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# Characterization of the negative elements involved in silencing the *bgl* operon of *Escherichia coli*: possible roles for DNA gyrase, H-NS, and CRP–cAMP in regulation

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## Summary

**The *bgl* operon of *Escherichia coli* is rendered cryptic and uninducible in wild-type cells by the presence of DNA structural elements that negatively regulate transcription. We have carried out a detailed analysis of the sequences implicated in negative regulation. Fine-structure deletion analysis of the upstream sequences showed the presence of at least two elements involved in silencing the promoter. Chemical probing of genomic DNA *in vivo* showed that a region of dyad symmetry, present upstream of the promoter, is hypersensitive to $KMnO_4$. The hypersensitive region detected corresponds to the potential cruciform structure implicated earlier in negative regulation. Enhancement of transcription from the wild-type promoter, observed in the presence of the gyrase inhibitor novobiocin, was absent in a mutant that carried point mutations in the inverted repeat. This observation suggests that the activation seen in a gyrase mutant is mediated by destabilization of the cruciform because of reduced supercoiling. Deletion of sequences downstream of the potential cruciform also resulted in an increase in transcription, indicating the presence of a second regulatory element. Measurement of transcription from the *bgl* promoter carrying the deletion, in a strain that has a mutation in the *hns* gene, indicated that this region is likely to be involved in binding to H-NS or a protein regulated by H-NS, which acts as a non-specific repressor. We also provide evidence which suggests that transcriptional activation by mutations at the cAMP receptor protein (CRP)-binding site is mediated partly by antagonization of the negative effect of H-NS by CRP–cAMP as a result of its increased affinity for the mutant site.**

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## Introduction

In *Escherichia coli,* operons involved in metabolic functions are mostly regulated by operon-specific regulators. However, the role of chromosomal organization in regulation is becoming increasingly apparent in many bacterial systems. This is exemplified in the case of the silencing of the cryptic *bgl* operon of *E. coli*, which is involved in the uptake and metabolism of the aromatic β-glucosides salicin and arbutin (Prasad and Schaefler, 1974). The operon is transcriptionally inactivated in wild-type cells by negative elements present upstream of the promoter (Lopilato and Wright, 1990; Singh *et al*., 1995; Schnetz, 1995). A predominant class of spontaneous mutations that result in activation of the operon consists of insertion of IS*1* and IS*5* within a 233 bp region near the promoter (Reynolds *et al*., 1981; Schnetz and Rak, 1992). Point mutations that improve the CRP-binding site within the *bgl* promoter also result in activation (Reynolds *et al*., 1986; Lopilato and Wright, 1990). Mutations in *gyrA* and *gyrB* genes and the *hns* gene also lead to enhanced transcription from the *bgl* promoter (Di Nardo *et al*., 1982; Higgins *et al*., 1988). Recently, a mutation in the gene *bglJ* has been identified which results in the activation of the operon (Giel *et al*., 1996). Subsequent to activation, the operon is inducible by β-glucosides and is regulated by antitermination of transcription (Mahadevan and Wright, 1987; Schnetz and Rak, 1988; Houman *et al*., 1990; Amster-Choder and Wright, 1992).

Our previous analysis (Singh *et al*., 1995) showed that one of the negative elements involved in silencing the operon is an inverted repeat present upstream of the promoter, which can extrude into a cruciform under torsional stress. Deletions of the element and point mutations within it were shown to enhance transcription from the promoter. As cruciforms are known to be stabilized at higher negative superhelical densities, their existence *in vivo* has been a subject of debate because of reduced torsional stress and slow kinetics of extrusion under physiological conditions. We present evidence for the existence of the cruciform *in vivo*. Our results also suggest that the mutations in the gyrase genes activate transcription by destabilizing the cruciform.

The H-NS protein, which is a major constituent of the bacterial nucleoid, has been shown to be a prokaryotic equivalent of a nucleoid-structuring protein. Lesions in the *hns* gene result in alterations in the overall topology of DNA as well as derepression of a number of genes (Ussery *et al*., 1994). It is still unclear whether H-NS is a regulatory protein or has only a passive structural role in modulating gene expression. The mechanism of activation of the *bgl* operon by mutations in the *hns* gene is not well understood. As the operon is sensitive to supercoiling, it has been speculated that the activation mediated by mutations in the *hns* gene may be due to alterations in supercoiling. It is quite conceivable, however, that H-NS may be involved in silencing by direct binding to DNA, resulting in the occlusion of RNA polymerase from the promoter. The genetic data presented here suggest that H-NS or an H-NS-regulated protein may silence *bgl* expression by interacting with specific sequences within the upstream region implicated in silencing. This conclusion is supported by recent *in vitro* experiments which suggest a negative role for H-NS in *bgl* transcription (Schnetz and Wang, 1996; Timchenko *et al*., 1996).

The activating point mutations at the CRP-binding site result in a sequence that is closer to the consensus binding site. The CRP–cAMP complex has been shown to bind with a higher affinity to the mutant site *in vitro* (Reynolds *et al*., 1986). Our data suggest that the CRP–cAMP complex may mediate transcriptional activation at two levels, one by acting as a positive regulator of transcription and another as an antagoniser of H-NS or a protein regulated by H-NS.

