Downregulation of the *Escherichia coli guaB* promoter by upstream-bound CRP

Seyyed I. Husnain[1#], Stephen J. W. Busby[2] and Mark S. Thomas[1]*

[1]School of Medicine and Biomedical Sciences, University of Sheffield, Beech Hill Road, Sheffield S10 2RX, UK.

[2]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

Running title: Regulation of the *E. coli guaB* promoter.

*Corresponding author. Tel: +44 (0)114 2712834. Fax: +44 (0)114 2713892. email: m.s.thomas@shef.ac.uk

[#]Present address: Department of Microbiology and Molecular Genetics, University of Texas Medical School, 6431 Fannin Street, Houston, Texas, TX77030.

ABSTRACT

The *Escherichia coli guaB* promoter (P_{guaB}) is responsible for directing transcription of the *guaB* and *guaA* genes that specify the biosynthesis of the nucleotide GMP. P_{guaB} is subject to growth rate-dependent control (GRDC), and possesses an UP element that is required for this regulation. In addition, P_{guaB} contains a discriminator, three binding sites for the nucleoid-associated protein, FIS, and putative binding sites for the regulatory proteins DnaA, PurR and CRP. Here, we show that the CRP.cAMP complex binds to a site located over 100 bp upstream of the *guaB* transcription start site, where it serves to downregulate P_{guaB}. The CRP-mediated repression of P_{guaB} activity increases in media that support lower growth rates. Inactivation of the *crp* or *cyaA* genes, or ablation/translocation of the CRP site, relieves repression by CRP and results in loss of GRDC of P_{guaB}. Thus, GRDC of P_{guaB} involves a progressive increase in CRP-mediated repression of the promoter as the growth rate decreases. Our results also suggest that the CRP.cAMP complex does not direct GRDC at P_{guaB}, and that at least one other regulatory factor is required for conferring GRDC on this promoter. However, PurR and DnaA are not required for this regulatory mechanism.

INTRODUCTION

The *Escherichia coli guaB* promoter (P_{guaB}) regulates transcription of the *guaBA* operon. The *guaB* and *guaA* genes encode inosine 5'-monophosphate dehydrogenase and guanosine 5'-monophosphate synthetase, respectively, and are required for synthesis *de novo* of guanosine 5'-monophosphate (GMP) from the common purine precursor, inosine 5'-monophosphate (46, 75). P_{guaB} responds to a variety of physiological signals. For example, the activity of P_{guaB} increases as a function of the cellular growth rate, such that *guaBA* mRNA forms an increasing fraction of total cell mass at higher growth rates (16, 33, 34). This form of regulation is referred to as growth rate-dependent control (GRDC) (17, 27). P_{guaB} is also subject to stringent control (16, 71), growth phase-dependent regulation (34), purine repression (16, 70), and its activity is coupled to the DNA replication cycle (73).

The multivalent regulation of P_{guaB} is reflected by the presence of a number of *cis*-acting regulatory sites that overlap this promoter. An UP element, located immediately upstream of the promoter -35 region, strongly enhances transcription and is required for GRDC of this promoter (28, 33). Three binding sites for the nucleoid-associated protein, FIS, have been identified centred near positions -11, +8 and +29, respectively, relative to the *guaB* transcription start site. Accordingly, FIS has been shown to repress transcription from P_{guaB} *in vitro*. However, FIS is not required for GRDC of P_{guaB} (34). P_{guaB} also contains a putative binding site for PurR that overlaps the core promoter region (16, 31; see Fig. 1). Consistent with this, IMP dehydrogenase activity is higher in a *purR* mutant strain and, unlike the situation in a wild type strain, this activity is not repressed by growth in the presence of high concentrations of guanine or guanosine (46, 48). Approximately 200 bp downstream

from the translation initiation codon for *guaB* is a consensus DnaA binding site that has been shown to bind DnaA *in vitro* and is required for DnaA-mediated repression of *guaB* transcription *in vivo* (73, 74). A second, non-consensus, DnaA site overlaps the *guaB* promoter. Although it does not bind DnaA *in vitro*, it may be required in concert with the downstream site for efficient DnaA-mediated downregulation of P_{guaB} *in vivo* (73, 74).

The cyclic AMP receptor protein (CRP) is a cyclic AMP (cAMP)-dependent global transcription regulator (for reviews, see 11, 45). It has been long established that, in the absence of exogenous glucose, CRP activates the expression of a large number of genes required for catabolism of alternative carbon sources by *E. coli* (for a review, see 41). More recent genomic studies have shown that CRP also activates transcription of genes that encode enzymes involved in central carbon metabolism and transporters of various alternative carbon sources (26, 29, 85). CRP activates transcription by binding to specific sites located upstream of promoters and contacting RNAP (11). Promoters that utilise CRP as the sole activator are categorised either as Class I or Class II (11). At Class I CRP-dependent promoters, homodimeric CRP binds DNA sites centred near positions -61.5, -71.5, -82.5 or -92.5, with respect to the transcription start site, and the downstream CRP monomer stimulates transcription by contacting the C-terminal domain of the RNAP α subunit (αCTD) through a surface-exposed loop known as Activating Region 1 (AR1, residues 156-164). At Class II CRP-dependent promoters, CRP binds to a site centred near position -41.5, and transcription is stimulated through interactions between αCTD and AR1 of the upstream CRP monomer, and between αNTD and an additional surface referred to as Activating Region 2 (AR2, residues 19, 21, 96 and 101) on the downstream CRP

monomer (11, 24, 76). At Class III promoters, optimum transcription activation is achieved by the binding of at least two CRP dimers or a combination of CRP and other regulatory protein(s) (11). CRP has also been shown to repress transcription of some genes, including the *crp* gene itself, and *cyaA*, encoding adenylate cyclase (2, 25, 26, 52, 85). Transcription repression by CRP can occur through the occupation of DNA sites that overlap core promoter regions (69) or by stabilisation of a transcriptional repressor or co-repressor bound to the promoter region (53, 77).

A putative binding site for CRP has been identified centred at position -117.5 relative to the start site for *guaB* transcription (Fig. 1). The CRP site matches the consensus at 17/22 positions, including 9/10 positions in the core binding motifs that are critical for CRP binding (9, 30, 35). Consistent with this, CRP has been shown to bind to a ~300 bp DNA fragment that extends to position -253 with respect to the P_{guaB} transcription start site (35). These authors proposed that CRP functions as an activator at P_{guaB}. In a separate transcriptomic study, *guaB* was identified as one of a number of genes that are subject to "CRP-dependent glucose activation" (26). We have previously shown that sequences located between positions -133 and -100, that include the putative CRP site, are required for GRDC of P_{guaB} (33). To resolve the apparent inconsistencies between some of these observations, we have carried out an investigation into the role of CRP at P_{guaB}. Here, we show that CRP binds to the putative CRP site located upstream of P_{guaB}, whereupon it serves to decrease promoter activity. Moreover, occupancy of this site by CRP is required for GRDC of P_{guaB}.

MATERIALS AND METHODS

Strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Promoter fragments were constructed by standard PCR techniques. Oligonucleotide primer sequences are shown in Supplementary Table 1. For promoter activity measurements *in vivo*, strains containing single copy promoter-*lacZ* transcriptional fusions were employed. All transcriptional fusions were carried on λ prophages and were constructed in the VH1000 genetic background using a system based on λimm21 (59, 68). Apart from P_{guaB} (-253 to +10), all P_{guaB}-*lacZ* fusions contained downstream endpoints at +36 with respect to the *guaB* transcription start site. The *cya1400*::*kan*, *crp*::*cat* and *purR6::*Tn*10* alleles were transferred into lysogenic VH1000 derivative strains by P1 transduction (51).

