H-NS mediated repression of the *Escherichia coli bgl* and *proU* operons

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Abbrevations

bp	base pair(s)
CAA	chloroacetaldehyde
cAMP	3'-5'-cyclic adenosine monophosphate
CRP	catabolite regulator protein
DRE	downstream regulatory element
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid.
FIS	factor for inversion stimulation
H-NS	histone-like nucleoid structuring protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
OD	optical density
ONPG	o-nitrophenyl-β,D-galactopyranoside
PNPG	p-nitrophenyl β-D-glucuronide
URE	upstream regulatory element
wt	wild type

I   Zusammenfassung

Das Protein H-NS ist für die Organisation des bakteriellen Chromosoms und die globale Genregulation bei der Antwort auf Stimuli der Umgebung und auf Stress wichtig. In Enterobacteriaceae wie *Escherichia coli* reprimiert H-NS ~5% aller Gene. Die Repression durch H-NS erfolgt durch Binding von H-NS in der Nähe eines Promotors und der Bildung eines ausgedehnten Nukleoproteinkomplexes, der die Transkriptionsinitiation hemmt. Die DNA-Bindespezifität von H-NS ist gering (H-NS bindet präferentiell AT-reiche gekrümmte DNA), dennoch werden einige Loci sehr spezifisch durch H-NS reprimiert. Beispiele sind das *E. coli bgl* und *proU* Operon. In beiden Loci, sind den Promotor flankierende regulatorische Elemente (upstream and downstream regulatory elements, URE bzw DRE) für die Repression notwendig. Im *bgl*-Locus bindet H-NS 600 bis 700 bp unterhalb des Promotors und in *proU* bindet H-NS 150 bis 300 bp unterhalb. Ergebnisse dieser Arbeit belegen, dass die Repression von *proU* und *bgl* durch die Bindung an URE und DRE kooperativ ist. Weiterhin wurde gezeigt, dass die Repression durch Bindung von H-NS an das DRE von der Transkriptionsrate abhängt und durch den Transkriptionsterminationsfaktor Rho sowie die ko-transcriptionelle Translation beeinflusst wird. Rho und die Translation können die Transkriptions-elongationsrate modulieren. Aufgrund weiterer Experimente konnte ausgeschlossen werden, dass H-NS als Prellbock ('roadblock') für die transkribierende RNA-Polymerase wirkt. Im *bgl* Operon vermindert H-NS die Transkriptionselongation nur 2-fach und in *proU* hat H-NS keinen Effekt auf die Elongation. Diese Experimente schließen CAA-Footprinting pausierender RNA-Polymerase-Transkriptionselongations-Komplexe, Northern Analysen, und Expressionsanalysen mit Hilfe eines Zwei-Reporter-Gen-Systems ein. In letzterem wurde das *bgl*- bzw. *proU*-DRE zwischen das *uidA* (β-Glucuronidase) Gen und dem *lacZ* (β-Galactosidase) Gen eingefügt. Weitere Experimente zur Analyse des *bgl*-DRE und umliegender Sequenzabschnitte zeigten, dass zwischen *bgl*-Promotor und H-NS Bindestelle eine intrinsische Pausenstelle für die Transkription kartiert. Die Deletion dieser Stelle hatte keinen Effekt auf die Repression von *bgl* durch H-NS. Jedoch ist der DNA-Abschnitt zwischen *bgl*-Promotor und *bgl*-DRE für die Repression wichtig.

Die Daten dieser Arbeit und laufende Experimente im Labor zeigen, dass bei Bindung von H-NS an das DRE die Transkriptionsinitiation am *bgl* und am *proU*-Promotor gehemmt wird. Vermutlich führt die Bindung von H-NS an das DRE zur Bildung eines reprimierenden Nukleoproteinkomplexes, der einen DNA-Abschnitt oberhalb des Promotors mit einschließt. Die so induzierte DNA-Schleifenbildung (DNA-Loop) führt zur Repression der Transkriptionsinitiation. Weiterhin belegt diese Arbeit die Bedeutung des Transkriptions-prozesses an sich, also der Transkriptions-Initiation und der Elongation, für die Effizienz der Repression durch H-NS. Eine effiziente Repression erfolgt nur bei geringer Transkriptionsrate, während bei einer Erhöhung der Transkriptionsrate der durch H-NS gebildete reprimierende Komplex vermutlich destabilisiert wird und die Loci maximal exprimiert werden.