## Results

### *Probing for secondary structures within the sequence upstream of the* bgl *promoter*

Earlier results from our laboratory indicated that one of the negative elements involved in the silencing of the *bgl* promoter is a region of dyad symmetry located between −148 and −114 (Fig. 1) which could extrude into a cruciform (Singh *et al*., 1995). For further confirmation of these observations, we used the chemical probe potassium permanganate ($KMnO_4$) to detect the presence of secondary structure within the *bglR* region. $KMnO_4$ is known to preferentially modify T and C residues in single-stranded DNA and has been successfully used for detecting sharply distorted DNA as well as DNA melted in an open complex *in vivo* (Sasse-Dwight and Gralla, 1991). Modification of the supercoiled plasmid carrying the wild-type promoter by $KMnO_4$ *in vitro,* followed by primer extension, showed the presence of a single-stranded region corresponding to the loop of the cruciform (Fig. 2A). The two adjacent

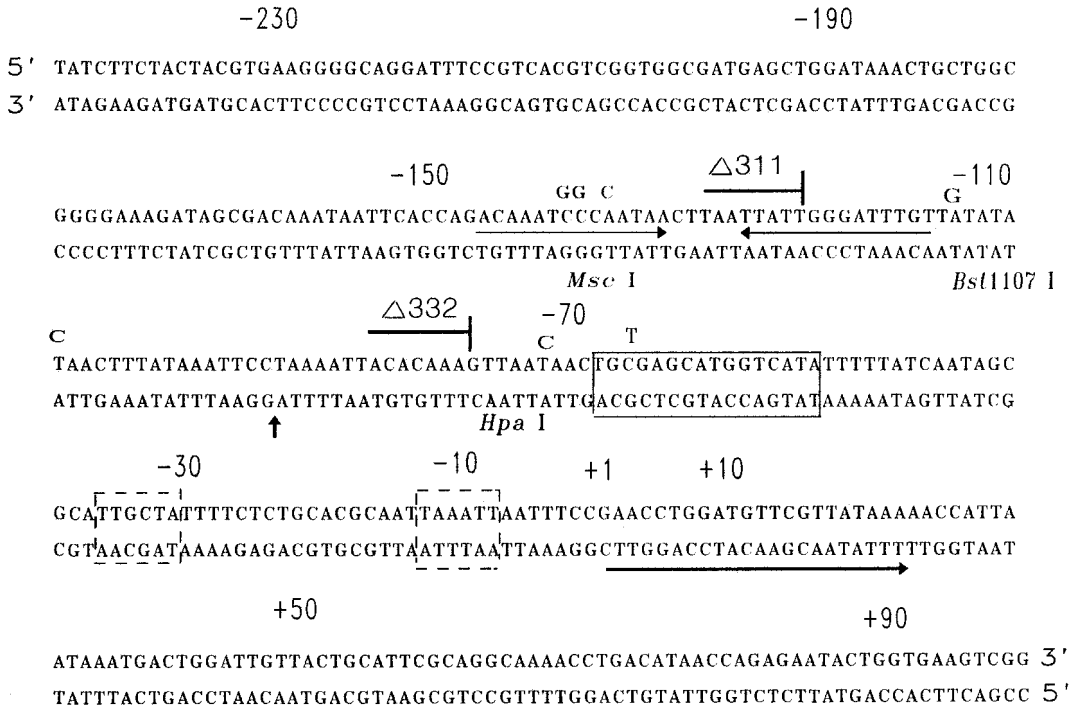


**Fig. 1.** Organization of the *bgl* promoter region. The *Msc*I, *Bst1107*I and *Hpa*I sites created and the C to T transition at the CRP-binding site are indicated. The end points of the large deletions Δ311 and Δ332 are indicated by vertical lines. The end point of the Δ332 deletion was incorrectly indicated in Singh *et al*. (1995). The position of the IS*1* insertion in pMN22AE is indicated by a vertical arrow. The transcription start site is indicated by a horizontal arrow.

