(pKU101)}\) | 6249 ± 219 | 5163 ± 249 |
| d            | TP2339-1    | pLG39ARS           | \(\text{glnHp1}\) | 1 | 1.8 ± 0.7 |
| TP2339-1    | pLG39CRP    | \(\text{glnHp1}\) | 1 | 56.6 ± 2.1 |
| TP2339-1    | pLG339CRP\^{H159L} | \(\text{glnHp1}\) | 1 | 3.0 ± 1.5 |
| TP2339-1    | pLG39CRP    | \(\text{glnHp1p2}\) | 1 | 1.0 ± 0.3 |
| TP2339-1    | pLG39CRP    | \(\text{glnHp1p2}\) | 1 | 3.1 ± 0.5 |
| TP2339-1    | pLG339CRP\^{H159L} | \(\text{glnHp1p2}\) | 1 | 0.9 ± 0.2 |

All strains were grown in M63 minimal medium with glycerol (0.4% w/v) as the sole carbon source, and 0.2% w/v glucose as nitrogen source (except c, 0.2mM ammonium as nitrogen source), in the absence/presence of exogenous cAMP (2mM). pLG39ARS is the negative control for CRP and its ARI mutant CRP\^{H159L} (see Table 1). a–c: Promoter expression is measured by \(\beta\)-galactosidase assays. d: level of the \(\text{glnHp1}\) and \(\text{glnHp1p2}\) expression from chromosome measured by real time RT-PCR in TP2339-1. M63 medium in the absence or presence of exogenous cAMP. The results showed that in the presence of exogenous cAMP, \(\text{glnHp1p2}\) can be activated 3-fold by wild-type CRP. This activation was abolished when ARI mutant CRP\^{H159L} replaced wild-type CRP (Table 2a).

In order to verify the CRP effect on \(\text{glnHp1}\) and \(\text{glnHp2}\) respectively, two parallel experiments were carried out. First, \(\text{in vivo}\) primer extension assays were carried out on both promoters from plasmid pKU550 (Figure 1). Second, a more quantifiable real time RT-PCR was carried out to verify the CRP effect on both \(\text{glnHp1}\) and \(\text{glnHp1p2}\) transcribed directly from chromosome (Table 2d). In both cases, the results showed that wild-type CRP activates \(\text{glnHp1}\), but represses \(\text{glnHp2}\). In contrast, CRP\^{H159L} does not affect the expression of \(\text{glnHp1}\) or \(\text{glnHp2}\). Quantitatively, the results obtained from real time RT-PCR correlate well with the results obtained from \(\beta\)-galactosidase assays (compare Table 2d with 2a). Therefore, it seems that the CRP effect on \(\text{glnHp1p2}\) is somewhat similar to that obtained with \(\text{glnAp1p2}\), where CRP also activates \(\sigma^{38}\)-dependent \(\text{glnAp1}\), and represses \(\sigma^{54}\)-dependent \(\text{glnAp2}\) (9). However, they are different in at least two ways. First, the overall output is different. In this case, CRP causes a 3–4-fold decrease of overall expression for \(\text{glnA}\) (9), but a 3-fold increase for \(\text{glnH}\) (Table 2a and d). Second, it is known that a classical CRP-binding site is located upstream of the \(\text{glnAp1}\) promoter (10,30), essential for CRP-dependent activation of \(\text{glnAp1}\) (9). In contrast, no obvious CRP-binding site can be found upstream of \(\text{glnHp1}\). CRP activates the transcription of \(\text{glnHp1}\) \(\text{in vivo}\) through its synergistic binding with Ec\^{70} (Figure 2).

The CRP effect is maximized through \(\text{glnHPQ}\)-mediated glutamine uptake

The above results, together with the results obtained from Maheswaran and Forchhammer (16), suggest that CRP increases expression of the \(\text{glnHPQ}\) operon, which in turn, increases glutamine uptake. It has been proposed that, as a consequence, the intracellular glutamine level is increased, and so elicits a nitrogen-excess signal. In this case, the GlnB signalling process leads to the dephosphorylation of NtrC, and subsequent de-activation of NtrC-P-dependent promoters such as \(\text{glnAp2}\) and \(\text{glnHp2}\) (16,31).