Measurement of transcription *in vivo*

Logarithmically growing cells containing chromosomally integrated P_{guaB}-*lacZ* transcriptional fusions were employed in the measurement of promoter activity as a function of growth rate. Cells from overnight cultures grown in medium supporting the lowest growth rate were inoculated to a starting OD600 of 0.02 into different media that supported a range of growth rates, and were grown with aeration at 37 °C. The set of culture media used for growing strains containing wild-type *crp* and *cyaA* alleles was based on M9 minimal medium and is referred to here as 'standard media' (33). The set of media used to grow strains containing deletions in the *crp* and/or *cyaA* genes is referred to as 'CRP media', and was M9 minimal medium containing one of the following (in order of increasing growth rate supported for wild-type strains):

0.4% (w/v) fructose, 0.4% (w/v) glucose, 0.4% (w/v) fructose + 20 amino acids, 0.4% (w/v) fructose + 1% (w/v) casamino acids, 0.4% (w/v) glucose + 20 amino acids or 0.4% (w/v) glucose + 0.8% (w/v) casamino acids. 20 amino acids comprised each amino acid at a final concentration of 20 μg/ml. All growth media also contained 5 μg/ml thiamine. Strains containing plasmids derived from pLG339 (i.e., pLG339ΔBS, pLG339CRP, pLG339CRP159L and pLG339CRP101E) were grown in the presence of 25 μg/ml kanamycin, and strains containing plasmids derived from pBR322 (i.e., pDU9, pDCRP and pHA7) were grown in the presence of 100 μg/ml ampicillin. cAMP sodium monohydrate (Sigma-Aldrich) was added to a final concentration of 5 mM, where included. Cultures were grown until OD600 0.40-0.45, whereupon the β-galactosidase activity was measured following disruption of cells by sonication (51, 80). [For measuring the effect of the Δ*purR* allele on transcription from P_{guaB} derivatives in bacteria growing in M9 medium containing glucose, cells were permeabilised by chloroform-SDS treatment.] All data points on GRDC plots represent the mean β-galactosidase activity (in Miller units) and mean growth rate from three independent experiments.

Measurement of transcription *in vitro*

For measurement of transcription *in vitro*, supercoiled plasmid DNA was used containing either the P_{guaB} (-133 to +36) promoter carried by plasmid pRLG770, or the synthetic CRP-dependent class II promoter, *CC(-41.5)*, carried by plasmid pSR. Multiple-round transcription reactions were performed as previously described, in the presence or absence of CRP, except that KCl was used at a concentration of 100 mM (33). Template was preincubated with 10 nM RNAP holoenzyme (Epicentre), 20 nM CRP, 200 μM cAMP, 200 μM each of CTP, and either ATP or GTP, and 10 uM UTP

for 10 minutes at 30 °C. The reaction commenced after addition of the initiating nucleotide (200 μM GTP for P_{guaB}, or 200 μM ATP for the *CC(-41.5)* promoter), and was allowed to proceed for 10 minutes at 30 °C. Reactions were terminated with an equal volume of stop solution (95% deionised formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Samples were fractionated in a 5.5% acrylamide gel containing 7 M urea, and transcript abundance was quantified using a FujiFilm FLA3000 phosphorimager

Electrophoretic mobility shift assay (EMSA)

DNA fragments containing P_{guaB} sequences were amplified from pUC19 by PCR using flanking pUC19-specific primers, as described previously (34). PCR products were digested with *Hin*dIII, and purified by crush-soaking in a solution containing 0.2 M NaCl, 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0), following electrophoresis in a 6% polyacrylamide gel (50). Fragments were labelled at the *Hin*dIII end using [α-^{32}P]-dATP (3000 Ci [1.11 x 10^{14} Bq]/mmol, MP Biomedicals) and DNA polymerase I Klenow fragment. Labelled DNA (final concentration 0.4 nM) was incubated at room temperature for 30 minutes in 10 μl of a buffer containing 20 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 50 mM potassium glutamate, 1 mM DTT, 10% (v/v) glycerol and, 20 μg ml^{-1} sonicated calf thymus DNA (GE Healthcare) and, where appropriate, 200 μM cAMP, in the absence or presence of 200 nM CRP. Samples were loaded onto a 6% polyacrylamide gel (37.5:1 acrylamide:bis-acrylamide) containing 7.5% (v/v) glycerol and 200 μM cAMP while running at ~15 V/cm, and electrophoresed for ~1 hour at 4 ^{o}C. Radiolabelled DNA was visualised using a FujiFilm FLA3000 phosphorimager.

DNase I footprinting

DNase I footprinting was performed as described previously, using an *Eco*RI-*Xho*I DNA fragment isolated from plasmid pBSG-253 (extending from positions -253 to +36 of the *guaB* promoter) and labelled at the *Xho*I end of the template strand with [γ-^{32}P]ATP (>7000Ci/mmol; MP Biomedicals) using T4 polynucleotide kinase (34). To facilitate CRP binding to DNA, reaction samples, each containing a final concentration of 2.5% (w/v) glycerol, were incubated with binding buffer (20 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 50 mM potassium glutamate, 1 mM DTT, 20 μg ml^{-1} sonicated calf thymus DNA) at room temperature for 30 minutes in the presence or absence of CRP at 200 nM and/or cAMP at 200 μM, and this was followed by digestion with DNase I. Samples were purified by phenol-chloroform extraction and ethanol precipitation, and DNA fragments were separated in a 6% poly?acrylamide-7 M urea sequencing gel. A Maxam-Gilbert G+A sequencing ladder was run alongside. Footprints were visualised using a FujiFilm FLA3000 phosphorimager.

SDS-PAGE and western blotting

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% resolving gel (29:1 acrylamide-bisacrylamide). Western blotting was carried out to measure CRP levels in strain VH1000G-133. A derivative strain (VH1000G-133*Δcrp*) that contained a deletion in the *crp* gene served as a negative control. For western blotting, cells were grown in media supporting different growth rates, and the OD600nm was measured periodically. Logarithmically growing cells at an OD600nm of ~0.35-0.45 were harvested by centrifugation, disrupted by sonication and the total protein concentration in the soluble fraction was determined using the RC DC protein assay kit (Bio-Rad). 2.4 μg of total protein was fractionated by SDS-

PAGE and transferred to a PVDF membrane by electroblotting. The membrane was blocked with StartingBlock PBS buffer (Pierce), and detection was performed using rabbit anti-CRP antiserum (a gift from H. Aiba), a secondary antibody conjugated to HRP (Caltag) and SuperSignal West Pico chemiluminescent substrate (Pierce). Protein bands corresponding to CRP were detected by autoradiography, and their intensity was quantified densitometrically.

RESULTS

Analysis of CRP binding to P_{guaB}

DNase I footprinting was employed to determine whether the putative CRP site located at position -117.5 is bound by purified CRP protein. The results show that CRP protects a region from -107 to -128 relative to the *guaB* transcription start site and this is consistent with the location of the predicted CRP site (Fig. 2). No other sequences between positions -253 to +36 were protected by CRP (result not shown).

Role of the CRP site in GRDC of P_{guaB}

A P_{guaB} promoter fragment with an upstream endpoint at -133 and a downstream endpoint at +36 with respect to the transcription start site (P_{guaB} (-133 to +36)), contains all the DNA sequence elements required for full GRDC of P_{guaB} (Fig. 1) (33). To determine whether the CRP site centred at position -117.5 is required for GRDC, cells containing single copy P_{guaB}-*lacZ* fusions in which the CRP site was present (P_{guaB} (-133 to +36)) or absent (P_{guaB} (-117 to +36)) were grown at different growth rates, and the β-galactosidase activity was measured.

In agreement with previous observations, transcription from P_{guaB} (-133 to +36) increased in media supporting higher growth rates (16) (Fig. 3A). As previously noted, this corresponded to a ~1.8-fold increase in promoter activity for a doubling of the growth rate (33). In contrast, the activity of P_{guaB} (-117 to +36) was maintained at a similar high level at each growth rate tested (Fig. 3B). The reason for this was due to a higher activity of P_{guaB} (-117 to +36), relative to P_{guaB} (-133 to +36), in bacteria growing in medium supporting low growth rates. This observation suggests that residues between positions -133 and -118, which includes the CRP binding site, contributes in some way to GRDC of P_{guaB}.

To determine whether the CRP binding site is required for GRDC of P_{guaB}, promoter derivatives containing single base pair mutations in the CRP site were analysed. CRP exhibits a high degree of specificity for a G base at position 7 (corresponding to a C at position 16) of the 22 bp consensus CRP site (aaaTGTGAtctagaTCACAttt) (core binding site in upper case; positions 7 and 16 underlined). Substitution of this residue by a C results in a large reduction in CRP binding to the consensus CRP site (30). Therefore, we introduced a G to C point mutation at the equivalent position in the P_{guaB} CRP site (i.e., position -122 relative to the *guaB* transcription start site) giving rise to P_{guaB} (-133 to +36, G-122C). Our results show that introduction of this substitution led to a complete loss of GRDC (Fig. 3C). Similar to the situation with P_{guaB} (-117 to +36), abolition of GRDC was due to an increase in P_{guaB} (-133 to +36, G-122C) activity, relative P_{guaB} (-133 to +36), which was most pronounced at low growth rates. CRP binding to the mutant site was assessed by EMSA. This showed that the substitution caused a significantly reduced CRP-P_{guaB} interaction (Fig. 3E). Our results strongly suggest that binding of CRP to the site located at -117.5 is

required for repression of P_{guaB} at low growth rates and that this plays a role in GRDC of P_{guaB}.