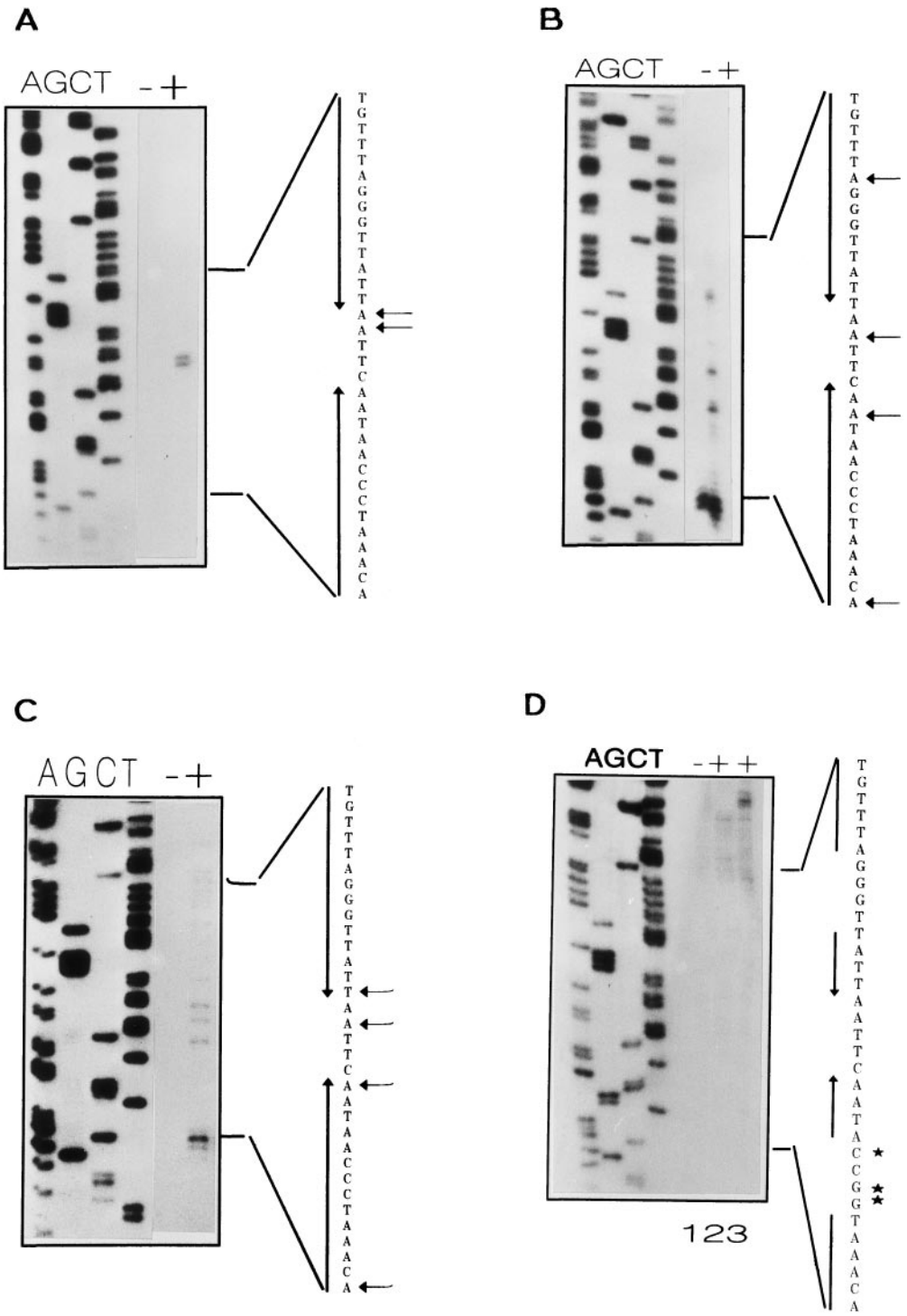


**Fig. 2.** Detection of secondary structures using $KMnO_4$ as a probe. Vertical arrows indicate the region of dyad symmetry. Horizontal arrows indicate the sites of modification. The sites actually correspond to the modified residue in the complementary strand. The absence and presence of $KMnO_4$ are indicated as (−) and (+), respectively.
A. Probing of pJS309 (*bglR*$^0$) *in vitro*.
B. Probing of pMN300 (*bglR*$^0$) *in vivo*.
C. Probing of the chromosomal *bglR*$^0$ *in vivo*.
D. Probing of pMN300DC which carries point mutations within the region of dyad symmetry. Lanes 1and 2, *in vitro*; lane 3, *in vivo.* The mutated bases are indicated by asterisks.

T residues in the loop region showed hypersensitivity to $KMnO_4$. Similar studies were carried out *in vivo* with the *bglR* region carried on a plasmid. In addition to the T residues in the loop region, additional hypersensitive sites in the vicinity of the cruciform, predominantly at the base of the cruciform, were seen *in vivo* (Fig. 2B). Interestingly, two strong bands could be seen corresponding to the base of the cruciform even in the absence of $KMnO_4$ treatment, possibly as a result of the polymerase pausing at the secondary structure. A pattern of hypersensitivity similar to that seen with the supercoiled plasmid DNA could be seen when the chromosomal *bglR* region was subjected to $KMnO_4$ modification (Fig. 2C). Hypersensitivity of the region could also be seen when primer extension of the complementary strand was carried out (data not shown). Similar treatment of a plasmid, carrying three point mutations that disrupt the dyad symmetry, did not reveal any hypersensitivity to $KMnO_4$ either *in vitro* or *in vivo* (Fig. 2D), indicating that the hypersensitivity seen in the wild-type promoter is directly related to the cruciform. The additional sites seen *in vivo* outside the loop in Figs 2B and 2C may arise as a consequence of proteins binding to the region which may modulate the kinetics of extrusion, resulting in intermediates that have a more open conformation *in vivo*. These studies confirm the presence of altered secondary structures in the region of dyad symmetry both *in vitro* and *in vivo*.

### *Effect of internal deletions on transcription*

Our earlier deletion analysis of the regulatory region suggested the presence of a second regulatory element downstream of the cruciform (Singh *et al*., 1995). A deletion that removed the dyad sequence (pMMΔ311) activated the operon and a deletion that removed additional sequences downstream (pMMΔ332) caused a further enhancement in activation. In an effort to understand whether this is an indirect effect of cruciform extrusion or a result of the presence of additional negative regulatory elements downstream, we carried out a fine-structure deletion analysis of the upstream sequence (Fig. 3A). An internal deletion extending from −114 to −147 (pMMΔMB), encompassing the region of the inverted repeat, resulted in fivefold higher levels of transcription compared to the wild type (Fig. 3B). Another internal deletion covering the sequences downstream of the cruciform from −73 to −114 (pMMΔBH) also resulted in four- to fivefold activation. This indicated the presence of a negative element that is distinct from the cruciform in regulating *bgl* expression. The deletion pMMΔMH, which covers both elements, showed activation which is higher compared to the individual deletions, but the effect was not additive, suggesting that the two negative elements may overlap.

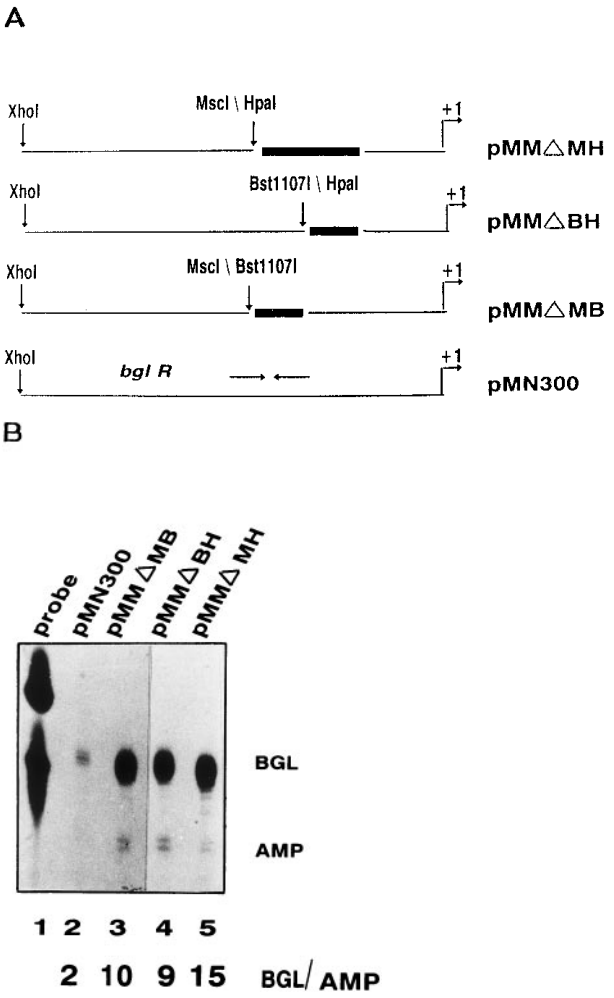


**Fig. 3.** Effect of internal deletions of *bglR* on transcription from the *bgl* promoter.
A. Structure of plasmids carrying the internal deletions. Thick lines indicate the region of the deletions.
B. Transcriptional analysis of the deletion. Lanes: 1, probe; 2, *bglR*$^0$; 3, *bglRΔMB*; 4, *bglRΔBH*; 5, *bglRΔMH*. The ratio of counts in the *bgl* and *amp* bands is indicated below each lane.