I   Summary

The histone-like nucleoid structuring protein H-NS is important in the organization of the bacterial chromosome and in global gene regulation in response to environmental stimuli and stress conditions. In Enterobacteriaceae such as *Escherichia coli* H-NS represses ~5 percent of all genes. Repression by H-NS is presumably mediated by binding of H-NS next to a promoter, and the formation of extended nucleoprotein complex, which inhibits transcription initiation. Although the specificity of binding of H-NS to DNA is low (it binds weakly specific to AT-rich curved DNA), some loci are very specifically repressed by H-NS including the *E. coli bgl* and *proU* operons. In both of these systems, upstream and downstream regulatory elements are required for efficient repression. In *bgl* H-NS binds 600 to 700 bp downstream to the promoter and in *proU* it binds 150 to 300 bp downstream. The analysis done here suggests that repression of *proU* and *bgl* by binding of H-NS to upstream and downstream regulatory elements is cooperative. Furthermore, it was shown that in the absence of the upstream regulatory element (URE), repression by H-NS binding to the downstream regulatory element (DRE) depends on the transcription rate. Termination factor Rho and co-transcriptional translation, which both modulate the transcription rate, were shown to also affect repression by H-NS *via* the DRE. Further experiments excluded, that H-NS acts as a roadblock to the transcribing RNA polymerase. In the *bgl* operon H-NS represses transcription elongation merely 2-fold and in *proU* it has no effect on elongation. These experiments include CAA-footprinting of stalled RNA polymerase transcription elongation complexes, Northern analysis, and a dual reporter gene system with the *bgl* and *proU* DRE, respectively, inserted in between *uidA* (β-glucuronidase) and *lacZ* (β-galactosidase). In addition, the analysis of structural components in *bgl* revealed an intrinsic transcription pause site located in between the promoter and the *bgl*-DRE. However, the deletion of the pause did not affect repression. Additional deletion analyses suggest that the DNA segment upstream of the *bgl*-DRE is important for repression.

The data shown here and ongoing experiments suggest that binding of H-NS to the DRE inhibits transcription initiation at the *bgl* and *proU* promoter, respectively. Possibly, H-NS bound to the DRE traps a DNA segment located upstream of the promoter resulting in DNA looping and repression of transcription initiation. Furthermore, the present work highlights the significance of the transcription rate and the process of transcription elongation in the modulation of H-NS mediated repression. Presumably, an increase in the transcription rate de-stabilizes the repressing complex formed by H-NS and thus causes full expression.

II.   Introduction

The bacterial histone-like nucleoid structuring protein (H-NS) was initially described as heat stable nucleoid associated protein (Falconi et al., 1988; Friedrich et al., 1988). H-NS is a small basic protein of 15.6KDa, present at around 20,000 molecules per genome equivalent and is highly conserved in gram negative bacteria (Falconi et al., 1988; Tendeng and Bertin, 2003; Ali et al., 1999; Azam and Ishihama, 1999). H-NS plays an important role as an architectural protein and also in transcriptional regulation (Dorman, 2004; Luijsterburg et al., 2006). By proteomic and genomic methods it was shown that H-NS affects approximately 5% of the *E. coli* genes, many of which play a role in adaptation to environmental stimuli (Bertin et al., 2001; Hommais et al., 2001). H-NS acts as a global transcriptional repressor since most of the genes reported to be regulated by H-NS are repressed (Dorman, 2004). H-NS also affects major DNA transactions, such as DNA replication, transposition, recombination, and constrains supercoils (Dorman, 2004; Rimsky, 2004; Rouquette et al., 2004). A wealth of information is available about H-NS and the genes it regulates, but the mechanism by which H-NS causes specific repression of transcription has remained a largely open question.