In a complementary experiment, an A to C point mutation was introduced at position 18 in the P_{guaB} CRP site (i.e., P_{guaB} (-133 to +36, A-111C)), generating a consensus core CRP binding site (Fig. 1). Stronger binding of CRP to P_{guaB} (-133 to +36, A-111C) was confirmed by EMSA (Fig. 3E). The stronger binding of CRP to the consensus CRP site resulted in a 30-50% decrease in P_{guaB} activity, depending on the growth rate (Fig. 3D). However, the fold change in activity of this promoter as a function of growth rate was not significantly different to that of P_{guaB} (-133 to +36). Thus, whereas for the wild type *guaB* promoter there was a ~1.8-fold increase in promoter activity as the growth rate doubled, a ~2-fold increase in activity was observed for the A-111C derivative. This result is consistent with our proposal that binding of CRP to the P_{guaB} upstream region serves to downregulate transcription.

Role of CRP and its activating regions in GRDC of P_{guaB}

To confirm that GRDC of P_{guaB} requires CRP, the activity of P_{guaB} (-133 to +36) in response to growth rate was measured in a *Δcrp* strain. In the absence of CRP, P_{guaB} exhibited an increase in activity at low growth rates relative to wild type cells, and there was no longer a positive correlation between the promoter activity and the growth rate (Fig. 4A). Moreover, as observed previously, the presence of the *Δcrp* allele resulted in a slow growth phenotype in all media tested (for example, see 15, 18, 56, 82). Normal GRDC of P_{guaB} was restored following introduction of a low copy number plasmid specifying CRP (Fig. 4B). These results are consistent with the observation that a functional CRP site is required for positive GRDC of P_{guaB}.

To ascertain the requirement for the RNAP contact sites on CRP, plasmids harbouring mutant *crp* alleles that encode derivatives containing inhibitory amino acid substitutions in AR1 or AR2, were introduced into the *Δcrp* strain harbouring P_{guaB} (-133 to +36), and the growth rate dependence of the promoter was measured. Control experiments with the CRP AR1 and AR2 variants demonstrated that this system could be used to measure the effects of inactivation of AR1 and AR2 at class I and class II CRP-dependent promoters *in vivo*, in the same strain background (results not shown; 12, 81). Our results show that inactivation of AR1 resulted in a decrease in P_{guaB} activity at all growth rates (Fig. 4C), whereas disruption of AR2 function gave rise to a more modest decrease in the activity of this promoter (Fig. 4D). However, the presence of either mutant CRP protein did not result in a significant change in GRDC of P_{guaB} in comparison to wild-type CRP. This is more obvious in plots of relative promoter activity versus growth rate (Supplementary Fig. 1).

Importance of CRP site location for GRDC of P_{guaB}

To test whether the location of the CRP site is important for repression of P_{guaB} and GRDC, the CRP site in P_{guaB} (-133 to +36) was translocated to a position centred at -106.5 (approximately one helical turn downstream of the original location) or -128.5 (approximately one helical turn upstream of the original location). In each case, the original CRP site was disrupted by point mutations in the core region that are unfavourable for CRP binding (Table 1) and the repositioned CRP site retained the 22 bp sequence of the original. The activity of these *guaB* promoter derivatives in bacteria growing at different rates was then measured in a wild type strain background. The results show that moving the P_{guaB} CRP site by one helical turn

upstream or downstream of the original location led to a complete loss of GRDC (Fig. 4E and 4F; see also Supplementary Fig. 1). This suggests that the distance between the CRP site and the P_{guaB} core promoter elements is important for repression of P_{guaB} (and GRDC) and/or that an adjacent or overlapping binding site for another transcription factor, that has been disrupted by re-positioning of the CRP site, may also play an important role in regulation of P_{guaB}.

Intracellular CRP concentration as a function of bacterial growth rate

If the cAMP·CRP complex acts as the primary sensor of growth rate for P_{guaB}, an inverse correlation between the intracellular concentration of CRP and/or cAMP and the cellular growth rate would be expected. For example, it has been shown previously that the intracellular concentration of CRP is lower in *E. coli* cells growing in the presence of glucose in comparison to cells utilising glycerol as sole carbon source (37). To examine whether the intracellular concentration of CRP varies with growth rate, western blotting was employed. The results show that at growth rates of <1.0 doubling per hour, the intracellular concentration of CRP is approximately twofold higher than at higher growth rates (Supplementary Fig. 2). However, as there does not appear to be a smooth inverse relationship between CRP abundance and growth rate, it is unlikely that the CRP concentration functions as a sensor of changes in the cellular growth rate for P_{guaB}.

In a complementary experiment, we asked whether artificially increasing the intracellular concentration of CRP can influence GRDC of P_{guaB}. To do this, we overexpressed the *crp* gene from its native promoter (plasmid pDCRP) and, separately, from the constitutive *bla* promoter (plasmid pHA7) in a Δ*crp* strain, and

measured the activity of P_{guaB} (-133 to +36) over a range of different growth rates. [Unlike the native *crp* promoter, the *bla* promoter is not subject to feedback regulation by CRP, or downregulation in the presence of glucose (36, 54).] P_{guaB} (-133 to +36) activity was also measured in cells containing a plasmid that lacked the *crp* gene (pDU9). Consistent with our previous observation, positive GRDC of P_{guaB} was not observed in the absence of *crp* (Fig. 5A). Overexpression of *crp* from pDCRP resulted in the restoration of normal GRDC to P_{guaB} (compare Fig. 5B and 5D). Thus, higher than normal levels of CRP do not result in repression of *guaB* promoter activity relative to that observed in wild type cells. Placing CRP under the control of the *bla* promoter also conferred GRDC on P_{guaB}, although the observed slope was steeper than for cells containing pDCRP (compare Fig. 5C and 5D). These results demonstrate that constitutively increased expression of *crp* does not result in constitutive repression of P_{guaB} activity.

Effect of manipulation of the intracellular cAMP concentration on GRDC of P_{guaB}

Previous studies indicate that addition of increasing concentrations of cAMP to the growth medium results in progressively increased CRP-mediated repression or activation of target promoters (20, 43, 62). This indicates that the intracellular concentration of cAMP can be manipulated by altering the amount of cAMP added to the medium. Various experimental observations suggest that an exogenous cAMP concentration of 0.5-1.0 mM restores intracellular cAMP to a physiologically functional level in adenylate cyclase-deficient, cAMP phosphodiesterase-proficient (i.e. Δ*cyaA*, *cpd*$^+$) strains, whereas addition of 5-10 mM cAMP to the culture medium results in higher than normal intracellular concentrations of cAMP (43, 83).

Therefore, to examine a possible role for the intracellular cAMP concentration in influencing regulation of P_{guaB}, we measured the activity of P_{guaB} (-133 to +36) in response to growth rate in a Δ*cyaA* strain growing in the absence and presence of 5 mM cAMP.

As expected, the presence of the Δ*cyaA* allele resulted in an increase in P_{guaB} activity at low growth rates relative to the wild-type strain background, and this abolished the positive correlation between P_{guaB} activity and the growth rate (Fig. 6A). Deletion of the *cyaA* gene also caused a reduction in the growth rates, as observed previously (1, 15, 22, 55). Addition of 5mM cAMP to media supporting the lowest growth rates caused Δ*cyaA* bacteria to grow faster than in cAMP-free media, whereas addition to media supporting the highest growth rates resulted in a decrease in the growth rate relative to cAMP-free media (similar observations have been reported previously (38, 43, 66, 83)). This gave rise to a more restricted range of growth rates (Fig. 6B). Despite the decreased range of growth rates, it is apparent that the relationship between the growth rate and P_{guaB} activity is similar to that observed in wild type cells growing in the absence of added cAMP (compare Fig. 6B with Fig. 5D). This observation suggests that although the CRP.cAMP complex is required for repression of P_{guaB}, and GRDC, variation in the intracellular concentration of cAMP in response to the prevailing carbon source is unlikely to dictate GRDC of this promoter.

cAMP did not restore normal GRDC to the P_{guaB} (-133 to +36, G-122C) promoter, which lacked a functional CRP site, nor did it result in repression of this promoter at low growth rates (compare Fig. 6C and 6D). Plots of relative activity of the wild type and mutant *guaB* promoters in response to cAMP clearly show that the mutant

promoter is essentially unresponsive to cAMP (compare Supplementary Fig. 3A and 3B). These results confirm that the cAMP-dependent effects observed at P_{guaB} (-133 to +36) are mediated by CRP binding to the P_{guaB} CRP site.