To further investigate the above proposal, the \(\text{glnHPQ}\) operon was deleted from \(\text{cya}\) and \(\text{crp}\) double mutant TP2339-1 to give a triple mutant strain designated BD4000. The expression of \(\text{glnAp2}\) (pKU101) was...
measured in the carbon poor medium in the presence of glutamine, in the presence/absence of cAMP. The results showed that the repression effect of wild-type CRP on the expression of glnA is reduced from 30-fold to 2-fold upon deletion of glnHPQ. As control, the ARI mutant CRP<sup>H159L</sup> has no such repression effect (Table 2b). We also noticed that such effect (Table 2b). This result indicates that there is another repression mechanism, which might not be related to the glutamine uptake. To further investigate this possibility, ammonium was used to replace the glutamine as the sole nitrogen source. Results showed that wild-type CRP, but not its ARI mutant CRP<sup>H159L</sup>, still represses glnAp2 by a factor of 2-fold in both TP2339-1 and BD4000 (Table 2c). This result supports the notion that alternative mechanism(s) may exist for CRP-mediated repression on glnAp2.

**CRP repressed the expression of glnAp2 directly in vitro**

To investigate whether CRP can repress glnAp2 directly, multiple-round transcriptional assays were carried out with different concentrations of CRP or its ARI mutant CRP<sup>H159L</sup>. Results showed that wild-type CRP can repress the transcriptional activity of glnAp2 by a factor of 2-fold. Moreover, when wild-type CRP was replaced by its ARI mutant CRP<sup>H159L</sup>, the repression effect was abolished (Figure 5). Also, this repression effect can be increased to 10-fold, when NtrC<sup>S160F</sup> (a constitutive active form of NtrC with a lower activity compared to NtrC-P) was used to replace wild-type NtrC-P (Figure 5), indicating that repression would be greater when a partially active form of NtrC exists in the cell (33).

To investigate the mechanism of the ARI-dependent repression effect on glnAp2, DNase I footprinting assays were carried out in vitro. In the absence of E<sup>S</sup><sup>54</sup>, CRP or its ARI mutant CRP<sup>H159L</sup> cannot occupy the upstream sequence of glnAp2 (lanes 9 and 10 of Figure 6). On the other hand, in the absence of CRP, full occupancy of E<sup>S</sup><sup>54</sup> on the core promoter of glnAp2 was observed (lane 13 of Figure 6). When CRP is present together with E<sup>S</sup> <sup>54</sup>, two additional protection regions were observed upstream of the binding site of E<sup>S</sup><sup>54</sup>. In contrast, when ARI mutant CRP<sup>H159L</sup> was used in the assay, these additional protections were not evident (compare lanes 11 and 12 of Figure 6).

The two additional protection regions are from −61 to −61 and from −63 to −71. According to the location and size of the protected regions, it is possible that CRP binds and bends DNA from −42 to −61 and αCTD of E<sup>S</sup> <sup>54</sup> binds DNA upstream. The binding and bending centre for CRP is at −51.5. Previous research by Huo et al. (22) had shown that when CRP was located at −50.5 on glnAp2, CRP and E<sup>S</sup> <sup>54</sup> lie on the same face of DNA helix. Presumably the DNA bending induced by CRP, places NtrC-P far from the activator-accessible face of the closed complex and in turn represses the expression of glnAp2. The contact between αCTD and the upstream DNA region supports the view that DNA bending exists. Taken together, CRP was recruited by E<sup>S</sup> <sup>54</sup> in an
ARI-dependent manner and repressed NtrC-P mediated activation of glnAp2 directly.

DISCUSSION

In this study, we have shown that there are two novel mechanisms for CRP to mediate nitrogen regulation. In one mechanism CRP can affect the PII-mediated signal transduction pathway, hence causing deactivation of $\sigma^{54}$-dependent gln promoters (for instance, glnAp2). Specifically, in vitro experiments show that glnHp1 promoter is a Class I-like CRP-dependent promoter. Through ARI–$\alpha$CTD interaction, CRP and E$\sigma^{70}$ bind synergistically to glnHp1 to form the closed complex (Figure 2). The synergy between CRP and E$\sigma^{70}$ activates glnHp1 (Table 2d). Despite glnHp2 repression (Figure 1), the overall enhanced glnHPQ expression (3-fold, see Table 2a and d) leads to increased glutamine uptake, leading to PII-UMP deuridylylation (Figure 4) and shuts down the Ntr response in consequence. The other mechanism is that CRP can change the topological arrangement of E$\sigma^{54}$, NtrC-phosphate and glnAp2 promoter, thus directly repressing the glnAp2 promoter (Figure 5). Specifically, CRP can be recruited to the glnAp2 promoter at position −51.5 through ARI–$\alpha$CTD interactions. This recruitment leads to DNA bending by CRP at −51.5, and hence inhibits the glnAp2 promoter activity (Figure 6). Taken together, our study reveals the mechanisms of CRP-mediated nitrogen regulation and presents two novel regulatory linkages between carbon metabolism and nitrogen assimilation in E. coli. The signal transduction pathway shown in our study illuminates the regulatory link between PII and carbon regulation (31). Although glutamine can be used as a sole carbon source supporting slow growth (34), it is surprising to find that the transport of glutamine is controlled by both global-carbon and global-nitrogen signal transduction pathways.

