

The histone-like nucleoid structuring protein H-NS represses the *Escherichia coli bgl* operon downstream of the promoter

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Summary

Specificity of repression by the histone-like nucleoid structuring protein and pleiotropic regulator, H-NS, is exceptionally high in case of the *Escherichia coli bgl* (β -glucoside) operon. Here we present evidence that H-NS represses the operon at two levels. The binding of H-NS to an upstream silencer results in an ~three-fold repression of the catabolite gene regulator protein (CRP) dependent *bgl* promoter. In addition, H-NS binds to a silencer region located approximately 600–700 base pairs downstream of the promoter, within the coding region of first gene, *bglG*, resulting in a ~sevenfold further decrease of expression. Repression by H-NS at the downstream silencer requires termination factor Rho and is reduced by translation of the *bglG* mRNA, but is independent of the promoter. This suggests that H-NS induces polarity of transcription by acting as a roadblock to the elongating RNA polymerase. The control of the *bgl* operon by H-NS at two levels results in a highly specific repression.

Introduction

The histone-like nucleoid structuring protein H-NS, a highly abundant 15.6 kDa protein, is an important pleiotropic regulator in the adaptation of the cellular physiology of enterobacteriaceae to external signals. It directly or indirectly affects expression of approximately 5% of the *E. coli* genes, many of which play a role in responses to environmental stimuli (Hommais *et al.*, 2001). H-NS binds to DNA with a preference for bent and AT-rich sequences (Ussery *et al.*, 1994; Schröder and Wagner, 2002). Structural and functional analyses suggest that H-NS is a dimer in solution. Extending from the initial binding (or nucle-

ation) site, H-NS forms oligomeric structures on the DNA, which prevent binding of RNA polymerase or which trap RNA polymerase at the promoter (Dame *et al.*, 2000; Badaut *et al.*, 2002; Esposito *et al.*, 2002; Dame *et al.*, 2002; Bloch *et al.*, 2003). Dimerization of the 136 amino acid H-NS protein requires the N-terminal ~45 amino acids, whereas the C-terminus encompasses the DNA binding domain. The central region of H-NS which is unstructured in solution, is required for oligomerization on the DNA (Shindo *et al.*, 1999; Esposito *et al.*, 2002; Bloch *et al.*, 2003).

How the activity of H-NS is modulated and how high specificity of repression by H-NS is achieved are largely open questions. The H-NS mediated repression of several genes is relieved by antagonistic positive transcription factors: for example the autoregulated *hns* gene is activated by the DNA-bending protein FIS (Falconi *et al.*, 1996), the CFA/I fimbrial system by CfaD (Jordi *et al.*, 1992), or the virulence gene *virB* by VirF (Colonna *et al.*, 1995). *In vitro* the formation of higher order structures of H-NS on the DNA is reduced at elevated temperatures (37°C versus 25°C) and by increased salt concentrations (Amit *et al.*, 2003). These physical properties of H-NS may explain the induction of several H-NS repressed virulence genes at 37°C and of the *proU* operon at high osmolarity (Higgins *et al.*, 1988; Beloin and Dorman, 2003). The *proU* operon, encoding an uptake system for the osmoprotectants glycine-betaine and proline, is repressed very specifically (~70-fold) by H-NS *in vivo*. However, up to date the highly specific repression was not reproducible *in vitro* (Jordi *et al.*, 1997; Jordi and Higgins, 2000). Similarly, the *bgl* operon, encoding the gene products necessary for the uptake and fermentation of aryl- β ,D-glucosides is repressed ~100-fold by H-NS (Higgins *et al.*, 1988; Lopilato and Wright, 1990; Schnetz, 1995; Caramel and Schnetz, 1998). For *bgl* no laboratory growth conditions are known which relieve silencing. Silencing is overcome by spontaneous mutations mapping close the CRP-dependent promoter including the deletion of an AT-rich silencer upstream of the promoter, integration of insertion elements, and point mutations that improve the CRP-binding site, indicating that H-NS represses the *bgl* promoter (Reynolds *et al.*, 1981; 1986; Lopilato and Wright, 1990; Schnetz and Rak, 1992; Schnetz, 1995; Mukerji and Mahadevan, 1997). However, as in case of *proU* *in*

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in vitro repression of the *bgl* promoter by H-NS is merely four- to fivefold (Schnetz and Wang, 1996). Interestingly, repression of both the *proU* locus and *bgl* requires *cis*-regulatory elements located upstream of the promoter and downstream within the transcribed region (Dattananda *et al.*, 1991; Overdier and Csonka, 1992; Owen-Hughes *et al.*, 1992; Schnetz, 1995).

Here we addressed the role of the downstream silencer in repression of the *bgl* operon by H-NS and how specificity of repression of the *bgl* operon by H-NS is achieved. The repression of the *bgl* operon by H-NS was analysed using chromosomally encoded *bgl-lacZ* fusion, which carry the *bgl* promoter including the upstream and the downstream silencer and its deletion derivatives. For analysis of the downstream silencer we used *bgl-lacZ* fusions, which carry a replacement of the *bgl* promoter and upstream silencer by the constitutive *lacUV5* promoter. Furthermore, the H-NS binding sites in *bgl* were mapped by electrophoretic mobility shift experiments. We present evidence that H-NS, in addition to repressing the promoter via the upstream silencer, induces a termination factor Rho-dependent polarity of transcription within the downstream silencer. The H-NS mediated polarity is enhanced when the co-transcriptional translation of the first gene encompassing the downstream silencer is impaired. The data suggest that H-NS, in addition to repressing the promoter, acts as a roadblock to the elongating RNA polymerase and thus silences the *bgl* operon at two levels.

Results

Repression of the *bgl* operon by H-NS

H-NS represses the wild-type *bgl* operon encoding the

positive regulator BglG, the β -glucoside specific permease BglF (or EII^{Bgl}), and the phospho- β ,D-glucosidase BglB (Fig. 1) (Defez and de Felice, 1981; Schnetz *et al.*, 1987; Higgins *et al.*, 1988). Accordingly, only background levels (<1 unit) of phospho- β -glucosidase activity are detectable in cultures grown to the exponential phase (OD₆₀₀–0.5) in NB medium containing 0.2% of the β -glucoside salicin as inducer (Fig. 1A). Upon introduction of an *hns* null allele (*hns-206::ApR*) (Dersch *et al.*, 1993) expression of the chromosomally encoded *bgl* operon strongly increases to 87 units (Fig. 1A). Similarly the expression level of *bgl* operon mutants, which escape silencing as a result of mutations mapping close to the *bgl* promoter, as for example a point mutation that improves the CRP-binding site is very high (135 units) (Fig. 1B). The expression of this activated *bgl* derivative is only marginally further increased in the *hns* mutant (170 units) (Fig. 1). These data are in agreement with other results. They suggest that the mutations mapping *in cis* to the promoter activate the operon by relieving the repression of the promoter by H-NS (Higgins *et al.*, 1988; Lopilato and Wright, 1990; Schnetz and Wang, 1996; Mukerji and Mahadevan, 1997).