Effect of CRP on P_{guaB} activity *in vitro*

Disruption of CRP binding to P_{guaB} results in derepression of P_{guaB} activity in medium supporting low growth rates and concomitantly abolishes GRDC. To determine whether CRP.cAMP is able to regulate P_{guaB} in the absence of other factors, transcription was measured *in vitro* from P_{guaB} (-133 to +36), in the presence and absence of purified CRP.cAMP. Results from multiple-round transcription assays show that P_{guaB} (-133 to +36) activity was not influenced by CRP.cAMP in the absence of other regulatory factors (Fig. 7). Varying the concentrations of the transcription initiating nucleotide (GTP), salt (KCl) or CRP did not enhance the responsiveness of P_{guaB} to CRP in multiple- or single-round reactions (results not shown). In control experiments, CRP.cAMP was able to activate transcription ~10-fold from the CRP-dependent *CC(-41.5)* promoter under the same conditions (Fig. 7). The impotence of CRP.cAMP in the *in vitro* system suggests that an additional factor is required for CRP-dependent downregulation of P_{guaB}.

Role of PurR in the regulation of P_{guaB}

A putative binding site for the regulatory protein, PurR, overlaps the *guaB* promoter (Fig. 1) (31, 48). Moreover, the activity of the *guaB* gene product, IMP dehydrogenase, in bacteria growing under conditions of purine repression is 3-fold higher in strains harbouring a null mutation in the gene encoding the PurR repressor (48). To assess the role of the PurR repressor in GRDC of the *guaB* promoter, we first

confirmed that P_{guaB} activity is repressed by this regulator. Thus, purine-mediated repression of P_{guaB} (-253 to +36), P_{guaB} (-37 to +36) or P_{guaB} (-253 to +10) were assessed *in vivo*. All three promoters were shown to be repressed by ~50% in the presence of high levels of exogenously added guanine, and this repression was entirely dependent on the presence of a functional *purR* gene (Fig. 8A and 8B). This result confirms that transcription of the *guaBA* operon is repressed by PurR and it is consistent with the predicted location of the PurR binding site. The activity of P_{guaB} (-253 to +36) in response to growth rate was then measured in the *purR* mutant background. The results show that there was no significant difference in GRDC of P_{guaB} in the presence or absence of PurR (Fig. 8C and 8D).

DISCUSSION

In this study, we have demonstrated that the CRP.cAMP complex binds to a site centred at position -117.5 relative to the P_{guaB} transcription start site, and we have shown that this interaction serves to downregulate P_{guaB}. Moreover, the degree of CRP-mediated repression progressively increases as the bacterial growth rate decreases. Thus, in a *crp* or *cya* mutant strain, or in a strain in which the P_{guaB} CRP site has been inactivated, P_{guaB} exhibits a marked increase in activity at low growth rates. As glucose supports relatively high growth rates, these results are consistent with a previous transcriptomic analysis in which it was observed that *guaB* transcription is subject to "CRP-dependent glucose activation" (26). However, our observation is not in agreement with a previous proposal that CRP activates P_{guaB} (35). The latter proposal was based on nutrient downshift experiments in which cells

were transferred, after washing, from a complex nutrient-rich medium to a glycerol-based medium containing casamino acids. In these experiments, the authors observed a rapid but short-lived increase in P_{guaB} activity following the downshift. This did not occur in a Δ*crp* strain. These experiments are difficult to interpret because the nutrient-rich medium also contains a source of purines, so cells are having to readjust not only to a change in carbon source, but also to purine availability. To avoid the possibility that purine repression would complicate interpretation of our results, we avoided use of media containing purines when examining the effect of media supporting different growth rates on P_{guaB} activity. In addition, our measurements were performed during steady state growth of the bacteria.

As the supply of guanine nucleotides must satisfy the demand for rRNA and tRNA biosynthesis, which are themselves subject to very tight growth rate-dependent control and constitute as much as 85% of the total RNA synthesised at high growth rates, it is not surprising that transcription of the *guaBA* operon should be subject to a growth rate-dependent control mechanism (10, 16, 33). However, the observed inverse relationship between CRP-mediated repression of P_{guaB} and the bacterial growth rate prompted us to reexamine whether P_{guaB} was in fact regulated according to the carbon source rather than as a function of the growth rate. Promoters that are subject to GRDC exhibit the same activity in cells growing at the same growth rate, irrespective of the nature of the carbon source. Supplementary Fig. 4 shows the activity of P_{guaB} in the presence of different carbon sources. As some of the carbon sources used give rise to a similar growth rate and P_{guaB} activity, it is clear that the activity of this promoter is influenced by the growth rate rather than the carbon source.

Our results also suggest that *E. coli* makes use of a mechanism that is distinct from that acting at stable RNA promoters, to confer growth rate dependence on P_{guaB}. Unlike the situation at rRNA and tRNA promoters, the mechanism at P_{guaB} employs CRP, and requires the participation of the UP element, thereby implying a role for αCTD in this process (33). However, our results do not suggest that GRDC of P_{guaB} is dictated by the intracellular concentration of the CRP.cAMP complex. Thus, manipulating the intracellular cAMP or CRP levels so that they remain constant at different growth rates does not abolish GRDC. This observation suggests the involvement of another factor that dictates GRDC of P_{guaB}. Furthermore, our *in vitro* results also suggest that at least one other factor is required for CRP-mediated repression of P_{guaB}. This is consistent with a model in which CRP-mediated repression and GRDC are facets of the same mechanism that requires at least one other regulatory factor.

How might binding of the CRP.cAMP complex to a site far upstream of the P_{guaB} core promoter region repress transcription? There are no known examples where CRP, acting alone or in concert with an UP element, modulates transcription from a site located more than 100 bp upstream of the transcription start site. However, there are examples where CRP bound at such distances has been shown to contact RNAP and regulate transcription at promoters that require the participation of additional regulatory proteins (i.e. Class III CRP-dependent promoters). For example, CRP can contact αCTD when bound at position -102.5/-103.5 at synthetic Class III promoters (8, 72). Moreover, at *acs*P2, a naturally occurring Class III promoter, the promoter-distal CRP site (CRP II) is centred at position -122.5, and CRP bound to this site can interact with αCTD (6). However, the CRP site at P_{guaB} is located on the opposite face

of the DNA helix in comparison to the CRP sites at these Class III promoters. Therefore, it is not clear that CRP would be in a position to interact with RNAP at P_{guaB}. Moreover, unlike the examples discussed above, the role of CRP at P_{guaB} is to downregulate transcription. One possibility is that CRP (with the aid of an unknown regulatory factor) competes with the UP element for αCTD, thereby recruiting αCTD to a location that is unfavourable for transcription from P_{guaB}. However, we demonstrated that GRDC of P_{guaB} was not altered significantly in strains producing AR1-defective CRP, and an alanine scanning analysis of αCTD did not implicate the AR1 contact site on αCTD (i.e. the 287 determinant) in regulation of this promoter (32, 63). In addition, the AR1 substitution exerted a repressive effect on P_{guaB}, something that would not be expected if the AR1-αCTD interaction was required to downregulate P_{guaB}. Furthermore, displacement of the CRP site from its native position by one turn of the DNA helix in either direction completely abolishes GRDC at P_{guaB}. At many promoters where CRP binding to upstream sites serves to regulate transcription by contacting αCTD, displacement of the CRP site by one helical turn from its optimal position does not completely abolish CRP activity (8, 12, 24, 44, 72). Thus, it is unlikely that direct interactions between CRP and RNAP play a role in GRDC of P_{guaB}.