1.   Structural features of H-NS

H-NS does not exhibit high affinity for specific sequences but binds preferentially to AT-rich and intrinsically curved DNA sequences (Dame et al., 2001; Rimsky et al., 2001; Schroder and Wagner, 2002; Ussery et al., 1994). The H-NS protein is 136 amino acids in length and can be divided into three structural parts. The N-terminal domain of H-NS extends up to the 65th amino acid residue (figure 1). The carboxy-terminal domain extends from amino acid residue 90 until the end of the protein. Both these domains (N- and C-terminal) are connected together by a highly flexible linker domain (Badaut et al., 2002; Bloch et al., 2003; Dorman et al., 1999; Esposito et al., 2002). To date the crystal structure of H-NS is unavailable, however the NMR structure of the N-terminal and the C-terminal regions have been resolved (Bloch et al., 2003; Shindo et al., 1999; Ueguchi et al., 1997; Shindo et al., 1995; Esposito et al., 2002). The functional significance of these individual domains was studied extensively using truncated H-NS proteins (Ueguchi et al., 1997; Ueguchi et al., 1996)(figure 1A). These results demonstrate that the C-terminal domain is involved in DNA binding and that the N-terminal domain is required for dimerization of the protein. The unstructured flexible domain is involved in formation of higher order oligomers of the protein (Badaut et al., 2002; Bloch et al., 2003; Esposito et al., 2002). The active from of H-NS is believed to be a dimer although higher oligomers have been observed in solution (Falconi et

al., 1988; Smyth et al., 2000). It is assumed that upon DNA binding lateral interaction of H-NS dimers allows the extension of H-NS binding from high affinity 'nucleation sites' to flanking low affinity sequences (Rimsky et al., 2001). The oligomerization property of H-NS is important for its role as transcriptional repressor (Badaut et al., 2002; Rimsky et al., 2001).
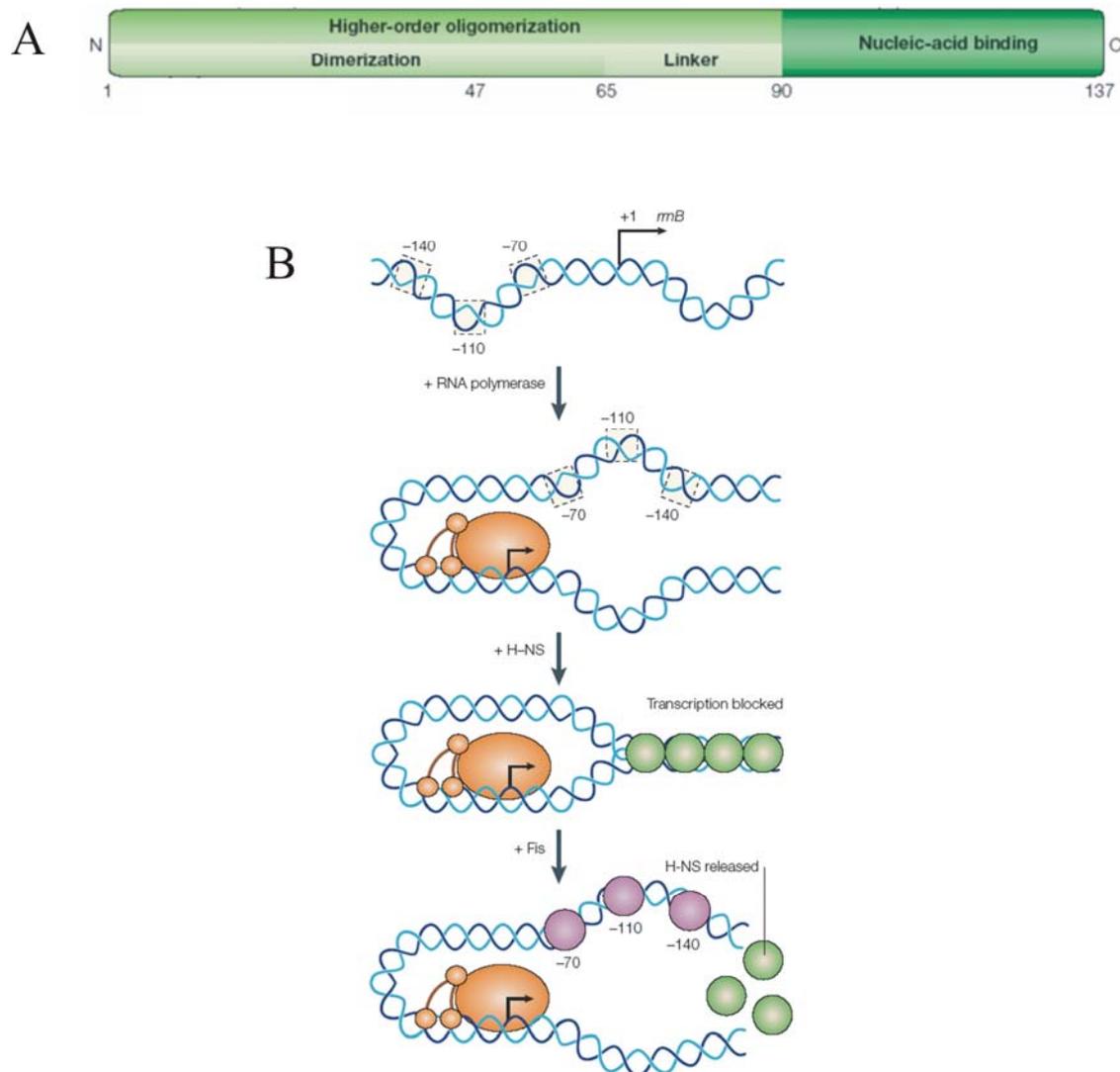
Figure 1. **Domain architecture of H-NS and repression of *rrnB P1* promoter by H-NS**: A) The domain structure of H-NS is shown schematically. The numbers indicate the amino acid residues. N and C refer to N and C-terminal end of H-NS. The dimerization, linker and nucleic acid binding domain are indicated. B) Schematic representation of H-NS mediated repression of the *rrnB P1* promoter. The *rrnB P1* promoter region along with the flanking regions are shown in blue. The RNA polymerase (shown in orange) binds to the promoter region and H-NS shown in green traps RNA polymerase at the promoter. The antagonist protein FIS binds to regions upstream to the promoter shown as dotted boxes and activates transcription presumably by disrupting the repression complex. The figure is modified from (Dorman, 2004).