### *Role of the* hns *gene in silencing*

Mutations in the *hns* gene result in the activation of the operon (Higgins *et al*., 1988; Hulton *et al*., 1990). However, it is still not clear whether H-NS mediates its effect directly by interacting with the *bglR* region or by altering the global level of supercoiling. In the case of regulation by a direct interaction with sequences upstream of the *bgl* promoter, a deletion of the target sequence is expected to result in activation. However, a mutation in *hns* is not expected to cause an additional increase in transcription in the deletion mutant. To test this possibility, the different deletion mutants described above were transformed into the *hns*$^+$ strain JF201 and the isogenic *hns* strain HNS201 (see Table 2 later) and their transcription levels were measured (Fig. 4). As *amp* transcription may be affected by

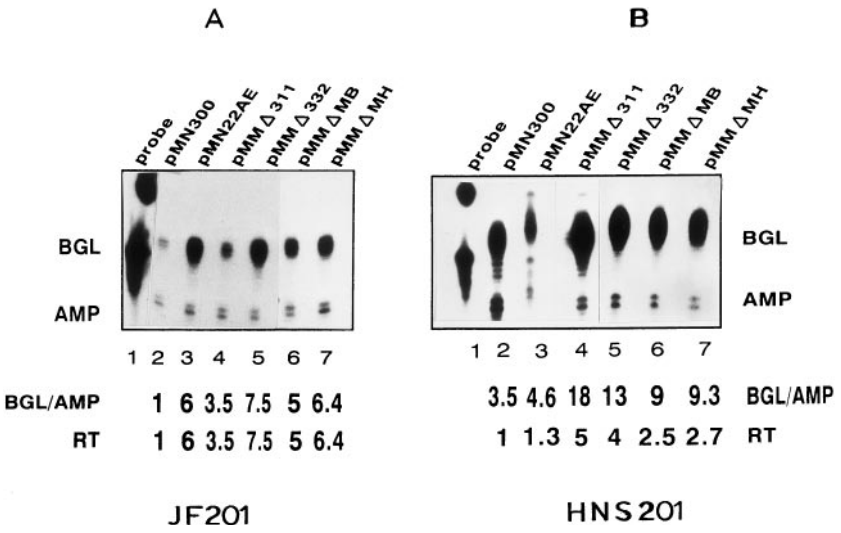


**Fig. 4.** Effect of H-NS on silencing *in vivo*. Analysis of transcription from plasmids carrying different *bglR* alleles in JF201 (*hns*$^+$) (A) and HNS201 (*hns*$^-$) (B) strains. Lanes: 1, probe; 2, *bglR*$^0$; 3, *bglR*::IS*1*; 4, *bglRΔ311*; 5, *bglRΔ332*; 6, *bglRΔMB*; 7, *bglRΔMH*. The constructs used also carried the *kan* gene. RT refers to relative transcription obtained by normalizing the bgl/amp ratio in each case to transcription from the *bglR*$^0$ promoter. A similar pattern of relative transcription was seen when the *kan* gene was used as an internal control (data not shown).

the *hns* status of the strain, the bgl/amp ratios in each case were normalized to the transcription from the wild-type construct in each strain so that the relative transcription (RT) values can be compared between the two strains. The sixfold difference in transcription between pMN22AE (*bgl*::IS*1*) and the plasmid pMN300 carrying the wild-type *bgl* promoter, seen in JF201, is reduced to 1.3-fold in the *hns* strain, indicating the enhancing effect of the *hns* mutation on transcription from the wild-type *bgl* promoter. The plasmids pMMΔ311 and pMMΔ332 (see above), which activate *bgl* transcription to different extents in JF201, do not show an appreciable difference in HNS201. A similar result is obtained with the plasmids pMMΔMB and pMMΔMH, carrying internal deletions. The increase in transcription in pMM332 and pMMΔMH in an *hns*$^+$ backgroud, compared to the plasmids pMM311 and pMMΔMB, can be related to the loss of sequences from −110 to −70 in the former. An increase in transcription can be observed in an *hns* strain even in the presence of this sequence. One possible explanation for this observation is that the sequence downstream of the cruciform is a potential target for interaction with the H-NS protein. An increase in transcription is mediated either by loss of the sequences or by a mutation in the *hns* gene. The high relative transcription seen in the case of the deletion mutants in the *hns* strain is likely to be related to the loss of the upstream negative element. The presence of the element in an *hns* strain may cause an enhanced negative effect due to altered supercoiling.

Further support for the possibility that the sequences downstream of the cruciform may act as a potential target for the H-NS protein comes from the observation that the deletion ΔBH, which results in a fivefold increase in transcription compared to the wild-type promoter in an *hns*$^+$ strain, does not show any differences from the wild-type promoter in an *hns* mutant (Fig. 5). These results suggest that H-NS, or a protein regulated by it, directly interacts with the region downstream to the inverted repeat and represses gene expression. The relative transcription seen in the pMMΔBH construct is similar to that of the wild type since the upstream negative element is unaltered in this construct. The same level of transcription is seen in the case of the plasmid pMN22AE carrying the insertion-activated promoter. The IS*1* insertion in this case may be mediating activation to a large extent by disrupting the potential H-NS-binding site, as the site of insertion is within the region between −110 and −70. The difference