An alternative possibility is that the 'missing factor' contacts RNAP at P_{guaB} in a manner that requires CRP. For example, it has been demonstrated that at some CRP-regulated promoters CRP can participate in cooperative interactions with other regulatory proteins that activate or repress transcription (14, 53, 79). Thus, at P_{guaB}, CRP could serve as a nucleation site for the assembly of a complex of transcription factors on the DNA, or it could allow remodelling of such an assembly, that sterically

blocks access to the UP element by αCTD. Steric hindrance of UP element utilisation as a regulatory mechanism has been observed previously for LexA at the P_{rstA} promoter of phage CTXΦ (58). Our results suggest that such a mechanism would not be dependent upon interactions between AR1 and the other regulatory protein(s), as has been observed at other promoters (47, 79). The previous observation that maximum *guaB* promoter activity can be achieved in the absence of sequences located upstream of -59 is inconsistent with a mechanism whereby CRP occludes an adjacent or overlapping binding site for a transcription factor that activates P_{guaB} (33).

Putative binding sites for the regulatory proteins DnaA and PurR have been identified within the core region of the *guaB* promoter (16, 31, 74). PurR mediates repression of genes required for the biosynthesis of purines in the presence of the corepressors guanine or hypoxanthine (49). It has been shown that the presence of guanine in the culture medium results in a similar degree of repression of P_{guaB} at all growth rates, and a *purR* mutation results in increased activity of the *guaB* gene product, IMP dehydrogenase, suggesting that PurR regulates P_{guaB} (16, 48). However, while confirming that PurR is responsible for guanine-mediated repression of P_{guaB}, our results rule out a role for PurR in GRDC at this promoter. Regarding DnaA, a second binding site for this protein, located in the *guaB* coding region, is also required for DnaA-mediated repression of P_{guaB} (73, 74). As the promoters used for assessment of GRDC in the current study contained only the upstream DnaA binding site, which does not serve to repress P_{guaB} in the absence of the downstream site, this argues against a role for DnaA in GRDC. Thus, the missing factor is unlikely to be PurR or DnaA.

The expression of many genes, including those encoding components of the translation apparatus, the RNAP-associated protein RapA, and Dam methylase, is subject to positive GRDC, a phenomenon characterised by an increase in the gene product/bacterial mass ratio as the growth rate increases (13, 19, 57, 60, 78). However, the responsiveness of individual growth rate-regulated promoters to the growth rate varies. Accordingly, there appear to be diverse mechanisms for implementing GRDC, and in some cases it is exerted at a post-transcriptional level (5, 13, 19, 57, 65). However, a complete understanding of the mechanism of GRDC at any single promoter remains elusive. In conclusion, our results implicate a role for the CRP.cAMP complex in GRDC of P_{guaB}, and highlights the diversity of mechanisms that can serve to modulate gene expression in response to growth rate.

ACKNOWLEDGMENTS

We are indebted to W. Ross, T. Gaal, H. Murray and R. L. Gourse (University of Wisconsin-Madison), and D. Browning, D. Grainger and D. Lee (University of Birmingham) for purified CRP protein, strains, plasmids and helpful discussions. We also thank H. Aiba (Nagoya University) for anti-CRP antiserum, strains and plasmids. This work was supported by a project grant awarded by the Wellcome Trust (grant number 073917) and a family Ph.D. sponsorship awarded to S.I.H., kindly provided by S. M. Husnain.

REFERENCES

1. **Ahmad, D., and E. B. Newman.** 1988. A deficiency in cyclic AMP results in pH-sensitive growth of *Escherichia coli* K-12. J. Bacteriol. **170:**3443-3447.

2. **Aiba, H.** 1983. Autoregulation of the *Escherichia coli crp* gene: CRP is a transcriptional repressor for its own gene. Cell **32:**141-149.

3. **Aiba, H., S. Fujimoto, and N. Ozaki.** 1982. Molecular cloning and nucleotide sequencing of the gene for *E. coli* cAMP receptor protein. Nucleic Acids Res. **10:**1345-1361.

4. **Alting-Mees, M. A., and J. M. Short.** 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. **17:**9494.

5. **Baker, H. V., and R. E. Wolf.** 1983. Growth rate-dependent regulation of 6-phosphogluconate dehydrogenase level in *Escherichia coli* K-12: β-galactosidase expression in *gnd-lac* operon fusion strains. J. Bacteriol. **153:**771-781.

6. **Beatty, C. M., D. F. Browning, S. J. W. Busby, and A. J. Wolfe.** 2003. CRP-dependent activation of the *Escherichia coli acs*P2 promoter by a synergistic class III mechanism. J. Bacteriol. **185:**5148-5157.

7. **Bell, A., K. Gaston, R. Williams, K. Chapman, A. Kolb,H. Buc, S. Minchin, J. Williams, and S. Busby.** 1990. Mutations that alter the ability of the *Escherichia coli* cyclic AMP receptor protein to activate transcription. Nucleic Acids Res. **18:**7243-7250.

8. **Belyaeva, T. A., V. A. Rhodius, C. L. Webster, and S. J. W. Busby.** 1998. Transcription activation at promoters carrying tandem DNA sites for the *Escherichia coli* cyclic AMP receptor protein: organisation of the RNA polymerase α subunits. J. Mol. Biol. **277:**789-804.

9. **Berg, O. G., and von P. H. Hippel.** 1988. Selection of DNA binding sites by regulatory proteins. II. The binding specificity of cyclic AMP receptor protein to recognition sites. J. Mol. Biol. **200:**709-723.

10. **Bremer, H., and P. P. Dennis.** 1996. Modulation of chemical composition and other parameters of the cell by growth rate, p.1553-1569. *In* F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger (eds.), Escherichia coli and Salmonella: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
11. **Busby, S., and R. H. Ebright.** 1999. Transcription activation by catabolite activator protein (CAP). J. Mol. Biol. **293:**199-213.
12. **Busby, S., D. West, M. Lawes, C. Webster, A. Ishihama, and A. Kolb.** 1994. Transcription activation by the *Escherichia coli* cyclic AMP receptor protein. Receptors bound in tandem at promoters can interact synergistically. J. Mol. Biol. **241:**341-352.
13. **Cabrera, J. E., and D. J. Jin.** 2001. Growth phase and growth rate regulation of the *rapA* gene, encoding the RNA polymerase-associated protein RapA in *Escherichia coli*. J. Bacteriol. **183:**6126-6134.
14. **Chahla, M., J. Wooll, T. Laue, N. Nguyen, and D. Senear.** 2003. Role of protein-protein bridging interactions on cooperative assembly of DNA-bound CRP-CytR-CRP complex and regulation of the *Escherichia coli* CytR regulon. Biochemistry **42:**3812-3825.
15. **D'ari, R., A. Jaffe, P. Bouloc, and A. Robin.** 1988. Cyclic AMP and cell division in *Escherichia coli*. J. Bacteriol. **170:**65-70.
16. **Davies, I. J., and W. T. Drabble.** 1996. Stringent and growth-rate-dependent control of the *gua* operon of *Escherichia coli* K-12. Microbiology **142:**2429-2437.

17. **Dennis, P. P., M. Ehrenber, and H. Bremer.** 2004. Control of rRNA synthesis in *Escherichia coli*: a systems biology approach. Microbiol. Mol. Biol. Rev. **68:**639-668.
18. **De Robertis, Jr., E. M., N. D. Judewicz, and H. N. Torres.** 1973. On the control mechanism of bacterial growth by cyclic adenosine 3', 5'-monophosphate. Biochem. Biophys. Res. Comm. **55:**758-764.
19. **Dong, H., L. A. Kirsebom, and L. Nilsson.** 1996. Growth rate regulation of 4.5 S RNA and M1 RNA the catalytic subunit of *Escherichia coli* RNase P. J. Mol. Biol. **261:**303-308.
20. **Epstein, W., L. B. Rothman-Denes, and J. Hesse.** 1975. Adenosine 3':5'-cyclic monophosphate as mediator of catabolite repression in *Escherichia coli*. Proc. Natl. Acad. Sci., USA **72:**2300-2304.
21. **Estrem, S. T., T. Gaal, W. Ross, and R. L. Gourse.** 1998. Identification of an UP element consensus sequence for bacterial promoters. Proc. Natl. Acad. Sci. USA **95:**9761-9766.
22. **Ezzel, J. W., and W. Dobrogosz.** 1975. Altered hexose transport and salt sensitivity in cyclic adenosine 3',5'-monophosphate-deficient *Escherichia coli*. J. Bacteriol. **124:**815-824.
23. **Gaal, T., M. S. Bartlett, W. Ross, C. L. Turnbough, Jr., and R. L. Gourse.** 1997. Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science **278:**2092-2097.
24. **Gaston, K., A. Bell, A. Kolb, H. Buc, and S. Busby.** 1990. Stringent spacing requirements for transcription activation by CRP. Cell **62:**733-743.