2.   Repression by H-NS

Recent genomic approaches revealed that H-NS binds specifically to approximately 250 loci in the *E. coli* chromosome and thus may regulate ~1000 genes (Oshima et al., 2006). However, the mechanism of regulation by H-NS has been studied in detail only for a very few genes. How the activity of H-NS is modulated and how high specificity of repression by H-NS is achieved are largely open questions.

Based on studies of the ribosomal *rrnB P1* promoter, binding of H-NS to an AT-rich curved DNA upstream of the promoter, allows H-NS to form a bridge to a DNA sequence downstream to the promoter (figure 1B). The DNA bridging by H-NS zips the two double strands that flank the promoter together and traps RNA polymerase at the promoter (Dame et al., 2002; Dame et al., 2005) (figure 1B). A similar mechanism of repression by H-NS has also been shown for the *hdeAB* promoter (Shin et al., 2005). This view of the mechanism of repression by H-NS was recently further substantiated using a biophysical approach, in which binding of H-NS to two single DNA molecules was analyzed (Dame et al., 2006). It was shown that one dimer of H-NS can bind to two DNA double strands and thus form a bridge between them. The bridge forming unit, one H-NS dimer, occupies one helical repeat of DNA (Dame et al., 2006).

For a few of the H-NS repressed loci it has been shown that binding of H-NS upstream and downstream to the promoter is crucial for repression. This has been first discovered for the *proU* operon, where H-NS binds upstream to the promoter and 150 to 300 bp downstream of the transcription start, and later for the *bgl* operon and the *hilA* operon (Olekhnovich and Kadner, 2006; Overdier and Csonka, 1992; Lucht et al., 1994a; Schnetz, 1995). All three loci are very specifically (more than 50-fold) repressed by H-NS (see below). Studies on an additional locus, the *eltAB* operon of the enterotoxic *Escherichia coli* (ETEC) showed that the operon is repressed by binding of H-NS to sequences downstream to the promoter. It was further shown that in this case H-NS does not affect open complex formation at the promoter but possibly promoter clearance or transcription elongation (Yang et al., 2005).

3.   Modulation of repression by H-NS

Many genes controlled by H-NS are specifically activated by proteins (transcription factors), which act as antagonist to H-NS (anti-repressors). The autoregulated *hns* gene is activated by another nucleoid associated protein FIS and by the cold shock protein CspA (Falconi et al., 1996; La et al., 1991). Studies on many other genes such as *cfaAB*, *pap,* and *coo*, which are repressed by H-NS, demonstrated activatation by *CfaD*, *PapB*, and *Rns* as anti-

repressor proteins, respectively (Forsman et al., 1992; Jordi et al., 1992; Murphree et al., 1997).