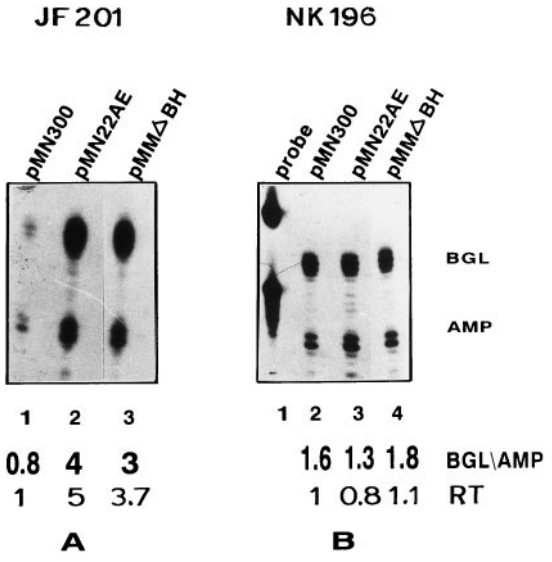


**Fig. 5.** Transcriptional analysis of pMMΔBH in JF201 (*hns*$^+$) (A) and NK196 (*hns*$^-$) (B).
A. Lanes: 1, *bglR*$^0$; 2, *bglR*::IS*1*; 3, *bglRΔBH*.
B. Lanes: 1, probe; 2, *bglR*$^0$; 3, *bglR*::IS*1*; 4, *bglRΔBH*. RT refers to relative transcription as described for Fig. 4.

in the absolute level of transcription from pMN22AE, shown in Figs 4B and 5B, may be related to the differences in the backgrounds of the two *hns* strains used.

### *Role of CRP–cAMP in* bgl *expression*

Point mutations within the CRP–cAMP-binding site, which result in higher affinity for CRP–cAMP, activate the operon (Reynolds *et al*., 1986; Lopilato and Wright, 1990). However, as the CRP–cAMP site is adjacent to the putative H-NS-binding site, it is conceivable that in the mutant, enhanced binding of CRP–cAMP may inhibit binding of H-NS, leading to activation. In the presence of both H-NS and CRP–cAMP, the relative affinities to their respective sites would determine the levels of transcription. To understand the mechanism of activation mediated by the point mutation at the CRP-binding site, transcriptional levels from the wild-type promoter (pMN300) and the promoter carrying the activating point mutation (pMM300CP) were measured in cells grown in the presence or absence of glucose (Fig. 6). Growth in the presence of glucose resulted in a twofold decrease in transcription in the strain JF201 carrying the plasmid pMN300, compared to growth in its absence. Measurement of transcription from pMM300CP in the same strain showed five- to sixfold higher levels in the absence of glucose compared to growth in glucose. When a similar transcriptional analysis from the two promoters was carried out in the *hns* mutant strain NK196, there were no differences in transcription from the wild-type and mutant promoters when cells were grown in the absence of glucose. Moreover, transcription from the plasmid pMM300CP in the *hns* strain was insensitive to glucose repression. Loss of H-NS function due to mutation therefore leads to CRP–cAMP independence. To eliminate

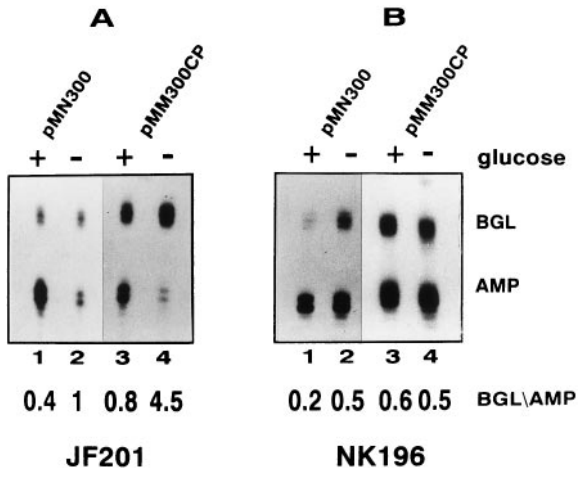


**Fig. 6.** Transcriptional analysis in the presence and absence of glucose from the wild-type promoter and the construct carrying a point mutation at the CRP-binding site in JF201(A) and NK196 (B). Lanes: 1 and 2, *bglR*$^0$; 3 and 4, *bglR-CP*. The presence and absence of glucose are indicated by (+) and (−), respectively.

the possibility that growth in glucose can affect transcription from the *amp* gene, transcriptional analysis was carried out using a construct that carried the *kan* gene. Relative transcription seen when the *amp* gene was used as a control was similar to that seen with the *kan* gene as an internal control (data not shown). These results indicate that transcriptional enhancement mediated by the point mutation within the CRP–cAMP-binding site may be a cumulative effect of activation by CRP–cAMP as well as antirepression of H-NS because of enhanced binding of CRP–cAMP.

The possibility of antirepression by CRP–cAMP was supported by additional experiments involving measurement of relative transcription from plasmids carrying deletions in the region upstream of the CRP–cAMP binding site, in the presence and absence of glucose. The results are shown in Table 1. In the case of deletions which do not extend into the putative H-NS-binding site (Δ311 and ΔMB), the increase in the levels of transcription when grown in succinate is three- to fourfold compared to growth in glucose. In contrast, the mutants Δ332 and ΔMH in which the deletions remove the putative H-NS-binding site show only a twofold higher level of transcription when grown in minimal succinate compared to growth in glucose. This is because the basal level in glucose itself is high in these mutants. The Δ332 deletion results in the maximum levels of activation (16-fold) in glucose compared to the plasmid pMN300 (*bglR*$^0$). As expected, the difference in transcription between pMMΔ332 and pMN300 in glucose is reduced (threefold) in an *hns* strain. The Δ332 deletion construct, when transformed into a *cya crp* double mutant (RF5.2), confers a Bgl$^+$ phenotype on indicator plates. RF5.2 transformants carrying the IS*1*-activated and the ΔMH deletion derivatives also show a weak Bgl$^+$ phenotype on indicator plates. The phenotype on plates is consistent with the transcriptional levels seen in the presence of glucose. These findings indicate that the loss of the putative H-NS-binding site results in partial glucose independence, suggesting once again that one of the roles of CRP–cAMP in transcriptional enhancement is to antagonize the negative effect of H-NS.