25. **Gonzalez-Gil, G., R. Kahmann, and G. Muskhelishvili.** 1998. Regulation of *crp* transcription by oscillation between distinct nucleoprotein complexes. EMBO J. **17:**2877-2885.

26. **Gosset, G., Z. Zhang, S. Nayyar, W. A. Cuevas, and M. H. Saier, Jr.** 2004. Transcriptome analysis of Crp-dependent catabolite control of gene expression in *Escherichia coli*. J. Bacteriol. **186:**3516-3524.

27. **Gourse, R. L., T. Gaal, M. S. Bartlett, J. A. Appleman, and W. Ross.** 1996. rRNA transcription and growth rate-dependent regulation of ribosome synthesis in *Escherichia coli*. Annu. Rev. Microbiol. **50:**645-677.

28. **Gourse, R. L., W. Ross, and T. Gaal.** 2000. UPs and downs in bacterial transcription initiation: the role of the alpha subunit of RNA polymerase in promoter recognition. Mol. Microbiol. **37:**687-695.

29. **Grainger, D. C., D. Hurd, M. Harrison, J. Holdstock, and S. J. Busby.** 2005. Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. Proc. Natl. Acad. Sci. USA **102:**17693-17698.

30. **Gunasekera, A., Y. W. Ebright, and R. H. Ebright.** 1992. DNA sequence determinants for binding of the *Escherichia coli* catabolite gene activator protein. J. Biol. Chem. **267:**14713-14720.

31. **He, B., K. Y. Choi, and H. Zalkin.** 1993. Regulation of *Escherichia coli glnB, prsA,* and *speA* by the purine repressor. J. Bacteriol. **175:**3598-3606.

32. **Husnain, S. I.** 2005. Ph.D. thesis, University of Sheffield.

33. **Husnain, S. I., and M. S. Thomas.** 2008a. The UP element is necessary but not sufficient for growth rate-dependent control of the *Escherichia coli guaB* promoter. J. Bacteriol. **190:**2450-2457.

34. **Husnain, S. I., and M. S. Thomas.** 2008b. Downregulation of the *Escherichia coli guaB* promoter by FIS. Microbiology **154:**1729-1738.
35. **Hutchings, M. I., and W. T. Drabble.** 2000. Regulation of the divergent *guaBA* and *xseA* promoters of *Escherichia coli* by the cyclic AMP receptor protein. FEMS Microbiol. Lett. **187:**115-122.
36. **Ishizuka, H., A. Hanamura, T. Inada, and H. Aiba.** 1994. Mechanism of the down-regulation of cAMP receptor protein by glucose in *Escherichia coli*: role of autoregulation of the *crp* gene. EMBO J. **13:**3077-3082.
37. **Ishizuka, H., A. Hanamura, T. Kunimura, and H. Aiba.** 1993. A lowered concentration of cAMP receptor protein caused by glucose is an important determinant for catabolite repression in *Escherichia coli*. Mol. Microbiol. **10:**341-350.
38. **Judewicz, N. D., E. M. De Robertis Jr., and H. N. Torres.** 1973. Inhibition of *Escherichia coli* growth by cyclic adenosine 3',5'-monophosphate. Biochem. Biophys. Res. Commun. **52:**1257-1262.
39. **Kilstrup, M., L. M. Meng, J. Neuhard, and P. Nygaard.** 1989. Genetic evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in *Escherichia coli*. J. Bacteriol. **171:**2124-2127.
40. **Kim, J., S. Adhya, and S. Garges.** 1992. Allosteric changes in the cAMP receptor protein of *Escherichia coli*: hinge reorientation. Proc. Natl. Acad. Sci. USA **89:**9700-9704.
41. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749-795.

42. **Kolb, A., D. Kotlarz, S. Kusan, and A. Ishihama.** 1995. Selectivity of the *Escherichia coli* RNA polymerase $E\sigma^{38}$ for overlapping promoters and ability to support CRP activation. Nucleic Acids Res. **23:**819-826.

43. **Kuhlman, T., Z. Zhang, M. H. Saier Jr., and T. Hwa.** 2007. Combinatorial transcriptional control of the lactose operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **104:**6043-6048.

44. **Law, E. C., N. J. Savery, and S. J. W. Busby.** 1999. Interactions between the *Escherichia coli* cAMP receptor protein and the C-terminal domain of the α subunit of RNA polymerase at Class I promoters. Biochem. J. **337:**415-423.

45. **Lawson, C. L., D. Swigon, K. Murakami, S. A. Darst, H. M. Berman, and R. H. Ebright.** 2004. Catabolite activator protein: DNA binding and transcription activation. Curr. Opin. Struct. Biol. **14:**10-20.

46. **Mehra, R. K., and W. T. Drabble.** 1981. Dual control of the *gua* operon of *Escherichia coli* K12 by adenine and guanine nucleotides. J. Gen. Microbiol. **123:**27-37.

47. **Meibom, K. L., L. Søgaard-Andersen, A. S. Mironov, and P. Valentin-Hansen.** 1999. Dissection of a surface-exposed portion of the cAMP-CRP complex that mediates transcription activation and repression. Mol. Microbiol. **32**:497-504.

48. **Meng, L. M., M. Kilstrup, and P. Nygaard.** 1990. Autoregulation of PurR repressor synthesis and involvement of *purR* in the regulation of *purB*, *purC*, *purL*, *purMN* and *guaBA* expression in *Escherichia coli*. Eur. J. Biochem. **187:**373-379.

49. **Meng, L. M., and P. Nygaard.** 1990. Identification of hypoxanthine and guanine as the co-repressor for the purine regulon genes of *Escherichia coli*. Mol. Microbiol. **4:**2187-2182.

50. **Meng, W., N. J. Savery, S. J. W. Busby, and M. S. Thomas.** 2000. The *Escherichia coli* RNA polymerase α subunit linker: length requirements for transcription activation at CRP-dependent promoters. EMBO J. **19:**1555-1566.

51. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork.

52. **Mori, K., and H.Aiba.** 1985. Evidence for negative control of *cya* transcription by cAMP and cAMP receptor protein in intact *Escherichia coli* cells. J. Biol. Chem. **260:**14838-14843.

53. **Nasser, W., R. Schneider, A. Travers, and G. Muskhelishvili.** 2001. CRP modulates fis transcription by alternate formation of activating and repressing nucleoprotein complexes. J. Biol. Chem. **276:**17878-17886.

54. **Okamoto, K., S. Hara, R. Bhasin, and M. Freundlich.** 1988. Evidence *in vivo* for autogenous control of the cyclic AMP receptor protein gene (*crp*) in *Escherichia coli* by divergent RNA. J. Bacteriol. **170:**5076-5079.

55. **Perlman, R. L., and I. Pastan.** 1969. Pleiotropic deficiency of carbohydrate utilization in an adenyl cyclase deficient mutant of *Escherichia coli*. *Biochem.* Biophys. Res. Comm. **37:**151-157.

56. **Perrenoud, A., and U. Sauer.** 2005. Impact of global transcriptional regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on glucose catabolism in *Escherichia coli*. J. Bacteriol. **187:**3171-3179.

57. **Pokholok, D. K., M. Redlak, C. L. Turnbough, Jr., S. Dylla, and W. M. Holmes.** 1999. Multiple mechanisms are used for growth rate and stringent

control of *leuV* transcriptional initiation in *Escherichia coli*. J. Bacteriol. **181:**5771-5782.

58. **Quinones, M., H. K. Kimsey, W. Ross, R. L. Gourse, and M. K. Waldor.** 2006. LexA represses CTXΦ transcription by blocking access of the α C-terminal domain of RNA polymerase to promoter DNA. J. Biol. Chem. **281:**39407-39412.

59. **Rao, L., W. Ross, J. A. Appleman, T. Gaal, S. Leirmo, P. J. Schlax, M. T. Record Jr., and R. L. Gourse**. 1994. Factor independent activation of *rrnB* P1. An "extended" promoter with an upstream element that dramatically increases promoter strength. J. Mol. Biol. **235:**1421-1435.