Repression of the *bgl* promoter by H-NS

To determine the efficiency of the H-NS mediated repression of the promoter we constructed promoter *lacZ* fusions, which carry the promoter up to position +25 relative to the transcription start followed by the *lacZ* gene. Four promoter variants were used: a wild-type promoter (wt) which carries the upstream AT-rich silencer sequence, a promoter allele which lacks the upstream silencer (Δ), an allele with an improved CRP binding site (CRP⁺), and

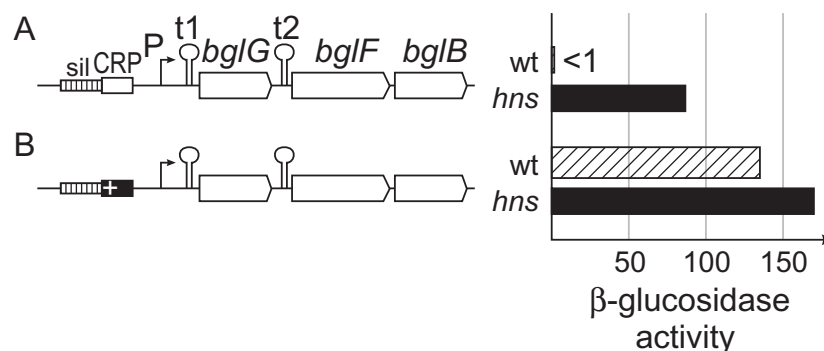


Fig. 1. Repression of the *bgl* operon by H-NS. The expression levels of the chromosomally encoded wild-type *bgl* operon and activated derivatives were determined in the wild-type and *hns* mutant strain background. The three genes of the *bgl* operon, *bglG*, *bglF* and *bglB* encode the antiterminator BglG, the β -glucoside specific permease EII^{Bgl}, and the phospho- β ,D-glucosidase BglB respectively. A. Strains CSH50 and its *hns* derivative S104 carry the wild-type *bgl* operon. CSH50 expressed background levels of β -glucosidase activity (<1 unit) (hatched bar), whereas the isogenic *hns* strain S104 expressed 87 units (black bar). B. Strains S432 and its *hns* derivative S982 carry a *bgl* derivative that is activated by a point mutation improving the CRP binding site (CRP⁺) (a C to T exchange at position –66). These strains directed 135 and 170 units of β -glucosidase activity in the wild type and *hns* mutant respectively. Expression levels were determined from cells grown in NB medium containing 0.2% salicin to OD₆₀₀ 0.5. Background levels were lower than 1 unit as determined using the Δ *bgl* strains S162 and its *hns* derivative S218. Deviations were <10%.

a double mutant which both lacks the upstream silencer and carries the improved CRP binding site (Δ , CRP⁺). These *Pbgl* + 25 *lacZ* fusions were integrated into the *attB* site of the chromosome (as described in *Experimental procedures*) and the expression level was determined in the wild-type strain (which is Δbgl and $\Delta lacZ$) and isogenic *hns* mutant derivatives (Fig. 2). All cultures were grown in NB medium to the exponential phase (OD₆₀₀–0.5). The wild-type promoter directed the expression of 175 units of β -galactosidase activity. The activity of a promoter that lacks the upstream silencer promoter is approximately 3.5-fold higher (600 units) (Fig. 2, compare A and B). In the *hns* strain the wild-type promoter is expressed to similar levels as the promoter lacking the upstream silencer (515 and 485 units, respectively) (Fig. 2A and B). Thus the wild-type promoter is repressed \sim threefold by H-NS, whereas the Δ promoter is not affected by H-NS (Fig. 2). Activation of the promoter by the improvement of the CRP-binding site leads to a \sim fivefold increase of the promoter activity (to 865 units), and a combination of both mutations (Δ and CRP⁺) increases the promoter activity \sim 7.5-fold (to 1300 units) (Fig. 2C and D). The activity of the CRP⁺ promoter increases \sim 1.5-fold in the *hns* mutant (from 865 to 1275 units) to the level of the Δ , CRP⁺ double mutant promoter, which is not affected by H-NS (Fig. 2C and D). These results support previous data indicating that the upstream silencer is necessary for repression of the promoter by H-NS and that efficient binding of CRP counteracts the H-NS-mediated repression of the *bgl* promoter (Schnetzer and Wang, 1996; Mukerji and Mahadevan, 1997). Furthermore, a promoter *lacZ* fusion that carries the promoter up to position +86 was used, to analyse whether additional sites mapping next to the promoter contribute to the H-NS mediated repression. In the wild-

type strain this *Pbgl* + 86 – *lacZ* fusion directed 154 units, and in the *hns* mutant the activity increased to 350 units. Thus the repression of the extended promoter fragment (*Pbgl* + 86) by H-NS is 2.5-fold and similar to the threefold repression of the shorter promoter (*Pbgl* + 25).

Role of the downstream silencer in repression by H-NS

The H-NS mediated silencing of the *bgl* operon is \sim 100-fold (Fig. 1), whereas repression of the promoter is merely \sim threefold (Fig. 2). Previously we have shown that a 'downstream silencer region' located within the leader and/or the first gene, *bglG*, is necessary for silencing of *bgl* when encoded on a plasmid (Schnetzer, 1995). Therefore, the expression level of a chromosomally encoded *bgl*–*lacZ* reporter, which carries the *bgl* promoter flanked by the upstream as well as the downstream silencer, was determined in the wild-type and *hns* mutant (Fig. 3). In the wild-type background this *Pbgl*–*t1*–*bglG*–*lacZ* fusion directs very low levels of β -galactosidase activity (5 units), whereas in the *hns* mutant the expression increases 110-fold to 550 units (Fig. 3A). However, expression of this *bgl*–*lacZ* fusion carrying the upstream and downstream silencer depends on antitermination of transcription by protein BglG at terminator *t1* located in the leader. We have shown recently, that the operon encoded antiterminator BglG is limiting at low transcription rates, which prevents expression of the operon, whereas above a threshold transcription rate, BglG antiterminates transcription resulting in a positive auto-regulatory feed-back loop and consequently efficient expression of the operon (Dole *et al.*, 2002). To monitor the role of H-NS in *bgl* regulation independently of BglG we used a *bgl*–*lacZ* fusion carrying a mutant terminator (*t1*-L) with three base