### *Role of gyrase in* bgl *transcription*

Mutations in *gyrA* and *gyrB* activate the operon in specific genetic backgrounds (Di Nardo *et al*., 1982). One attractive possibility is that this activation is mediated by destabilization of the cruciform as a result of reduced negative superhelicity in these strains. To explore this possibility, transcriptional levels from the wild-type promoter (pMN300) and a construct with a disrupted cruciform (pMN300DC) were compared in the presence and absence of the *gyrB* inhibitor novobiocin (Fig. 7). A twofold increase in transcription from the wild-type plasmid was seen in the

**Table 1.** Effect of glucose on transcription from different *bglR* alleles.

| | Relative Transcriptional Levels | | | | |
|---|---|---|---|---|---|
| | JF201 (*hns*$^+$) | | HNS201 (*hns*$^-$) | | Bgl phenotype in RF 5.2 (Δ*cya* Δ*crp*) |
| | Glucose | | Glucose | | |
| | + | − | + | − | |
| *bgl* R$^0$(WT) | 1[a] | 2 | 1[b] | 3 | − |
| *bgl* R ::IS*1* | 7 | 24 | 2 | 6 | + |
| Δ 311 | 3 | 9 | ND | ND | − |
| Δ 332 | 16 | 32 | 3 | 7 | ++ |
| Δ MB | 3 | 11 | ND | ND | − |
| Δ MH | 6 | 12 | ND | ND | + |

The numbers represent the relative levels of transcription from the constructs in the presence and absence of glucose obtained by normalizing the bgl/amp ratio in each case to transcription from the *bglR*$^0$ promoter in glucose. The superscripts a and b refer to relative transcription in JF201 (*hns*$^+$) and HNS201 (*hns*$^-$) respectively. The corresponding constructs are illustrated on the left-hand side. The phenotype of each of the constructs transformed in RF5.2 (Δ*crp*, Δ*cya*) is indicated. The (+) indicates a red colour on MacConkey plates supplemented with 1% salicin and (−) indicates a white colour on the same plates. ND, not determined.

presence of novobiocin. The plasmid pMN300DC showed a twofold higher level of transcription compared to the wild type in the absence of novobiocin, but there was no additional increase in transcription in its presence. This experiment was carried out in the presence of glucose (absence of CRP) in an *hns* mutant since these elements are affected by, or affect, supercoiling. Thus, destabilization of the cruciform by a mutation renders the promoter insensitive to novobiocin, supporting the argument that mutations in gyrase influence transcription by altering the stability of the cruciform.

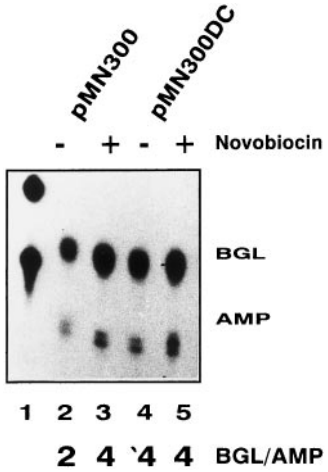


**Fig. 7.** Effect of novobiocin on transcription from the wild-type promoter and a mutant carrying a disrupted cruciform. Lanes: 1, probe; 2 and 3, *bglR*$^0$ (wild type); 4 and 5, *bglR-DC*. Cells were treated with novobiocin (200 μg ml$^{-1}$) for 30 min at $A_{550} = 0.4$ prior to RNA isolation. (+) and (−) indicate the presence and absence of novobiocin, respectively.

## Discussion

The inability of wild-type *E. coli* cells to utilize β-glucoside is directly related to a low level of transcription from the *bgl* promoter, brought about by the presence of upstream negative elements. In this report, we have attempted to characterize the elements involved in silencing the promoter and to integrate the mechanisms of transcriptional enhancement brought about by the different activating mutations.

In an earlier report, we had shown that one of the elements involved in silencing the *bgl* promoter is an inverted repeat which can extrude into a cruciform and negatively regulate transcription (Singh *et al*., 1995). A computer-assisted search for inverted repeats that could form stable cruciforms *in vivo* revealed only two sequences in a 324 146 bp region of the *E. coli* chromosome (Scroth and Shing Ho, 1995). One of them is the inverted repeat present upstream of the *bgl* promoter. This is consistent with the *in vitro* as well as *in vivo* results reported here. The cruciform is stable even at higher temperatures *in vitro* (M. Mukerji and S. Mahadevan, unpublished). The region of the loop shows hypersensitivity to the single-strand-specific chemical probe $KMnO_4$ both *in vivo* as well as *in vitro*. However, additional regions in the stem

seem to be in an open conformation *in vivo*. This could be due to the association of the cruciform with proteins *in vivo* distorting the DNA. Alternatively, the additional bands may represent intermediates formed during cruciform extrusion. Probing of a construct in which the dyad symmetry of the inverted repeat is disrupted does not show hypersensitivity in the region of the cruciform, indicating that the bands seen *in vivo* are related to the cruciform. The similar pattern of sensitivity seen in the plasmid and the chromosome indicates that the extrusion is not an artifact of plasmid supercoiling. A specific deletion of the inverted repeat results in four- to fivefold activation. These results demonstrate that the cruciform exists *in vivo* and is involved in modulating transcription from the *bgl* promoter.