H-NS has been reported to form heterodimers with other proteins suggesting additional modes of H-NS activity modulation. Genetic and biochemical studies on StpA, an intraspecies homologue of H-NS have shown that H-NS and StpA form heterodimer complexes *in vivo* (Cusick and Belfort, 1998; Free et al., 1998; Williams et al., 1996). Both H-NS and StpA share similar domain structures and StpA can complement some *hns* mutant phenotypes (Free et al., 2001; Shi and Bennett, 1994). Another example of this group includes the *Shigella flexineri* serotype 2a Sfh protein, which shares 59% sequence homology with H-NS. This protein also has the capability to partially complement *hns* mutant phenotypes (Doyle and Dorman, 2006; Beloin et al., 2003; Deighan et al., 2003).

H-NS activity may further be modulated by its interaction with H-NS like proteins such as Hha, H-NS$_T$ and Ler (H-NS$_T$ and Ler are found in enteropathogenic *E. coli*) (Williamson and Free, 2005; Nieto et al., 2002; Juarez et al., 2000; Haack et al., 2003). All these proteins share homology to different domains of H-NS and affect the gene expression by interaction with H-NS, but the molecular mechanism is not understood. Hha and H-NS$_T$ share similarity with the N-terminal domain of H-NS. Therefore they are proposed to heterodimerize with H-NS and affect the oligomerization of H-NS thus modulating the repression by H-NS (Garcia et al., 2005; Rodriguez et al., 2005; Williamson and Free, 2005). The Ler protein which shares similarity with the C-terminal DNA binding region of H-NS was proposed to affect H-NS binding in the LEE locus in enteropathogenic *E. coli* (Haack et al., 2003; Sperandio et al., 2000; Elliott et al., 2000).

Most of the genes regulated by H-NS respond to environmental stimuli and some studies have been done on the modulation of the physical properties of H-NS based on change in conditions. *In vitro* the addition of osmolytes such as NaCl or KCl and increased temperature affects the binding of H-NS to DNA (Amit et al., 2003). Although the H-NS:DNA interaction was affected by a change in osmolarity or temperature, a change in DNA structure upon these conditions cannot be ruled out. Furthermore H-NS was reported to be modified post transcriptionally by poly-(R)-hydroxybutyrate but the physiological significance remains enigmatic (Reusch et al., 2002).

4. The *bgl* and *proU* operons of *E. coli*

The *bgl* and *proU* operon in *E. coli* are repressed by H-NS with exceptionally high specificity, and in both loci H-NS binding upstream and downstream of the promoter is required for effective repression (Bertin et al., 1990; Mukerji and Mahadevan, 1997; Schnetz

and Wang, 1996; Schnetz, 1995; Lucht et al., 1994b; Overdier and Csonka, 1992; Druger-Liotta et al., 1987). The *bgl* operon encodes gene products necessary for the uptake and fermentation of aryl-β,D-glucosides; it is repressed ~100 fold by H-NS (Higgins et al., 1988; Lopilato and Wright, 1990; Schnetz, 1995). The *bgl* operon consist of six genes namely *bglG*, *F*, *B*, *H*, *I*, and *K*. Two Rho-independent transcriptional terminators, *t1* and *t2*, flank the first gene of the operon *bglG*, which encodes an antiterminator, BglG (Mahadevan and Wright, 1987; Schnetz and Rak, 1988; Schnetz et al., 1987)(figure 2). To date no conditions are known that activate the *bgl* operon, yet the operon is highly conserved among *E. coli* population (Neelakanta, 2005) (Thirupathy, pers. communication). Silencing by H-NS is overcome by spontaneous mutations, which map close the CRP-dependent promoter including the deletion of an AT-rich regulatory region upstream of the promoter, integration of insertion elements, and point mutations that improve the CRP-binding site. Activation by these mutations indicate that H-NS represses the *bgl* promoter (Reynolds et al., 1986; Reynolds et al., 1981; Schnetz, 1995; Schnetz and Rak, 1992; Lopilato and Wright, 1990; Mukerji and Mahadevan, 1997). However, *in vitro* repression of the *bgl* promoter by H-NS is merely 4 to 5 fold (Schnetz and Wang, 1996). The sequence flanking the promoter upstream and downstream were reported to be necessary for efficient repression by H-NS and based on this a silencing nucleoprotein complex formation by H-NS in *bgl* was proposed (Schnetz, 1995). The repression of *bgl* by H-NS is modulated by various other proteins such as FIS, BglJ, LeuO, Lon, Hfq and DnaKJ (Caramel and Schnetz, 1998; Dole et al., 2004a; Giel et al., 1996; Madhusudan et al., 2005; Ueguchi et al., 1998). However, the mechanism of specific repression by H-NS is not clearly understood.