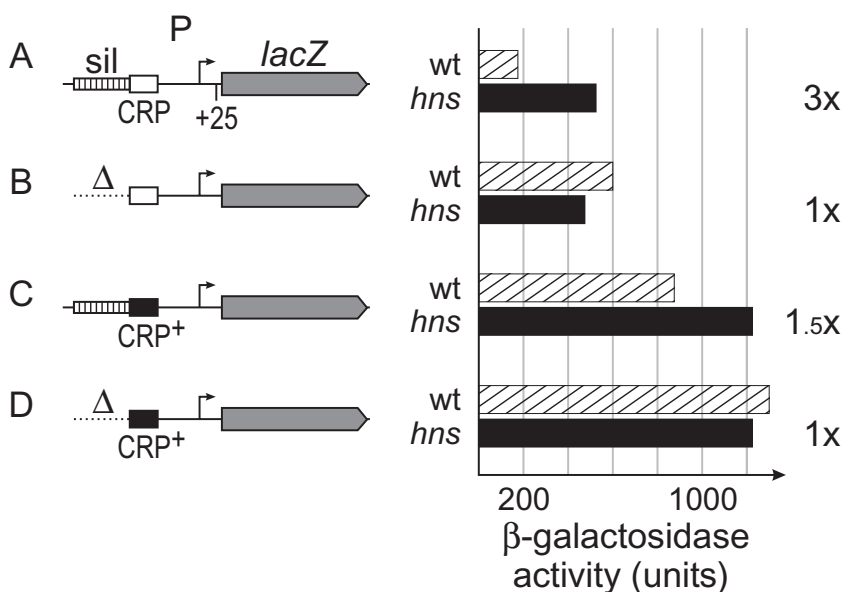


Fig. 2. Repression of the *bgl* promoter by H-NS. To determine the activity of the *bgl* promoter in the wild-type (hatched bars) and *hns* mutant (black bars) the *lacZ* gene was fused 25 base pairs downstream of the transcription start site. In addition to (A) the wild-type promoter, the activities of derivatives were determined, which (B) carry a deletion (Δ , including position –77 and upstream) of the upstream silencer (sil) (C) an improved CRP binding site (CRP⁺), or (D) both mutations together (Δ , CRP⁺). Cultures were grown in NB medium to OD₆₀₀ 0.5. (A) strains S1213, and S1471 (*hns*) directed 175 and 515 units of β -galactosidase activity (B) strains S1211 and S1473 (*hns*) directed 605 and 485 units (C) strains S1215 and S1475 (*hns*) directed 865 and 1275 units, and (D) strains S1217 and S1477 (*hns*) directed 1300 and 1230 units. To the right of each pair of values the fold increase in activity of each promoter allele in the *hns* mutant is given. Deviations were <10%.

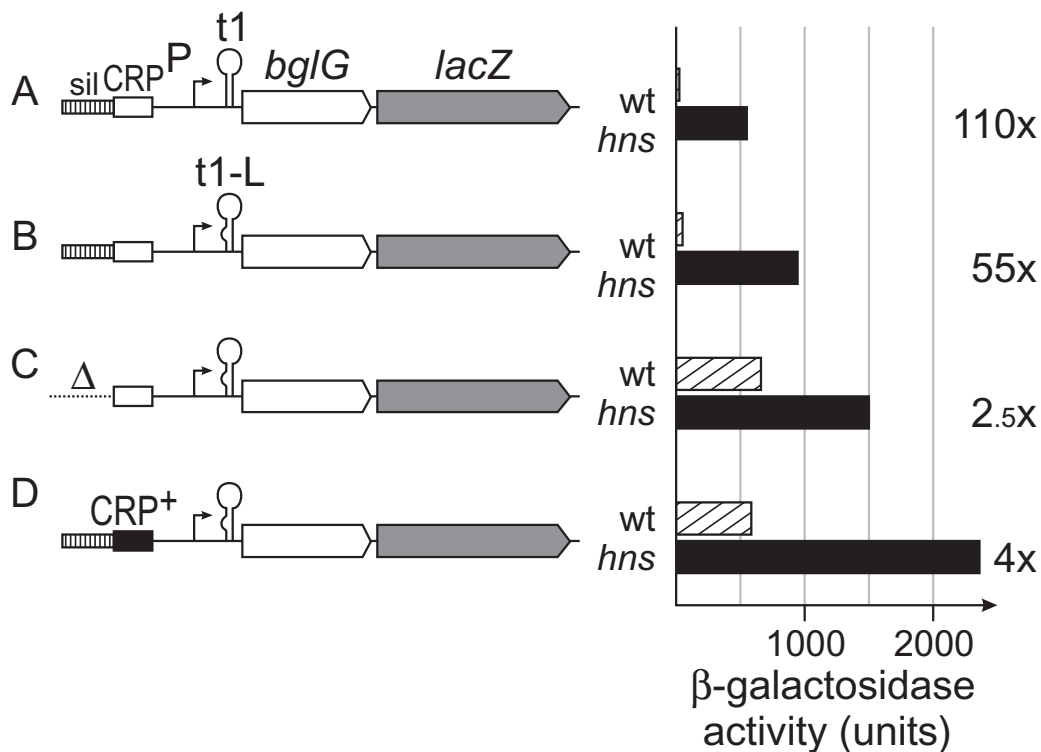


Fig. 3. A downstream silencer enhances the repression of *bgl* by H-NS. (A) The expression level directed by the wild-type *bgl* promoter which is flanked by the upstream silencer (sil) and the downstream silencer including the leader with terminator t1 and the *bglG* gene was determined in the wild-type (hatched bars) and *hns* mutant (black bars). The *lacZ* gene was fused 3' of *bglG*. In addition (B) a similar *bgl-lacZ* fusion carrying terminator mutant *t1-L* was analysed, as well as two derivatives of it which carry promoter alleles activated (C) by the deletion of the upstream silencer (Δ) and (D) an improved CRP binding site (CRP⁺), respectively. In these three constructs antitermination by BglG is not required for expression of *lacZ*. To the right of each pair of values the fold-increase in activity of each promoter allele in the *hns* mutant is given. (A) strains S940 and S1040 (*hns*) direct 5 and 550 units of β -galactosidase activity respectively. (B) strains S1142 and S1467 (*hns*) directed 17 and 945 units (C) strains S1146 and S1469 (*hns*) directed 660 and 1500 units, and (D) strains S1144 and S1514 (*hns*) directed 585 and 2360 units.

exchanges that disrupt the stem loop structure of the terminator (Dole *et al.*, 2002). The respective *Pbgl-t1-L-bglG-lacZ* fusion directs 17 units of β -galactosidase activity in the wild-type strain (Fig. 3B). In the *hns* mutant the expression level increases ~55-fold to 945 units (Fig. 3B). As expected, the H-NS mediated silencing is lower when the expression is BglG independent (Fig. 3, compare A and B). Nevertheless, these data show that the H-NS mediated repression of the wild-type *Pbgl-t1-bglG-lacZ* as well as the terminator mutant *Pbgl-t1-L-bglG-lacZ* fusion is very similar to that of the intact *bgl* operon (Figs 1 and 2).

Promoter independent repression via the downstream silencer

To further analyse the downstream silencer we replaced the *bgl* promoter by the constitutive *lacUV5* promoter. This *lacUV5-t1-L-bglG-lacZ* fusion, which carries the leader and *bglG* in between the *lacUV5* promoter and *lacZ*, was integrated into the chromosome and the expression level was determined in the wild-type and *hns*

mutant. The expression level in the wild-type strain is 240 units of β -galactosidase activity, whereas in the *hns* mutant 1100 units were detected (Fig. 4A). Likewise, a *lacUV5-bglG-lacZ* fusion which lacks the leader expressed 490 units in the wild-type and 1325 units in the *hns* mutant (Fig. 4C). Interestingly, the expression level of a $\Delta Pbgl-t1-L-bglG-lacZ$ fusions which carries the H-NS independent *bgl* promoter $\Delta Pbgl$ lacking the upstream silencer also increases ~2.5-fold in the *hns* mutant (from 660 to 1500 units) (Fig. 3C). Likewise, a *bgl-lacZ* fusion with a promoter that is derepressed by an improved CRP-binding site is expressed at ~fourfold higher levels in the *hns* strains (585 and 2350 units, Fig. 3D). As a control, the expression level directed by a *lacUV5-t1-L-lacZ* fusion which carries the *bgl*-leader in between the *lacUV5* promoter and *lacZ* but lacks the *bglG* coding region (encompassing the downstream silencer, see below) is not affected by H-NS (Fig. 4E). These data demonstrate that the three- to fourfold H-NS mediated repression via the downstream silencer is independent of the leader (Fig. 4E) and the promoter (compare Fig. 3C and D with Fig. 4A and C).