60. **Rasmussen, L. J., A. Lobner-Olesen, and M. G. Marinus.** 1995. Growth-rate-dependent transcription initiation from the *dam* P2 promoter. Gene **157:**213-215.

61. **Ross, W., J. F. Thompson, J. T. Newlands, and R. L. Gourse.** 1990. *E.coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. EMBO J. **9:**3733-3742.

62. **Sa, J. H., M. A. Namgung, C. J. Lim, and J. A. Fuchs.** 1997. Expression of the *Escherichia coli* thioredoxin gene is negatively regulated by cyclic AMP. Biochem. Biophys. Res. Commun. **234:**564-567.

63. **Savery, N. J., G. S. Lloyd, S. J. W. Busby, M. S. Thomas, R. H. Ebright, and R. L. Gourse.** 2002. Determinants of the C-terminal domain of the *Escherichia coli* RNA polymerase alpha subunit important for transcription at class I cyclic AMP receptor protein-dependent promoters. J. Bacteriol. **184:**2273-2280.

64. **Savery, N. J., G. S. Lloyd, M. Kainz, T. Gaal, W. Ross, R. H. Ebright, R. L. Gourse, and S. J. W. Busby.** 1998. Transcription activation at Class II CRP-dependent promoters: identification of determinants in the C-terminal domain of the RNA polymerase α subunit. EMBO J. **12:**3439-3447.

65. **Schneider, D. A., W. Ross, and R. L. Gourse.** 2003. Control of rRNA expression in *Escherichia coli*. Curr. Opin. Microbiol. **6:**151-156.
66. **Setty, Y., A. E. Mayo, M. G. Surette, and U. Alon.** 2003. Detailed map of a cis-regulatory input function. Proc. Natl. Acad. Sci. USA **100:**7702-7707.
67. **Shah, S., and A. Peterkofsky.** 1991. Characterisation and generation of *Escherichia coli* adenylate cyclase deletion mutants. J. Bacteriol. **173:**3238-3242.
68. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85-96.
69. **Spassky, A., S. Busby, and H. Buc.** 1984. On the action of cyclic AMP-cyclic AMP receptor protein complex at the *E. coli* lactose and galactose promoter regions. EMBO J. **3:**43-50.
70. **Spibey, N., and W. T. Drabble.** 1981. Construction and characterization of *guaB-lacZ* fusions in *Escherichia coli* K12. J. Gen. Microbiol. **126:**497-501.
71. **Srivatsan, A., and J. D. Wang.** 2008. Control of bacterial transcription, translation and replication by (p)ppGpp. Curr. Opin. Microbiol. **11:**100-105.
72. **Tebbutt, J., V. A. Rhodius, C. L. Webster, and S. J. W. Busby.** 2002. Architectural requirements for optimal activation by tandem CRP molecules at a class I CRP-dependent promoter. FEMS Microbiol. Lett. **210:**55-60.
73. **Tesfa-Selase, F., and W. T. Drabble.** 1992. Regulation of the *gua* operon of *Escherichia coli* by the DnaA protein. Mol. Gen. Genet. **231:**256-264.
74. **Tesfa-Selase, F., and W. T. Drabble.** 1996. Specific binding of DnaA protein to a DnaA box in the *guaB* gene of *Escherichia coli* K12. Eur. J. Biochem. **241:**411-416.

75. **Tiedeman, A. A., and J. M. Smith.** 1984. Isolation and characterization of regulatory mutations affecting the expression of the *guaBA* operon of *Escherichia coli* K-12. Mol. Gen. Genet. **195:**77-82.

76. **Ushida, C., and H. Aiba.** 1990. Helical phase dependent action of CRP: effect of the distance between the CRP site and the -35 region on promoter activity. Nucleic Acids Res. **18:**6325-6330.

77. **Valentin-Hansen, P., L. Sogaard-Andersen, and H. Pedersen.** 1996. A flexible partnership: the CytR anti-activator and the cAMP-CRP activator protein, comrades in transcription control. Mol. Microbiol. **20:**461-466.

78. **Van Delft, J. H., H. M. Verbeek, P. J. De Jong, D. S. Schmidt, A. Talens, and L. Bosch.** 1988. Control of the tRNA-*tufB* operon in *Escherichia coli* 1. rRNA gene dosage effects and growth-rate-dependent regulation. Eur. J. Biochem. **175:**355-362.

79. **Wade, J. T., T. A. Belyaeva, E. I. Hyde, and S. J. W. Busby.** 2001. A simple mechanism for co-dependence on two activators at an *Escherichia coli* promoter. EMBO J. **20:**7160-7167.

80. **Wanner, B. L., R. Kodaira, and F. C. Neidhardt.** 1977. Physiological regulation of a decontrolled *lac* operon. J. Bacteriol. **130:**212-222.

81. **West, D., R. Williams, V. Rhodius, A. Bell, N. Sharma, C. Zou, N. Fujita, A. Ishihama, and S. Busby.** 1993. Interactions between the *Escherichia coli* cyclic AMP receptor protein and RNA polymerase at class II promoters. Mol. Microbiol. **10:**789-797.

82. **Wright, L. F., and C. J. Knowles.** 1977. The interrelated effects of cyclic AMP and growth rate on the respiratory system and glycolytic enzyme synthesis of *Escherichia coli* K-12. FEMS Microbiol. Lett. **1:**259-263.

83. **Wright, J. M., and S. M. Boyle.** 1982. Negative control of ornithine decarboxylase and arginine decarboxylase by adenosine-3',5'-cyclic monophosphate in *Escherichia coli*. Mol. Gen. Genet. **186:**482-487.

84. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

85. **Zheng, D., C. Constantinidou, J. L. Hobman, and S. D. Minchin.** 2004. Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. Nucleic Acids Res. **32:**5874-5893.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/Description[a]	Promoter designation[b]	Source or reference
Bacterial strains			
DM0068	MG1655*Δcrp::cat*	-	40
SØ5052	*thi⁻ lacZ608 purR6::*Tn*10 rpsL999*	-	39
SP850	*E14- relA1 spoT1 Δcya1400::kan thi-1*	-	67
VH1000	MG1655 *lacZ lacI pyrE*$^+$	-	23
VH1000G-253	VH1000/λ [P_{guaB} (-253 to +36)]-*lacZ*	P_{guaB} (-253 to +36)	34
VH1000G-253*ΔpurR*	*purR6::*Tn*10* derivative of VH1000G-253	P_{guaB} (-253 to +36)	This study
VH1000G-25310	VH1000/λ [P_{guaB} (-253 to +10)]-*lacZ*	P_{guaB} (-253 to +10)	27
VH1000G-25310*ΔpurR*	*pur6::*Tn*10* derivative of VH1000G-253	P_{guaB} (-253 to +10)	This study
VH1000G-133	VH1000/λ [P_{guaB} (-133 to +36)]-*lacZ*	P_{guaB} (-133 to +36)	34
VH1000G-133G7C	VH1000/λ [P_{guaB} (-133 to +36, G-122C)]-*lacZ*	P_{guaB} (-133 to +36, G-122C)	This study
VH1000G-133A18C	VH1000/λ [P_{guaB} (-133 to +36, A-111C)]-*lacZ*	P_{guaB} (-133 to +36, A-111C)	This study
VH1000G-133CRPUP	VH1000/λ harbouring P_{guaB} (-133 to +36)-*lacZ*, with CRP site displaced 11 base pairs further upstream[c,d]	P_{guaB} (CRP -128.5)	This study
VH1000G-133CRPDN	VH1000/λ harbouring P_{guaB} (-133 to +36)-*lacZ*, with CRP site displaced 11 base pairs further downstream[d]	P_{guaB} (CRP -106.5)	This study
VH1000G-133*Δcrp*	*crp::cat* derivative of VH1000G-133	P_{guaB} (-133 to +36)	This study
VH1000G-133*Δcya*	*cya1400::kan* derivative of VH1000G-133	P_{guaB} (-133 to +36)	This study
VH1000G-133G7C*Δcya*	*cya1400::kan* derivative of VH1000G-133G7C	P_{guaB} (-133 to +36, G-122C)	This study
VH1000G-117	VH1000/λ [P_{guaB} (-117 to +36)]-*lacZ*	P_{guaB} (-117 to +36)	This study
VH1000G-37	VH1000/λ [P_{guaB} (-37 to +36)]-*lacZ*	P_{guaB} (-37 to +36)	34
VH1000G-37*ΔpurR*	*pur6::*Tn*10* derivative of VH1000G-37	P_{guaB} (-37 to +36)	This study
Plasmids			
pBluescript II KS	*E. coli*-specific cloning vector (Ap^R)	-	4
pUC19	*E. coli*-specific cloning vector (Ap^R)	-	84