The *proU* operon in *E. coli* consists of three genes, *proV*, *proW* and *proX*, and encodes a high affinity glycine-betaine uptake system, which is essential for cell survival in media of high osmolarity (Gowrishankar, 1989; May et al., 1986). In *E. coli* two promoters P1 and P2 have been identified for *proU*, which are located 250 and 60 bp respectively upstream to the *proV* gene (Gowrishankar, 1989; Lucht et al., 1994a; Rajkumari et al., 1996b) (figure 2). The induction of the *proU* operon by osmotic shock is remarkable, and the level of expression correlates with the osmolarity of the medium (May et al., 1986; Higgins et al., 1988; Gowrishankar, 1985; Gowrishankar, 1986). Trans-acting mutations that activate the *proU* operon map in *topA* (Topoisomerase I) and in the *hns* gene (Higgins et al., 1988). The repression of *proU* by H-NS is also highly specific and requires the presence of flanking sequence upstream and downstream to the promoter (Druger-Liotta et al., 1987; Barr et al., 1992; Overdier and Csonka, 1992; Lucht et al., 1994b; Rajkumari et al., 1997; Jordi and

Higgins, 2000). *In vitro* studies done in *proU* were not able to reproduce the highly specific repression by H-NS observed *in vivo* (Jordi et al., 1997b; Jordi and Higgins, 2000). Furthermore it was shown that binding of H-NS to the downstream regulatory region affects the open complex formation at the promoter (Jordi and Higgins, 2000). The mechanism of osmotic induction of *proU* is not clearly understood. One model suggests that the accumulation of intracellular potassium glutamate ions upon osmotic shift activates transcription initiation at the *proU* promoter (Higgins et al., 1988). Another model explains the modulation of gene expression conferred by change in DNA topology during osmotic stress (Hulton et al., 1990; Owen-Hughes et al., 1992). As in the case of *bgl* the precise mechanism of specific repression by H-NS in *proU* remains enigmatic.
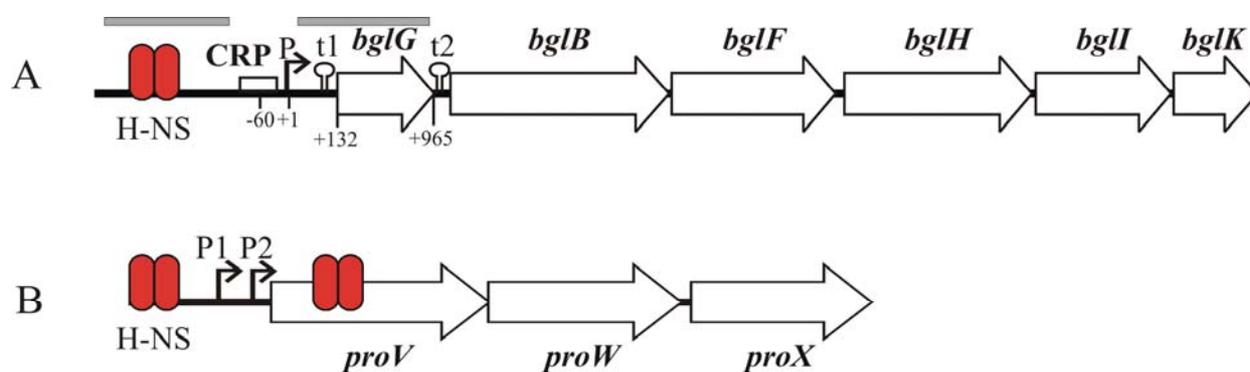
Figure 2. The *E. coli bgl* and *proU* operons. A) Schematic showing *bgl* operon with the promoter (P), the CRP binding site (CRP), the Rho independent terminators (t1 and t2) and the structural genes (*bglG,B,F,H,I* and *K* respectively). It was proposed that H-NS (shown in red ovals) binds to the AT-rich sequence upstream to the promoter and forms an extended repression complex, which is indicated by grey bar (Caramel and Schnetz, 2000; Schnetz, 1995). B) The *E. coli proU* operon contains three structural genes *proV*, *proW* and *proX*, and is driven by promoters P1 and P2. The binding site of H-NS upstream and downstream to the promoter is shown by red ovals.