Mutations in the *gyrA* and *gyrB* genes which result in decreased supercoiling activate the operon. It is well documented that initiation of transcription from a majority of bacterial promoters is affected by levels of supercoiling (Pruss and Drlica, 1989). Extrusion of a cruciform reduces the overall superhelical density by an amount equal to the twist contained in it. As cruciform extrusion is energetically favoured by negative supercoiling, a decrease in supercoiling due to inhibition of the *gyr* genes would destabilize it. In the case of the *bgl* promoter, this apparently results in an increase in transcription initiation from the promoter. Novobiocin, an inhibitor of GyrB (Lockshon and Morris, 1983), was used to study the role of supercoiling in regulation of transcription from the *bgl* operon. The level of activation is twofold greater from the wild-type promoter in the presence of novobiocin relative to transcription in its absence. However, there is no additional increase in transcription from a promoter carrying a mutation in the inverted repeat upon treatment with novobiocin. In other words, inhibition of gyrase does not have an effect in the case of a promoter that has a disrupted cruciform. This result strongly suggests that the effect of gyrase mutations on *bgl* transcription is mediated by destabilization of the cruciform structure, consistent with our earlier observation that relaxation of the wild-type template results in transcriptional activation *in vitro* (Singh *et al*., 1995). A similar effect of novobiocin was seen on transcription from a synthetic promoter in which the −10 region contained a sequence which could extrude into a cruciform at lower negative superhelical densities (Horwitz and Loeb, 1988). Thus it is not the lowering of superhelical density *per se*, but the destabilization of the cruciform which causes transcriptional activation.

In a recent report it was shown that the *bgl* promoter is activated *in vitro* by increasing values of negative superhelical densities (Schnetz and Wang, 1996). However, it is difficult to compare these results with our observations, for several reasons. As the internal control used by Schnetz and Wang is sensitive to changes in superhelical density, comparison of the levels of transcription corresponding to different values of superhelical density becomes difficult. The authors have also used single-round transcription, which may not be a true reflection of the steady-state RNA levels *in vivo* as well as *in vitro,* as structural elements sensitive to supercoiling are present upstream of the promoter. Transcription itself generates substantial negative supercoiling upstream and positive supercoiling downstream (Wu *et al*., 1988; Tsao *et al*., 1989). Negative supercoiling is greatest near the promoter and this may be sufficient to drive DNA structural transitions (Ellison *et al*., 1987). Supercoiling generated by transcription would favour extrusion of the cruciform, which might in turn reduce transcription reinitiation. This would be undetected in a single-round assay.

The *bgl* operon is activated by mutations in the *hns* gene. However, as mutations in the *hns* gene have been shown to have a pleiotropic effect it was not known until recently whether derepression of gene expression is a result of direct binding or a consequence of alterations in the global levels of supercoiling. Our analyses indicate that the region downstream of the cruciform may be a putative binding site for direct interaction with H-NS or a protein under its regulation. This is because the downstream deletion which shows four- to fivefold higher levels of transcription in an $hns^+$ strain compared to the wild type has comparable transcriptional levels in the *hns* mutant. H-NS has been shown to bind preferentially to AT-rich/curved DNA sequences (Yamada *et al*., 1990; Owen-Hughes *et al*., 1992). The region implicated in H-NS binding by our deletion analysis, which contains short A and T tracts in a periodic arrangement, shows a retarded mobility in a two-dimensional gel, indicative of the presence of an intrinsic curvature in the sequence (Timchenko *et al*., 1996). Moreover, though direct binding of H-NS to this region was not demonstrated, the authors have shown that the mouse nuclear protein Btcd, a functional analogue of H-NS, binds tightly to this sequence. The protein can also repress the activated *bgl* operon in an *hns* mutant, both at the phenotypic as well as the transcriptional level. The extent of repression mediated by Btcd was comparable to that of H-NS *in vivo*. Repression of transcription from the *bgl* promoter *in vitro* in the presence of cell extracts has also been reported (Schnetz and Wang, 1996). Repression, absent when the extract is derived from an *hns* mutant strain, was restored on addition of purified H-NS.

What is the role of H-NS in silencing of the *bgl* promoter? H-NS has been shown to constrain negative supercoils *in vitro* and to bind preferentially to curved DNA sequences at sub-saturating concentrations (Tupper *et al*., 1994). This would result in an alteration of the topology of DNA and could affect promoter function. Binding of H-NS has also been shown to be co-operative, resulting in the compaction of DNA into a complex nucleoprotein

structure. Such a structure, in the vicinity of a promoter, would prevent binding of RNA polymerase and would also reduce the accessibility of transcriptional factors (Ussery *et al*., 1994).

The region implicated in the binding of H-NS is adjacent to the CRP–cAMP-binding site; therefore binding of one might inhibit binding of the other. The effect of growth in succinate in the case of mutants carrying deletion of the H-NS-binding site was twofold greater relative to growth in glucose in terms of *bgl* transcription. This corresponds to the direct effect on transcription as a result of the binding of CRP–cAMP. However, in the deletions in which the putative sites for H-NS binding have not been removed, the extent of activation by CRP–cAMP is three- to fourfold. This indicates that CRP–cAMP binding might affect the binding of H-NS; the activation seen is the result of the positive effect of CRP–cAMP binding as well as its anti-H-NS function. This is further substantiated by the observation that transcription from a promoter with a point mutation at the CRP-binding site is five- to sixfold higher compared to the wild-type promoter in the presence of H-NS; however, in an *hns* mutant there are no differences in the levels of transcription from the wild-type and the mutant promoters. Thus, the presence of a higher affinity CRP–cAMP-binding site leads to an alleviation of the negative effect of H-NS. In addition, there is no appreciable glucose repression in the promoter carrying the CRP-binding-site mutation in the *hns*$^-$ strain. Mutants that have lost all of the H-NS-binding sites upstream of the CRP site show a Bgl$^+$ phenotype in a *crp cya* strain. These observations are consistent with the results of *in vitro* experiments in which activation from the *bgl* promoter is CRP dependent in the presence of H-NS but no CRP dependence is seen in the absence of H-NS (Schnetz and Wang, 1996). The anti-H-NS action of positive regulators has also been reported in other systems (Forsman *et al*., 1992; Jordi *et al*., 1992).