The dynamic range of expression of glnHPQ from the ‘off’ state to the ‘on’ state is greater when it has one $\sigma^{54}$-dependent promoter and one $\sigma^{70}$ promoter. The $\sigma^{54}$-dependent promoter is able to give higher levels of transcription than the $\sigma^{70}$-dependent one could, but still is repressible by carbon status through action of CRP. In the presence of glutamine, the product of these two tandem promoters provides a novel feedback to the glnAp2 expression, through PII-NtrBC in the genetic cascade controlling nitrogen assimilation, other than GlnK-AmtB (35). The complicated regulatory mechanisms described above are involved in a related physiological pathway. To manage the nitrogen deficiency, E. coli uses NtrC/Nac system to scavenge for nitrogen-containing compounds as a first line of defense against nitrogen starvation (36). To manage the carbon deficiency, E. coli uses CRP to activate various carbon source transporter systems (37–39) (You et al. unpublished data). Results obtained from this study indicate that CRP also accelerates transport of glutamine, even in the absence of glutamine in the medium, and represses the synthesis of glutamine to efficiently balance the carbon metabolism and nitrogen assimilation. When the relevant key signals in the promoter sequences are searched...
for in silico, the CRP inhibitory effect on glutamine biosynthesis appears to be also encountered in other enterobacteria (such as Klebsiella pneumoniae, Shigella flexneri, Enterobacter gergoviae, Erwinia carotovora and Salmonella enterica serovar Typhimurium), although the details of the regulation might differ. Interestingly in S. typhimurium, a bona fide CRP-binding site ('caaTGTGAaagtGCACAgat', which has a score of 10.99 bits, according to methods described before (29)) is found centred at -27.5 upstream of glnAp2. This suggests that competition may occur between CRP and Ec54 for binding to the promoter region. As there is no Nac protein found yet in S. typhimurium (40), it would be interesting to know if a tighter regulatory linkage exists.

As a global regulator, CRP is estimated to interact at ≈200 regulatory regions in E. coli. Zheng et al. (41) had revealed 152 hitherto unknown CRP regulons involved in energy production, amino acid metabolism, nucleotide metabolism and ion transport systems by run-off transcription/microarray analysis (ROMA). Moreover, Grainger et al. (42) had shown that CRP also interacts with thousands of weaker sites across the whole E. coli chromosome other than CRP-binding sites at known CRP regulated promoters by chromatin immuno-precipitation and high-density microarrays (ChIP-chip). We demonstrate that, physiologically, CRP tends to use weak affinity DNA-binding sites for transcriptional activation or repression by a regulated recruitment mechanism (43). It would be interesting to see if cross-talk between carbon metabolism and the other major areas of metabolism are mediated through mechanisms similar to those identified in our studies.

ACKNOWLEDGEMENTS

We are grateful to S. Wigneshweraraj for NtrC, σ54, and K. Forchhammer for GlnB-specific antibodies. We thank C.H. You and B.Y. Nan for the E. coli macroarray assays, Y.T. Zhang for the help of real time RT-PCR assays, Z.X. Tian for bioinformatics analysis in other enterobacteria. This research was supported by the 973 National Key Basic Research Program on Nitrogen Fixation in China (No. 2001CB108900 to Y.P.W., as Chief Scientist of the program), the NSFC (No. 30407040, and Outstanding Young Scholar Award (No. 39925017) to Y.P.W.) and the Program of Introducing Talents of Discipline to Universities, No. B06001. The stay of Y.X.H. in France was supported by the Ministère des Affaires Etrangères. Open Access publication charges for this paper were waived by Oxford Journals.

Conflict of interest statement. None declared.

REFERENCES

1. Monod, J. (1942) Recherches sur la croissance des cellules bactériennes. Ph.D. thesis, Actualités scientifiques et industrielles, Hermann Paris.

2. Saier,M.H., Ramsayer,T.M. and Reizer,J. (1996). In Neidhardt,F.C., Curtiss,R., III, Ingraham,J.L., Lin,E.C.C., Low,K.B., Magasanik,B., Reznikoff,W.S., Riley,M., Schaechter,M. and Umbarger,H.E. (eds), Escherichia coli and Salmonella: Cellular and Molecular Biology, 2nd edn. Am. Soc. Microbiol., Washington, DC, Vol.1, pp. 1325–1343.