pMSB1	Derivative of promoter cloning plasmid, pRS1553, for construction of *lacZ* fusions (Ap^R)		-	59
pRLG770	Derivative of pKM2 for *in vitro* transcription reactions (Ap^R)		-	61
pSR	pBR322 derivative for *in vitro* transcription reactions (Ap^R)		-	42
pBSG-253	pBluescript II KS containing P_{guaB} (-253 to +36)			
pRLGgua-133	pRLG770 containing P_{guaB} (-133 to +36)	P_{guaB} (-133 to +36)		34
pSR/CC(-41.5)	pSR containing Class II CRP-dependent promoter CC(-41.5)	*CC(-41.5)*		64
pLG339ΔBS	pLG339 derivative containing *Bam*HI-*Eco*RI-*Sal*I linker (Km^R)		-	7
pLG339CRP	pLG339ΔBS derivative containing *crp*		-	7
pLG339CRP159L	pLG339ΔBS derivative containing *crpH159L*		-	81
pLG339CRP101E	pLG339ΔBS derivative containing *crpK101E*		-	12
pDCRP	pBR322 derivative carrying wild-type *crp* gene under the control of the *crp* promoter (Ap^R)		-	7
pDU9	Derivative of pDCRP with *crp* gene deleted		-	7
pHA7	pBR322 derivative carrying wild-type *crp* under the control of the *bla* promoter (Ap^R)		-	3

[a] Ap^R, ampicillin resistance. Km^R, kanamycin resistance. Architecture of promoters are shown within square brackets. SUB sequence is a DNA sequence that contributes minimally to transcription through contacts with αCTD (21).

[b] Designations of promoter derivatives referred to in the text are given, where appropriate. Coordinates are relative to the *guaB* transcription start site.

[c] Although derived from P_{guaB} (-133 to +36), upstream displacement of the CRP site in this promoter derivative means that native P_{guaB} sequences extend to position -117 and the upstream boundary of the CRP site is located 139 bp upstream of the transcription start site.

[d] Sequences corresponding to the original CRP upstream half-site in P_{guaB} (CRP -106.5) (positions -125 through to -121), or the original CRP downstream half-site in P_{guaB} (CRP -128.5) (positions -114 to -110) were inactivated by substitution with the sequence 5'-GACTG-3'. In each case, wild type P_{guaB} sequences flanking the inactivated half site and the repositioned CRP site were not altered.

FIGURE LEGENDS

FIG. 1. DNA sequence of the *E. coli guaB* promoter. The sequence of P_{guaB} from positions -180 to +40 relative to the *guaB* transcription start site is shown. A putative CRP binding site centred at position -117.5, and the UP element are shown in bold and underlined. The putative binding site for PurR centred at -24.5, and the discriminator are underlined. The core promoter elements (-35 and -10 regions) and the initiating nucleotide are shown in bold. The core binding motifs in the CRP site that contribute most strongly to the interaction with CRP are overlined (30). Positions of single base changes pertinent to this study are indicated by an 'X' below the substituted base, with the introduced base shown below the X. For clarity, binding sites for FIS and DnaA are not shown.

FIG. 2. Identification of the CRP binding site at P_{guaB} by DNase I footprinting. DNase I footprinting was performed using a DNA fragment containing P_{guaB} (-253 to +36) radiolabelled at the downstream end (relative to the *guaB* transcription start site) on the template strand. DNase I footprinting was performed in the presence or absence of CRP with or without cAMP, as indicated. The region protected by CRP is indicated by the grey bar to the right of the gel. A G+A DNA sequencing ladder was run alongside DNase I-treated samples. Nucleotide positions are shown relative to the *guaB* transcription start site.

FIG. 3. Requirement of the CRP site for GRDC of P_{guaB}. GRDC of (A) a wild type P_{guaB} derivative, with endpoints at -133 and +36, compared to (B) a derivative with an upstream endpoint at -117 (P_{guaB} (-117 to +36)), (C) a derivative with an inactivating G to C point mutation at position 7 of the CRP site (P_{guaB} (-133 to +36, G-122C)) or

(D) a derivative with an improved CRP site harbouring an A to C mutation at position 18 of the CRP site (P_{guaB} (-133 to +36, A-111C)). Strains harbouring fusions of these promoters to *lacZ* were grown at different growth rates using the 'standard media' set, whereupon the β-galactosidase activity was determined. The standard errors (not shown) were no more than 10% in each case. (E) CRP-DNA interactions at each of the above promoters was probed by EMSA. Promoter designations are as follows: -133, P_{guaB} (-133 to +36); -117, P_{guaB} (-117 to +36); G-122C, P_{guaB} (-133 to +36, G-122C); A-111C, P_{guaB} (-133 to +36, A-111C).

FIG. 4. Involvement of CRP in GRDC of P_{guaB}. The activity of P_{guaB} in response to growth rate was measured in a *crp* deletion strain harbouring a P_{guaB} (-133 to +36)-*lacZ* fusion and (A) a plasmid lacking a *crp* gene (pLG339ΔBS), or a plasmid encoding (B) wild type CRP (pLG339CRP), (C) CRPH159L (pLG339CRP159L) or (D) CRPK101E (pLG339CRP101E). Cells were grown using 'CRP media', whereupon the β-galactosidase activity was determined. The effect of the bacterial growth rate on the activity of P_{guaB} derivatives harbouring a CRP site displaced by (E) 11 bp downstream of the original location (P_{guaB} (CRP -106.5)), or (F) 11 bp upstream of the original location (P_{guaB} (CRP -128.5)), was determined in the wild type strain background (VH1000), using 'standard media'.

FIG. 5. Effect of CRP overproduction on GRDC of P_{guaB}. The activity of P_{guaB} (-133 to +36) was measured in a Δ*crp* strain containing (A) a plasmid that did not encode CRP (pDU9), or a plasmid expressing *crp* under the control of (B) the *crp* promoter (pDCRP) or (C) the *bla* promoter (pHA7), growing in 'CRP media', whereupon the β-galactosidase activity was determined. For comparison, GRDC of

the same P_{guaB} derivative was measured in a *crp*$^+$ strain (D), growing under the same conditions.

FIG. 6. Effect of exogenously added cAMP on GRDC of P_{guaB}. The effect of growth rate on the activity of P_{guaB} (-133 to +36) was measured in a Δ*cyaA* background, in (A) the absence or (B) presence of exogenously added cAMP (5 mM). In control experiments, the effect of growth rate on a promoter lacking a functional CRP site (i.e., P_{guaB} (-133 to +36, G-122C)) was assessed under identical conditions (panels C and D). Cells were grown at different cellular growth rates using 'CRP media', whereupon the β-galactosidase activity was determined.

FIG. 7. Effect of CRP on P_{guaB} activity *in vitro*. Multiple-round transcription reactions were performed on P_{guaB} (-133 to +36) and on the Class II CRP-dependent promoter, *CC(-41.5)*, using 10 nM RNAP holoenzyme in the presence or absence of 20 nM CRP and 200 μM cAMP, as indicated. Transcripts are indicated by the arrows shown on the left of the transcription gel. P_{guaB} activities were determined from the transcript abundance under each condition and are presented in a bar graph as the mean (with standard deviation) relative to P_{guaB} in the absence of CRP (activity = 1). The mean was calculated using data from three independent experiments.

FIG. 8. Role of PurR in regulation of P_{guaB}. The wild type *E. coli* strain (A) and an isogenic strain harbouring a deletion within the *purR* gene (B), each containing *lacZ* fusions to P_{guaB} (-37 to +36), P_{guaB} (-253 to +10), or P_{guaB} (-253 to +36), as indicated, were grown in M9 minimal medium with 0.2% (w/v) glucose and 5 μg/ml thiamine in the presence (grey bars) or absence (black bars) of 60 μg/ml guanine. At mid-

exponential growth phase the β-galactosidase activity was determined. Each bar represents the mean β-galactosidase activity (with standard deviation) in Miller units, and was calculated using data obtained from three independent experiments. The effect of growth rate on the activity of P_{guaB} (-253 to +36) was measured in the wild type strain (C) and in the *purR* mutant (D) growing in.'standard media'.