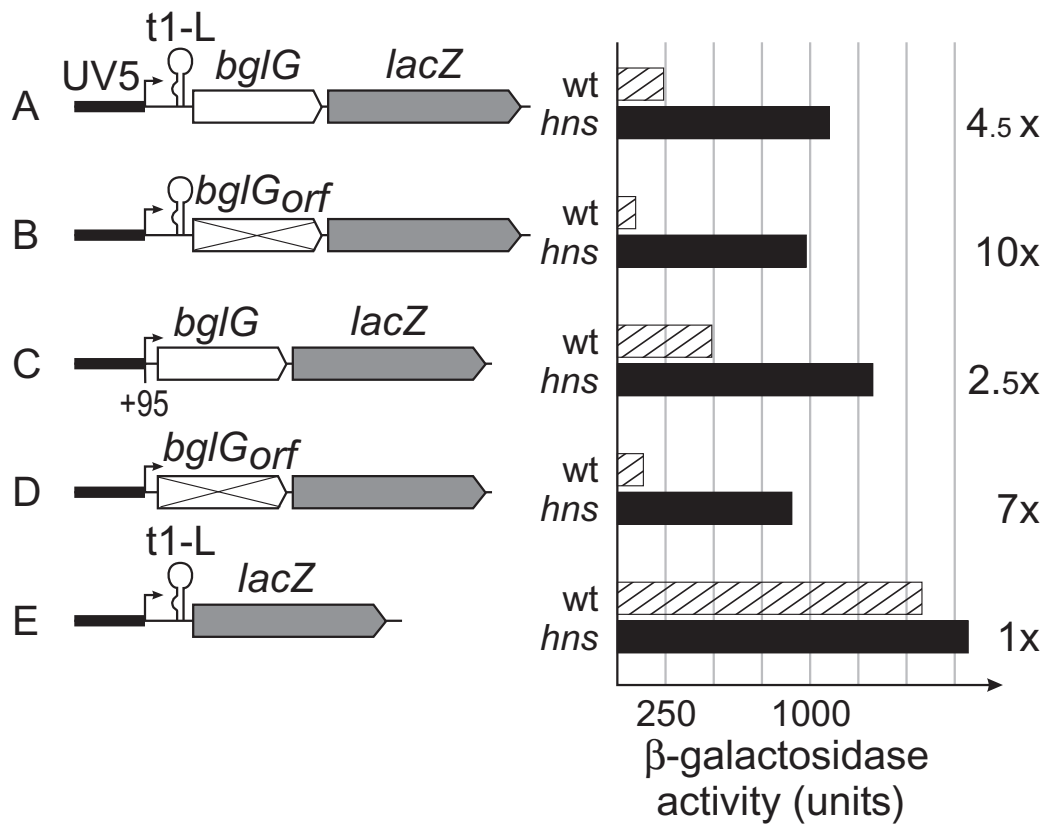


Fig. 4. H-NS mediated repression via the downstream silencer. The influence of the downstream silencer was analysed by its insertion in between the constitutive *lacUV5* promoter (UV5) and the *lacZ* gene. (A) The insertion of a leader *bglG* fragment (with terminator mutant t1-L) resulted in the expression of 240 units in strain S1097, and 1100 units in its isogenic *hns* derivative S1309. (B) In the isogenic *bglGorf* construct the *bglG* codons 1, 3 and 27 were mutated to exclude translation of *bglG*. The activity was 94 units in S1189, and 980 units in the *hns* derivative S1252. (C) Insertion of a *bglG* or (D) *bglGorf* fragment from position +95 and lacking the leader resulted in (C) 490 units in S1193 and 1325 units in S1256 (*hns*) (D) 135 units in S1195 and 905 units in S1258. (E) The presence of the leader (position +1 to +131) caused similar expression levels in the wild-type (S1191) and *hns* mutant (S1254) 1570 and 1820 units respectively.

Translation of *bglG* reduces the H-NS mediated repression via the downstream silencer

It is possible that repression by H-NS via the downstream silencer affects transcription initiation at any promoter (*bgl* and *lacUV5* were tested) located upstream. However, another possibility is that H-NS hinders transcription elongation. Transcription elongation can also be influenced by the efficiency of the co-transcriptional translation of the mRNA (Carter and Newton, 1969). To further narrow down on the downstream silencer we mutated the start codon and two additional ATG triplets (codon 3 and 27) of *bglG* to prevent translation.

When the translation of *bglG* was excluded the H-NS mediated repression was more efficient (Fig. 4). In the wild-type strain background the *lacUV5-t1-L-bglGorf-lacZ* fusion (Fig. 4B) directed 94 units of β -galactosidase activity (compare to 240 units when *bglG* can be translated, Fig. 4A). Likewise, a *lacUV5-bglGorf-lacZ* fusion lacking the leader directed only 135 units of β -galactosidase activity (Fig. 4D, compare to 490 units, when *bglG*

can be translated Fig. 4C). Remarkably, in the *hns* mutant, preventing translation of *bglG* has very little effect. All four constructs carrying the *bglG* downstream silencer in between the *lacUV5* promoter and *lacZ* directed similar levels of β -galactosidase activity in the *hns* mutant (compare Fig. 4A and B, 1180 units 980 units, respectively, and Fig. 4C and D, 1325 units and 905 units in case of the *bglG* and *bglGorf* constructs lacking the leader). Thus the H-NS mediated repression is enhanced when the *bglG* mRNA can not be translated. These data suggest that H-NS induces polarity within the *bglG* coding region.

Termination factor *Rho* is required for efficient downstream repression by H-NS

In general polarity is the result of a pause in transcription elongation allowing termination factor *Rho* to catch up with RNA polymerase and to terminate transcription elongation. To address the question whether downstream repression by H-NS can be the result of H-NS acting as a roadblock to the elongating RNA polymerase and subse-

quent termination of transcription by Rho, we determined the expression level directed by the downstream reporter *lacUV5–bglGorf–lacZ* (Fig. 5A) in a temperature sensitive *rho-702(ts)* mutant and a *hns rho-702(ts)* double mutant at the permissive (28°C) and non-permissive temperatures (42°C). For comparison the expression level in the wild-type and the *hns* mutant was determined when grown at identical temperature conditions.