3. Kolb,A., Busby,S., Buc,H., Garges,S. and Adhya,S. (1993) Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem., 62, 749–795.

4. Zhou,Y., Zhang,X. and Ehrlich,R.H. (1993) Identification of the activating region of catabolite gene activator protein (CAP); isolation and characterization of mutants of CAP specifically defective in transcription activation. Proc. Natl. Acad. Sci. U.S.A., 90, 6081–6085.

5. Lawson,C.L., Swigon,D., Murakami,K.S., Darst,S.A., Berman,H.M. and Ehrlich,R.H. (2004) Catabolite activator protein: DNA binding and transcription activation. Curr. Opin. Struct. Biol., 14, 10–20.

6. Reitzer,L.J. and Magasanik,B. (1986) Transcription of glnA in E. coli is stimulated by activator bound to sites far from the promoter. Cell, 45, 785–792.

7. Wyman,C., Rombel,I., North,A.K., Bustamante,C. and Kustu,S. (1997) Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. Science, 275, 1658–1661.

8. De Carlo,S., Chen,B., Kondrashkina,E., Hoover,T.R., Nogales,E. and Nixon,B.T. (2006) The structural basis for regulated assembly of the transcriptional activator NtrC. Genes Dev., 20, 1485–1495.

9. Tian,Z.X., Li,Q.S., Buck,M., Kolb,A. and Wang,Y.P. (2001) The CRP-cAMP complex and downregulation of the glnAp2 promoter provides a novel regulatory linkage between carbon metabolism and nitrogen assimilation in Escherichia coli. Mol. Microbiol., 41, 911–924.

10. Reitzer,L.J. and Magasanik,B. (1985) Expression of glnA in Escherichia coli is regulated at tandem promoters. Proc. Natl. Acad. Sci. U.S.A., 82, 1979–1983.

11. Magasanik,B. (1996). In Neidhardt,F.C., Curtiss,R., III, Ingraham,J.L., Lin,E.C.C., Low,K.B., Magasanik,B., Reznikoff,W.S., Riley,M., Schaechter,M. and Umbarger,H.E. (eds), Escherichia coli and Salmonella: Cellular and Molecular Biology, 2nd edn. Am. Soc. Microbiol., Washington, DC, Vol.1, pp. 1344–1356.

12. Willis,R.C., Iwata,K.K. and Furlong,C.E. (1975) Regulation of glutamine transport in Escherichia coli. J. Bacteriol., 122, 1032–1037.

13. Claverie-Martin,F. and Magasanik,B. (1991) Role of the integration host factor in the regulation of the glnHp2 promoter of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A., 88, 1631–1635.

14. Ninfa,A.J. and Atkinson,M.R. (2000) PII signal transduction proteins. Trends Microbiol., 8, 172–179.

15. Ninfa,A.J. and Jiang,P. (2005) PII signal transduction proteins: sensors of alpha-ketoglutarate that regulate nitrogen metabolism. Curr. Opin. Microbiol., 8, 168–173.

16. Maheswaran,M. and Forchhammer,K. (2003) Carbon-source-dependent nitrogen regulation in Escherichia coli is mediated through glutamine-dependent GlnB signalling. Microbiology, 149, 2163–2172.

17. Jiang,P. and Ninfa,A.J. (1999) Regulation of autophosphorylation of Escherichia coli nitrogen regulator II by the PII signal transduction protein. J. Bacteriol., 181, 1906–1911.

18. Pioszak,A.A., Jiang,P. and Ninfa,A.J. (2000) The Escherichia coli PII signal transduction protein regulates the activities of the two-component system transmitter protein NRII by direct interaction with the kinase domain of the transmitter module. Biochemistry, 39, 13450–13461.

19. Atkinson,M.R., Blauwkap,M.T.A., Bondarenko,V., Studitsky,V. and Ninfa,A.J. (2002) Activation of the glnA, glnK, and nac promoters as Escherichia coli undergoes the transition from nitrogen excess growth to nitrogen starvation. J. Bacteriol., 184, 5358–5363.

20. Jiang,P., Peliska,J.A. and Ninfa,A.J. (1998) Enzymological characterization of the signal-transducing uridylyltransferase/ uridylyl-removing enzyme (EC 2.7.7.59) of Escherichia coli and its interaction with the PII protein. Biochemistry, 37, 12782–12794.