5.   Salient features of transcription in *E. coli*

Since repression by H-NS is tightly coupled to the transcription process essential features of the transcription cycle are briefly summarized here. The transcription cycle is composed of three broadly classifiable steps namely initiation, elongation and termination. Each of this process involves complex mechanisms and is regulated by various factors (reviewed in (Mooney et al., 1998) (see figure 3). Upon promoter engagement by RNA polymerase, the DNA duplex of the promoter is melted by ~12bp. The melted region extends from -10 to +1 relative to the transcription start site (deHaseth and Helmann, 1995). This process may be accompanied by a RNA polymerase conformational change and is termed 'open complex' formation (Browning and Busby, 2004). After the open complex has formed transcription is initiated and RNA of 7 to 9 bp is synthesized. In this 'initial transcription complex' RNA polymerase remains strongly associated with the promoter. Initial

transcription may result in the release of the short RNA transcripts, which is termed 'abortive transcription', or it may result in clearing of the promoter and transition to the transcription elongation phase. This requires overcoming the interaction of RNA polymerase to the promoter DNA (Tadigotla et al., 2006; Kapanidis et al., 2006). The regulation and kinetics of these steps determine the efficiency of promoter clearance, and thus the 'strength' of a promoter see review from (Mooney et al., 1998). After promoter clearance the RNA polymerase-DNA-RNA complex is stabilized by RNA polymerase-DNA contacts, RNA polymerase-RNA contacts, and by the RNA-DNA hybrid until it reaches the termination signal. Transcription elongation complexes serve as a important targets for regulatory factors (Greive and von Hippel, 2005; Borukhov et al., 2005).
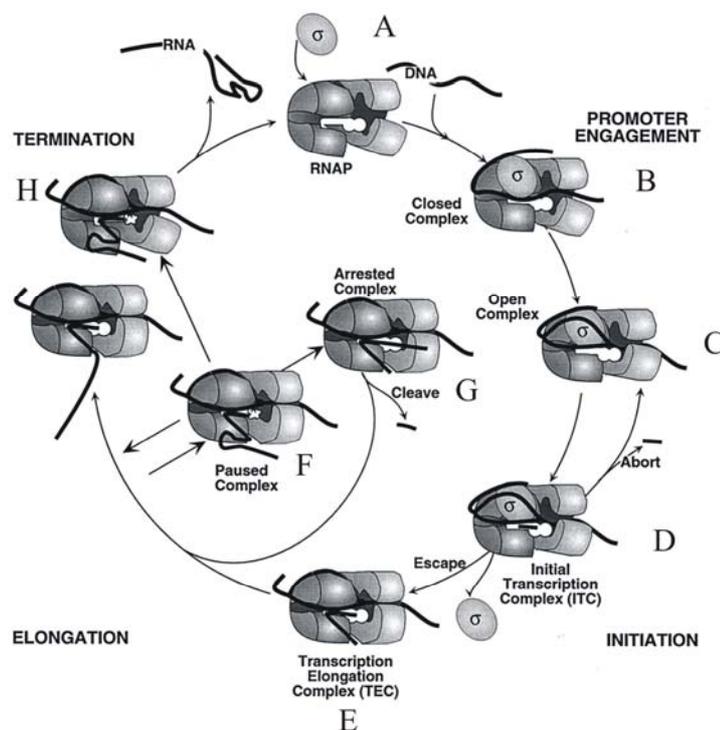
Figure 3. The transcription cycle in *E. coli*: A) The preinitation complex with RNA polymerase (RNAP), DNA, σ factor. B) The promoter engagement where the RNA polymerase-σ factor holoenzyme binds to the promoter DNA. C) The open complex formation where the duplex DNA melts in the promoter region. D) Formation of the initial transcription complex, with the addition of the first nucleotide. E) The transcription elongation complex where RNA polymerase translocates along the DNA concomitantly transcribing RNA. F) RNA polymerase becomes paused: in this case due to a RNA secondary structure as shown in the figure. The paused complex either can move into arrested complex (G) or can lead to termination (H) depending upon various conditions. G) The paused complex becomes arrested and is unable to translocate further until other cellular factors act to release the protruding RNA. H) Termination of transcription, where the RNA polymerase finally will dissociate from the DNA releasing the RNA. Figure modified from (Mooney et al., 1998).