In the wild-type background the *lacUV5–bglGorf–lacZ* reporter directed 130 units of β -galactosidase activity at 28°C and 100 units at 42°C (Fig. 5B). In the *rho-ts* mutant the expression level increased to 260 units at 28°C and to 390 units, i.e twofold at the permissive and fourfold at the non-permissive temperature of 42°C (Fig. 5B). However, in the *hns* background the *rho-ts* mutation had no effect. In both the *hns* mutant and the *hns rho* double mutant similar β -galactosidase levels were detected, which were 1015 and 1025 units, respectively, at 28°C, and 815 and 865 units at 42°C (Fig. 5B). These results demonstrate that Rho is important for H-NS to repress *bgl* via the downstream silencer supporting the model that H-NS acts as a roadblock to the elongating RNA polymerase as a prerequisite for Rho-mediated termination.

Binding of H-NS to the *bgl* operon DNA

To characterize the sites involved in the regulation of *bgl* by H-NS we performed electrophoretic mobility shift

assays with a series of *bgl* fragments. The fragments and the results of the DNA shift assays are shown in Fig. 6. Fragment I which includes the upstream silencer and promoter (position –164 to +25 relative to the transcription start) was efficiently shifted by H-NS. No binding of H-NS was detected for fragments II (position +132 to +459) and IV (position +669 to +965), which map within the *bglG* coding region. However, fragment III (position +450 to +737) was efficiently bound by H-NS. The use of smaller fragments IIIa, IIIb, and IIIc (Fig. 6) allowed mapping the H-NS binding sites to fragment IIIc (position +609 to +737). A very weak shift was also seen with fragment IIIb (positions +532 to +650). Specific competition experiments further support the result that H-NS binds to fragment IIIc. Binding to fragment IIIc could be competed by fragment I encompassing the upstream silencer and vice versa (data not shown). In addition, all fragments were analysed for bends using the bend.it prediction tool at <http://www.icgeb.trieste.it/dna/> (Munteanu *et al.*, 1998). Planar bends were predicted for the –115 to –68 and +615 to +700 regions located within fragments I and IIIc, but not in any other *bgl* fragment between positions –450 and +965.

Discussion

The approximately 100-fold repression of the *bgl* operon by H-NS requires an AT-rich and presumably bent silencer

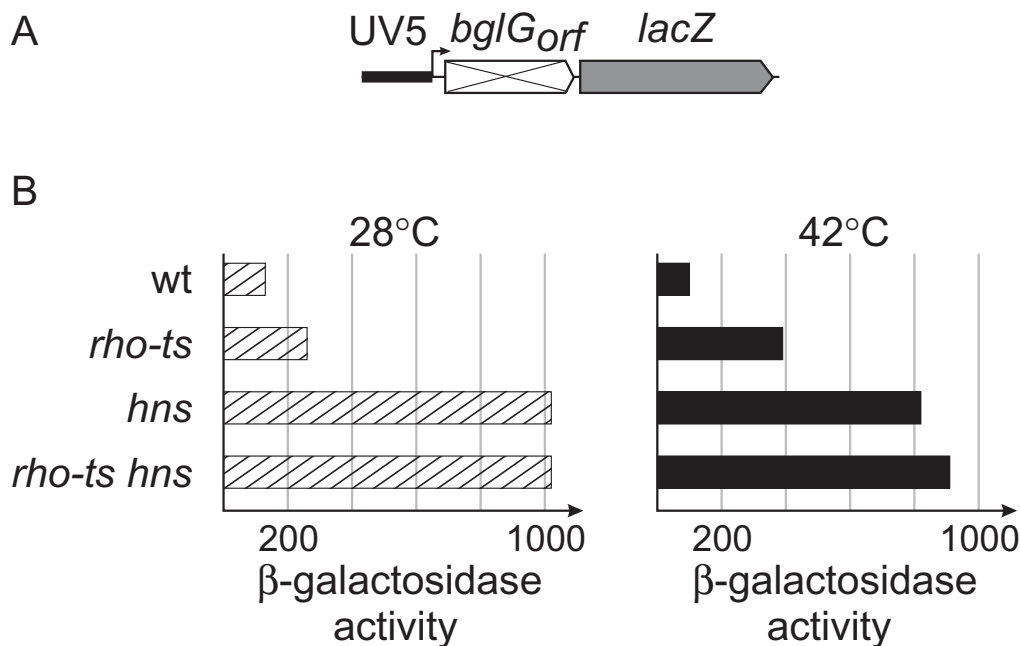


Fig. 5. Termination factor Rho is required for the H-NS mediated repression via the downstream silencer. The expression level directed by the downstream silencer reporter construct *lacUV5–bglGorf–lacZ* (A) was determined in the wild-type, *rho-ts(702)* mutant, *hns* mutant, and the *rho-ts(702) hns* double mutant of cells grown at 28°C and at 42°C in LB medium. (B) At 28°C 130 units of β -galactosidase activity were determined in the wild-type, 260 units in the *rho* mutant, 1015 in the *hns* mutant, and 1025 in the *hns rho* double mutant. At 42°C 100 units of β -galactosidase activity were determined in the wild-type, 390 units in the *rho* mutant, 815 in the *hns* mutant, and 890 in the *hns rho* double mutant.