In this study we have made an attempt to characterize the elements that are involved in silencing the *bgl* promoter. These elements mediate their effect by reducing the accessibility of the RNA polymerase to the promoter, either by the formation of a nucleoprotein complex or by altering DNA topology. Mutations that disrupt these elements lead to activation of the operon. Even in wild-type strains, conditions that alter DNA supercoiling or those that reduce the levels of functional H-NS protein may lead to transient activation. As the apparent function of the operon is the uptake and catabolism of β-glucosides, it is unclear whether any naturally occurring substrate can bring about these changes in the cell. In the presence of a sophisticated mechanism for regulation by antitermination of transcription, the evolutionary significance of this mode of regulation remains an enigma.

## Experimental procedures

### *Plasmid constructions*

Most of the plasmids used in this study were constructed by modification of the plasmid pMN300, which contains the wild-type *bgl* operon cloned in pBR322, and its derivatives (Singh *et al*., 1995). The plasmid pJS309 used for *in vitro* probing experiments is a subclone containing the 1.4 kb *Xho*I–*Hin*dIII promoter fragment from pMN300 cloned at the *Sal*I–*Hin*dIII sites of pUC19. The plasmid pMMΔMB was constructed by introducing a *Bst*1107I site at the −110 position (Fig. 1) in pMM300DC, which has an *Msc*I site within the inverted repeat (Singh *et al*., 1995), and subsequently deleting the sequences between the two sites by digestion with the two enzymes followed by ligation of the blunt ends. This deletion was cloned back into its homologous site in pMN300. The plasmid pMMΔMH was constructed similarly by introducing an *Hpa*I site at −73 position in the derivative containing the *Msc*I site; this was followed by digestion with *Msc*I and *Hpa*I and ligation of the flush ends. The mutant pMMΔBH was constructed by deleting the sequences between the *Bst*1107I site at −110 and the *Hpa*I site at −73. All of the restriction sites were created by site-directed mutagenesis (Kunkel, 1985).

The plasmid pMM300CP carrying the point mutation within the CRP site was constructed by introducing a C to T transition at position −67 (Fig. 1) by site-directed mutagenesis. The plasmid pMN300DC, a derivative of pMM300DC carrying the point mutations disrupting the cruciform (Singh *et al*., 1995), has the complete *bgl* operon cloned in pBR322. The kanamycin-resistant derivatives of pMN300, pMN22AE, pMM311, pMM332, pMMΔMB and pMMΔMH were made by introducing a 1.2 kb *kan* gene fragment from pUC4K (Pharmacia) at the *Stu*I site at +1337. The *kan*-containing plasmids were used in one set of transcription experiments to enable selection of transformants in the HNS201 background.

### *Bacterial strains*

The *E. coli* K-12 strains used in this study are listed in Table 2. The *hns* mutant strain HNS201 was constructed by transducing the *hns*::*amp* allele from the parent strain PD32 (Dersch *et al*., 1993) into JF201. The strain NK196 was constructed by transducing the *bgl* deletion from JF201 into the *hns* mutant AE196 using linkage to *tna*::Tn*10*.

**Table 2.** Genotypes of the relevant bacterial strains.

| Strain | Description | Source |
|---|---|---|
| JF201 | F$^-$ Δ*lacX74* Δ(*bgl–pho*)*201 ara thi gyrA* | Reynolds *et al.* (1986) |
| PD32 | F$^-$ *araD139* Δ(*argF–lac*) *U169 deoC1 flb5301 relA1 rpsl150 ptsF25 rbsR hns206*::Ap$^R$ | Dersch *et al.* (1993) |
| HNS201 | JF201, *hns206*::Ap$^R$ | This work |
| AE196 | HfrHP07, *lacZ* (Am) *relA thi spoT bglY196(hns)* | A. Wright |
| NK196 | AE196, Δ(*bgl–pho*)*201* | This work |
| RF5.2 | F$^+$, Δ*lacU169* Δ*crp45* Δ*cya2* | A. Wright |

### *Probing for secondary structure* in vivo *and* in vitro

The procedure used for chemical probing for secondary structure was similar to that of Sasse-Dwight and Gralla (1991). Specifically for *in vitro* probing, the DNA template was prepared using the alkaline lysis procedure (Sambrook *et al*., 1989), suspended in 10 mM Tris, 1 mM EDTA, pH 8.0, and treated with 1 mM $KMnO_4$ at 20°C for 15 min. The reaction was terminated by the addition of β-mercaptoethanol, followed by phenol extraction and ethanol-precipitation. The template was further purified by precipitation with polyethylene glycol (PEG). The hypersensitive sites were detected by primer extension using a 5′-end-labelled oligonucleotide complementary to the top strand. The primer was extended using the Klenow fragment of DNA polymerase I (Sasse Dwight and Gralla, 1991). The resulting extension products were analysed on a 6% acrylamide–urea gel. The bands were visualized by autoradiography. The same primer was used for generating the sequence ladder of the unmodified template by the chain termination method. A hypersensitive site would correspond to the modified residue in the complementary strand. For *in vivo* probing, the strains were grown in minimal medium supplemented with 0.4% succinate. Cells were grown to an $A_{550}$ value of 0.7, pelleted and resuspended in 0.1 M $PO_4$ buffer (pH 7.4), and treated with 5 mM $KMnO_4$ for 20 min at 20°C. The reaction was terminated using β-mercaptoethanol and the plasmid/chromosomal DNA was isolated. The modified templates were subjected to linear polymerase chain reaction (PCR) in a thermal cycler, using the same primer as above and *Taq* DNA polymerase. The products were analysed as above.

### *Analysis of transcripts*

The method used for the estimation of transcription was essentially the same as that described earlier (Singh *et al*., 1995). Briefly, RNA was isolated from appropriate strains by the hot-phenol method and was hybridized with 5′-end-labelled oligonucleotides specific for the *bgl* and *amp* transcripts. Both of the probes have non-complementary nucleotides at their 3′ end. As a result, after S1 digestion of the hybridized samples and subsequent electrophoresis on a 12% acrylamide–urea gel, bands corresponding to the protected transcripts could be visualized on an autoradiogram. The gel pieces corresponding to these bands were cut and radioactive counts measured using a scintillation counter. The ratio of the counts corresponding to the *bgl* and *amp* bands was used for quantification of transcription.

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