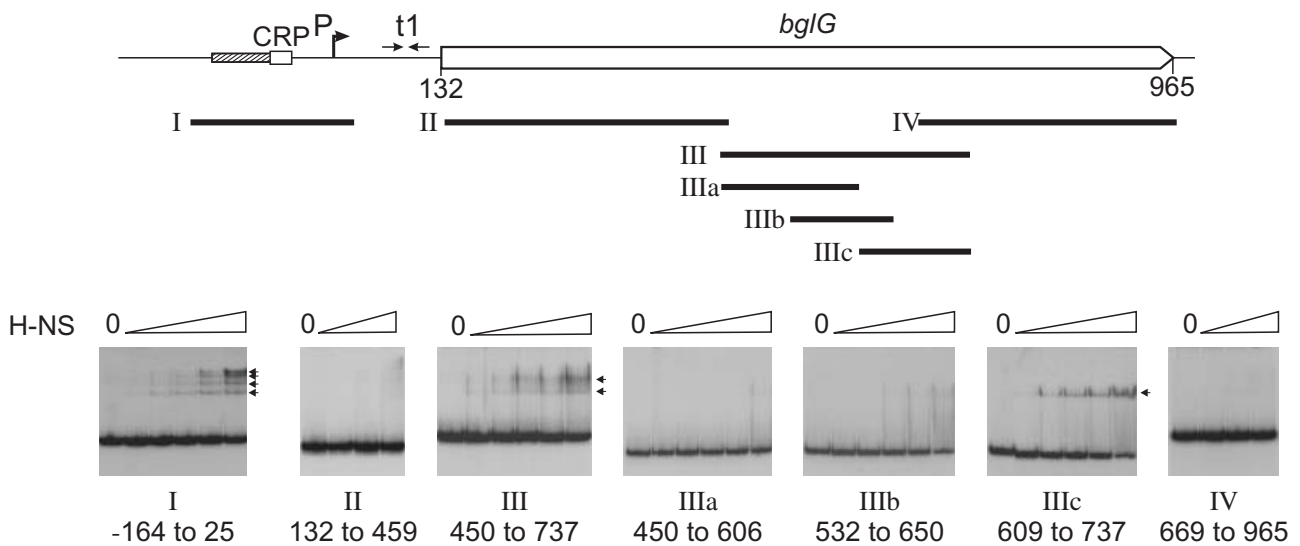


Fig. 6. Mapping of H-NS binding sites in *bgl*. The binding sites of H-NS within the downstream silencer were mapped by electrophoretic mobility shift experiments. In addition binding of H-NS to the upstream silencer promoter was analysed. Top: the structure of the *bgl* regulatory region including the upstream silencer (sil) and the downstream region with terminator t1 and *bglG* is shown schematically. Fragments I, II, III, IIIa, IIIb, IIIc, and IV used in the mobility shift experiments are represented by horizontal bars. Lower part: the different [32 P]-labelled fragments whose map positions are given relative to the *bgl* transcription start site were incubated with increasing concentrations of H-NS (50 nM, 75 nM, 100 nM, 150 nM, and 200 nM) and then separated on acrylamide gels which were run at 4°C. For fragments II and IV only the shifts with H-NS concentrations of 50 nM, 100 nM, and 200 nM are shown. Shifted bands are marked with arrows. The fragments were completely shifted at concentration of H-NS being 400 nM and higher (not shown). The concentration of the labelled fragment was 0.2 nM (see *Experimental procedures* for details).

sequence located directly upstream of the CRP-dependent *bgl* promoter, and a downstream silencer located within the coding region of the first gene, *bglG*. Our data confirm that H-NS represses transcription initiation at the promoter by binding to the upstream silencer. In addition, we found that H-NS binds to a stretch of DNA located 600–700 base pairs downstream of the promoter, which is predicted to be bent. Repression by H-NS *via* the downstream silencer requires termination factor Rho, is enhanced when translation of the *bglG* gene is prevented, and is promoter independent. These results suggest that H-NS in addition to repressing transcription initiation represses transcription elongation by inducing polarity (Fig. 7).

H-NS binds specifically to the bent and AT-rich *bgl* upstream silencer and promoter fragment, as shown by gel electrophoresis mobility shift assays. In addition, *in vivo* expression studies using the *lacZ* reporter gene

fused at position +25 to the promoter demonstrated that the upstream silencer is necessary for the repression of transcription initiation at the *bgl* promoter by H-NS. Repression of the promoter is approximately threefold. This result is in agreement with *in vitro* studies where the transcription initiation at the promoter was repressed four- to sixfold by H-NS (Schnetz and Wang, 1996), and with quantification of the amount of *bgl* leader mRNA present in the wild-type and an *hns* mutant (Mukerji and Mahadevan, 1997).

In addition to the upstream silencer, a downstream silencer is necessary for full silencing of *bgl* by H-NS (Schnetz, 1995). H-NS binds within the coding region of the *bglG* gene approximately ~600–700 base pairs downstream of the transcription initiation site (Fig. 6). This region is predicted to be bent (Munteanu *et al.*, 1998). In addition, we show that H-NS represses transcription through the *bglG* coding region encompassing the down-

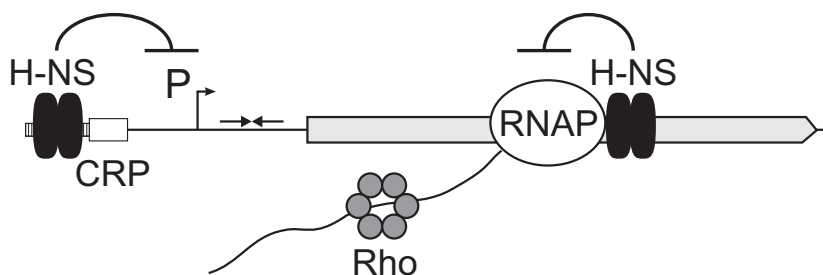


Fig. 7. Model of the H-NS mediated repression of the *bgl* operon at two levels. H-NS binds upstream of the promoter and represses transcription initiation. In addition, H-NS binds within the coding region of the first gene, approximately 600–700 bp downstream of the transcription initiation site, where it induces a Rho-dependent repression, which is likely to be mediated by stalling transcription elongation.

stream silencer. The degree of repression is affected by the translation of the *bglG* gene. Without translation of *bglG* the H-NS mediated repression was ~sevenfold, while translation of *bglG* reduced the repression to ~threefold, suggesting that the access of a protein to the mRNA or the secondary structure of the mRNA is important in this process. When we tested whether H-NS binds also to the *bglG* mRNA using *in vitro* transcribed RNA no binding was detectable (data not shown). Furthermore, we demonstrate that in a *rho* mutant, which encodes a temperature-sensitive termination factor Rho protein, the repression by H-NS is reduced and is merely twofold at the non-permissive temperature, whereas the mutation of *rho* has no effect in the *hns* mutant. These data suggest that H-NS acts as a roadblock to the elongating RNA polymerase, allowing termination factor Rho to catch up with the paused RNA polymerase and to terminate transcription, in agreement with the current picture of transcription elongation and termination (Severinov, 2000; Landick, 2001). In addition, it is possibly that the mRNA protruding of an H-NS stalled transcription elongation complex is more efficiently degraded.

Previously it was assumed that the 100-fold silencing of the *bgl* operon by H-NS (Fig. 1) is mediated by repression of the promoter. This assumption was drawn because of the fact that mutations mapping *in cis* to the promoter and preventing repression of the promoter by H-NS completely relieve silencing of the operon (see *Introduction*), which raises the question of why the downstream repression is masked, when repression of the promoter is relieved. This may have several reasons: first, the expression of the operon requires the operon-encoded specific antiterminator BglG. We have shown recently, that BglG is limiting at low transcription rates, which accentuates repression of *bgl* by H-NS. Second, above a threshold transcription rate, BglG actively antiterminates transcription, and positively auto-regulates its own expression, which results in the amplification of a moderate increase of the transcription initiation rate into a large increase of the expression of the structural genes of the operon (Dole *et al.*, 2002; Schnetz, 2002). Third, once the threshold required for antitermination by BglG is overcome, the level of transcription of the *bglG* coding region increases. It has been shown recently that increased transcription enhances read-through at a roadblock (Epshtein *et al.*, 2003). Fourth, BglG stimulates its translation (Nix, Dole and Schnetz, unpublished), and thus the Rho-mediated termination is likely to be reduced. Fifth, every positive auto-regulatory system requires a mechanism that limits expression. In case of BglG this is generated by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). BglG is activated by the transfer of a phosphate group from the general PTS protein HPr, and it is negatively regulated in the absence of β -glucosides by its phosphorylation by the operon

encoded β -glucoside specific permease EII^{Bgl} (Görke and Rak, 1999). High expression levels of the operon and concomitant expression of EII^{Bgl} deplete the PTS of phosphoryl groups resulting in less efficient activation of BglG by the HPr mediated phosphorylation, which thus generates an upper limit for BglG activity and expression of the operon (Görke and Rak, 1999). Six, it is possibly that the H-NS mediated repression of transcription initiation upstream at the promoter and of transcription elongation downstream within *bglG* is synergistic. H-NS forms extended oligomeric structures on the DNA as has been shown by DNA footprinting studies, by atomic force microscopy and force extension measurements (Lucht *et al.*, 1994; Dame *et al.*, 2000; 2002; Rimsky *et al.*, 2001; Badaut *et al.*, 2002; Amit *et al.*, 2003). These extended structures seem not to have a defined size but usually cover around 200–500 bp. It has also been shown that H-NS can trap two DNA strands together with the intervening DNA forming a loop (Dame *et al.*, 2000; 2002). Thus it is possible that the upstream and downstream repressing complexes mutually enhance each other. Taken together, within the natural context of the *bgl* operon the relief of the H-NS mediated silencing of the promoter masks the H-NS mediated repression at the downstream silencer due to increased transcription and translation of *bglG*, positive autoregulation of BglG, and limitation of the maximum expression level of the operon by the PTS. For a similar reason, in a previous study repression of *bgl* by H-NS in the presence of only one *bgl* silencer may not have been detected as a plasmidic reporter system was used (Schnetz, 1995).

It is generally assumed that H-NS represses expression by forming extended nucleoprotein complexes that interfere with transcription initiation. The proposed mechanism of repression by H-NS acting as a roadblock to the elongation RNA polymerase at the *bgl* downstream silencer is the first example of a novel mechanism. It is possible that other H-NS repressed genes are also regulated by this mechanism.

Experimental procedures

Strains and plasmids

The genotypes of the *E. coli* strains are listed in Table 1. All experiments were performed using isogenic *E. coli* K12 CSH50 derivatives [CSH50 is *ara* Δ (*gpt-lac*) *ara* *thi*; (Miller, 1972)]. Transductions were performed using phage T4G77 (Wilson *et al.*, 1979). The transduction of the *rho-702*(ts) allele was confirmed by PCR amplification and sequencing of the fragment encompassing the *rho* gene. In the *rho-702*(ts) allele we found three mutations: an A to G exchange of the first base of codon 158 resulting in an amino acid change from threonine to alanine, an G to A exchange of the first base of codon 224 causing an amino acid change from glutamate to isoleucine, and a G to A exchange of the first

Table 1. *E. coli* K-12 strains.

Strain	Relevant genotype or structure ^a	Construction ^b /reference
CAG18431	F ⁻ , λ ⁻ , rph-1, ilvD500::Tn10	CGSC#7462
CSH50	<i>bgl</i> ^P Δ(<i>lac-pro</i>) <i>ara thi</i>	Miller (1972)
HD152	F ⁻ , <i>thr-33</i> , λ ⁻ , <i>trpE9829</i> (Am), <i>serU126</i> (ts, AS), <i>his-2130</i> , <i>tyrA15</i> (Am), <i>thyA707</i> , IN(<i>rrnD-rrnE</i>)1, <i>rho-702</i> (ts)	CGSC#6106
M182 <i>stpA</i> ::Tc ^R	Δ(<i>lacI</i> POZYA)74 <i>galU galK strA stpA</i> ::Tc ^R	Zhang <i>et al.</i> (1996)
PD32	MC4100 <i>hns-206</i> ::Ap ^R	Dersch <i>et al.</i> (1993)
S104	CSH50 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S162	CSH50 Δ <i>bgl</i> -AC11	Caramel and Schnetz (1998)
S218	S162 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S219	S162 <i>stpA</i> ::Tc ^R	x T4G77(M182 <i>stpA</i> ::Tc ^R)
S236	S157 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S432	CSH50 <i>bgl</i> -CRP spontaneous Bgl ⁺ mutant	Schnetz (2002)
S541	S539 Δ <i>bgl</i> -AC11 Δ <i>lacZ</i> -Y217	Dole <i>et al.</i> (2002)
S940	S541 <i>attB</i> ::[SpecR <i>P</i> _{<i>bgl</i>} <i>t1 bglG lacZ</i>]	x pKESD8; Dole <i>et al.</i> (2002)
S982	S432 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1040	S940 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1097	S541 <i>attB</i> ::[SpecR <i>lacUV5 t1-L bglG lacZ</i>]	x pKESD28; Dole <i>et al.</i> (2002)
S1142	S541 <i>attB</i> ::[SpecR <i>P</i> _{<i>bgl</i>} <i>t1-L bglG lacZ</i>]	x pKESK11; Dole <i>et al.</i> (2002)
S1144	S541 <i>attB</i> ::[SpecR <i>CRP</i> ⁺ <i>P</i> _{<i>bgl</i>} <i>t1-L bglG lacZ</i>]	x pKESK12; Dole <i>et al.</i> (2002)
S1146	S541 <i>attB</i> ::[SpecR Δ <i>P</i> _{<i>bgl</i>} <i>t1-L bglG lacZ</i>]	x pKESK13; Dole <i>et al.</i> (2002)
S1189	S541 <i>attB</i> ::[SpecR <i>lacUV5 t1-L bglGorf lacZ</i>]	x pKESD47
S1191	S541 <i>attB</i> ::[SpecR <i>lacUV5 t1-L(+130) lacZ</i>]	x pKESD36
S1193	S541 <i>attB</i> ::[SpecR <i>lacUV5 (+95)bglG lacZ</i>]	x pKESD48
S1195	S541 <i>attB</i> ::[SpecR <i>lacUV5 (+95)bglGorf lacZ</i>]	x pKESD49
S1211	S541 <i>attB</i> ::[SpecR Δ <i>P</i> _{<i>bgl</i>} (+25) <i>lacZ</i>]	x pKEKB25
S1213	S541 <i>attB</i> ::[SpecR <i>P</i> _{<i>bgl</i>} (+25) <i>lacZ</i>]	x pKEKB30
S1215	S541 <i>attB</i> ::[SpecR <i>CRP</i> ⁺ <i>P</i> _{<i>bgl</i>} (+25) <i>lacZ</i>]	x pKEYK1
S1217	S541 <i>attB</i> ::[SpecR Δ, <i>CRP</i> ⁺ <i>P</i> _{<i>bgl</i>} (+25) <i>lacZ</i>]	x pKEYK2
S1252	S1189 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1254	S1191 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1256	S1193 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1258	S1195 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1309	S1097 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1467	S1142 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1469	S1146 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1471	S1213 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1473	S1211 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1475	S1215 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1477	S1217 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1514	S1144 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1516	S1503 <i>hns</i> ::Ap ^R	x T4G77(PD32)
S1946	S541 <i>ilvD</i> ::Tn10	x T4G77(CAG18431)
S1956	S1946 <i>rho-702</i> (ts) <i>ilvD</i> ⁺	x T4G77(HD152)
S1995	S1956 <i>attB</i> ::[SpecR <i>lacUV5 (+95)bglGorf lacZ</i>]	x pKESD49
S1997	S1995 <i>hns</i> ::Ap ^R	x T4G77(S102)

a. The relevant genotype of the constructed CSH50 derivatives refers to the *bgl*, *lac* and *hns* loci. Mutations *CRP*⁺ (a C to T exchange in the CRP binding site at position -66, relative to the transcription start), and Δ (a deletion of the upstream silencer, including position -77) cause activation of the *bgl* promoter. Terminator mutation *t1-L* carries a 3 bp exchange (+72 to +74) in the left stem of the termination loop disrupting terminator *t1* (Dole *et al.*, 2002). The *lacUV5* promoter (positions -40 to +1) was fused to *bgl* (position +1 or +95 as indicated) as described (Dole *et al.*, 2002). In the *bglG* gene mutation *bglGorf* ATG codons 1, 3 and 27 were replaced by GCG. Positions of fusions to *lacUV5* and *lacZ*, respectively, are given in brackets, e.g. *P*_{*bgl*}(+25), indicate that the *lacZ* gene was fused downstream of base pair +25 to the *bgl* promoter, and (+95)*bglG* indicates that the *bglG* fragment including base pair at position +95 (to +1421) was fused to *lacZ*.

b. x: Transductions and integrations into *attB* were performed as described in *Experimental procedures*.

base of codon 304 causing an alanine to threonine exchange. Thus *rho-702*(ts) encodes a Rho-T158A-E224I-A304T mutant protein. The integration of the *bgl-lacZ* reporter constructs into the chromosomal phage lambda attachment site *attB* was performed as described (Diederich *et al.*, 1992; Dole *et al.*, 2002). Briefly, strain S541 or mutant derivatives of it, harbouring the temperature sensitive integrase expressing plasmid pLDR8 (Diederich *et al.*, 1992) were transformed with re-ligated origin-less *Bam*HI fragments carrying *attP*, an Ω spectinomycin resistance cassette (Prentki and Krisch, 1984), and the respective *bgl-lacZ* reporter gene fusion

(Table 1). Integrations were selected on LB spectinomycin plates at 42°C. Independent colonies were tested by PCR to verify the integration of a monomer into *attB* and the integrity of the *bgl* promoter region. Two independent clones were used in the enzyme assays.

Plasmids were constructed according to standard techniques (Sambrook and Russell, 2001). All constructed plasmids carry the pACYC177 origin and *neo* gene vector backbone (Chang and Cohen, 1978), *attP*, an omegon-spectinomycin resistance cassette (Prentki and Krisch, 1984), and the respective *bgl-lacZ* reporter gene fusions. Site-specific

mutations and fusion of *bgl* and *lac* sequences were introduced by PCR. All regions of plasmids that were derived from PCR fragments were sequenced. The relevant structures of the plasmids are schematically shown in the figures and the structures of the fragments which were integrated into *attB* are listed in Table 1. Details of constructions and compiled sequences of the plasmids are available upon request. Media and plates were used as described (Dole *et al.*, 2002). Antibiotics were added to 25 µg ml⁻¹ kanamycin, 50 µg ml⁻¹ ampicillin, 15 µg ml⁻¹ chloramphenicol, 50 µg ml⁻¹ spectinomycin, and 12.5 µg ml⁻¹ tetracycline final concentration, where necessary.

Determination of β -galactosidase and β -glucosidase activities

Enzyme activities were determined from cultures grown to exponential phase in NB medium (Difco), since the *hns* strains grow poorly in minimal medium. The cultures were inoculated from fresh over-night cultures and harvested after approximately 3 h of growth at 37°C at an OD₆₀₀ of 0.5. For induction of the *bgl* operon salicin (0.2%) was added to the exponential culture. The β -galactosidase and β -glucosidase assays were performed as described (Schaeffler and Maas, 1967; Miller, 1972; Dole *et al.*, 2002). The enzyme activities were determined at least three times and standard deviations were less than 10%.

Purification of H-NS and DNA mobility shift assay

H-NS was overexpressed and purified free of StpA using strain S219 (*stpA::Tc^R*) and plasmid pFDY400, which carries the *hns* gene under control of the *tac* operator promoter as described (Owen-Hughes *et al.*, 1992; Schnetz and Wang, 1996). For repression the Lac repressor was provided from a second plasmid pFDX500, a pACYC-derivative carrying the *lacI^R* gene (Schnetz *et al.*, 1990) (B. Rak and M. von Reutern, pers. comm.). Transformants of strain S219 with plasmids pFDX500 and pFDY400 were grown in LB2Y (10 g yeast extract (Difco), 10 g tryptone (Difco), 5 g NaCl per litre) containing 25 µg ml⁻¹ kanamycin and 50 µg ml⁻¹ ampicillin. At an OD₆₀₀ of 0.8 the expression of H-NS was induced with 1 mM IPTG (isopropyl- β ,D-thiogalactoside), and one hour after induction the cells were harvested. The cell pellet was washed in 100 mM KCl, 30 mM Tris-HCl pH 7.2, 7 mM 2-mercaptoethanol, 10% glycerol, and resuspended in 100 mM NH₄Cl, 30 mM Tris-HCl pH 7.2, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 7 mM 2-mercaptoethanol (1 ml buffer per 100 ml culture volume). Then the cells were lysed by sonification, the lysates were cleared by two consecutive centrifugations at 15000rpm for 20 min (SA600 Sorvall), and loaded onto a phospho-cellulose (P11, Whatman) column (volume 30 ml, equilibrated in 100 mM NH₄Cl, 30 mM Tris-HCl pH 7.2, 1 mM EDTA, 10% glycerol, 7 mM 2-mercaptoethanol). The proteins were eluted using an NH₄Cl gradient (100 mM to 500 mM) (120 ml). H-NS elutes at approximately 300 mM NH₄Cl. The fractions containing H-NS were pooled, diluted with 30 mM Tris-HCl pH 7.2, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 7 mM 2-mercaptoethanol to 100 mM NH₄Cl, and loaded onto a Q Sepharose (Sigma) column (4 ml) equil-

ibrated in 100 mM KCl, 30 mM Tris-HCl pH 7.2, 1 mM EDTA, 10% glycerol, 7 mM 2-mercaptoethanol. H-NS was eluted using a KCl gradient (100 mM to 500 mM KCl) (50 ml). H-NS elutes at approximately 300 mM KCl. Then the purified H-NS was concentrated to 600 ng ml⁻¹ (~40 pMol µl⁻¹) in 75 mM KCl, 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1.5 mM 2-mercaptoethanol and 20% glycerol using a Centricon centrifugational filter unit (cut-off 3000).

For shift experiments fragments were generated by PCR and agarosegel purified. Approximately 5 pmol of each fragment was labelled at the 5' end with T4 polynucleotide kinase and adenosine 5'-[γ -³²P]-triphosphate (5000 Ci mmol⁻¹). Non-incorporated nucleotides were removed using a Nick Sephadex™ G50 column (AmershamBiosciences). In the binding assays ~2 fmol of the labelled fragment (15000–30000 cpm, 0.2 nM final concentration) were incubated with various amounts of H-NS in 20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol in 10 µl for 15 min at 30°C, and separated on a 7.5% acrylamide, bis-acrylamide (29.2 : 0.8) gel in 0.5 × TBE (i.e. 45 mM Tris-borate pH 8.3, 1 mM EDTA), 2.5% glycerol which was run in the cold-room.

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