Transcription elongation is not a uniform process; it is marked by various punctuations caused by pausing, arrest and transcription termination. Pausing involves RNA polymerase to isomerize from the rapidly translocating complex to alternative conformation where RNA chain extension becomes reversibly inhibited (Dalal et al., 2006; Herbert et al.,

2006; Landick, 2006; Tadigotla et al., 2006). Pausing is also a prerequisite for transcription termination or anti-termination and occurs by at least two mechanisms. Firstly, RNA secondary structures at the exit channel of RNA polymerase induce pausing and prevent further nucleotide addition (Artsimovitch and Landick, 2000). Secondly, pausing occurs by elements, which act as physical barriers to RNA polymerase translocation. These include roadblocks by DNA-binding proteins, misincorporated substrates, DNA lesion and special DNA sequences (Fish and Kane, 2002). Both of these pausing, depending on various conditions, can lead to transcription termination. Pausing of transcription elongation can play a role in gene regulation, for example as a means to allow synchronization of transcription and translation (Landick et al., 1996), and the binding of cofactors, which modify transcription and facilitate co-transcriptional folding of transcripts (Artsimovitch and Landick, 2002; Palangat et al., 1998; Bailey et al., 1997; Richardson and Greenblatt, 1996).

6. Aim of the Thesis

The aim of the thesis is to study the mechanism of repression by H-NS in the *Escherichia coli bgl* and *proU* operon with special emphasis on the regulation by H-NS bound to the downstream regulatory elements.

III.   Results

1.   H-NS represses the *bgl* operon upstream and downstream to the promoter

Part of data in this section has been published (Dole,S., Nagarajavel,V., and Schnetz,K. (2004). The histone-like nucleoid structuring protein H-NS represses the *Escherichia coli bgl* operon downstream of the promoter. Mol. Microbiol. *52*, 589-600).

Specificity of repression of the *E.coli bgl* operon by H-NS is exceptionally high. This specific repression requires regulatory elements located upstream and downstream to the promoter (Dole, 2001; Schnetz, 1995). Biochemical experiments have shown binding of H-NS to the upstream regulatory element (URE) (Dole, 2001). To analyze whether H-NS binds specifically to the downstream regulatory element (DRE) or whether repression *via* the downstream regulatory element is indirect, a systematic approach to study the H-NS binding in *bgl* operon was carried out.

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

The *bgl* operon is effectively silenced only when the upstream and downstream regulatory elements are together (Dole, 2001; Schnetz, 1995).To characterize the H-NS binding regions in the *bgl* operon, DNA electophoretic mobility shift assays (EMSA) were performed. Briefly, 5' end labeled DNA fragments corresponding to different regions of the *bgl* operon were incubated with increasing concentrations of H-NS. The H-NS:DNA complexes were resolved on 7.5% non-denaturing polyacrylamide gels and the shifts were examined by autoradiography. The fragments used and the results of the shifts are shown in figure 4. Fragment I, which includes the upstream regulatory element (URE) and the *bgl* promoter (-165 to +25 relative to the transcriptional start site) was efficiently shifted by H-NS. Fragment Ia, which includes the core promoter region from position -75 to +25 was also shifted by H-NS, but with weaker affinity compared to fragment I (figure 4). No binding was detected for fragment II (position +132 to +459) and fragment IV (position +669 to +965), which map within the *bglG* coding region. However, fragment III (position +450 to +737) was efficiently shifted by H-NS (figure 4). To further narrow down the binding site on fragment III, smaller PCR fragments were used for shifts (IIIa, IIIb, IIIc). The results show specific binding of H-NS to the fragment IIIc (position +609 to +737); a weak binding was also observed with fragment IIIb (position +532 + 650) (figure 4). Fragment V (position +1 to +131) which maps within the leader sequence of the *bgl* operon was weakly shifted by H-NS. The specificity of

the binding was further confirmed by competitive shift experiments. In these, binding of H-NS to fragment I and IIIc, respectively, was effectively competed for by unlabelled fragment I and IIIc, and not by non-specific competitor DNA (Appendix, figure 18).
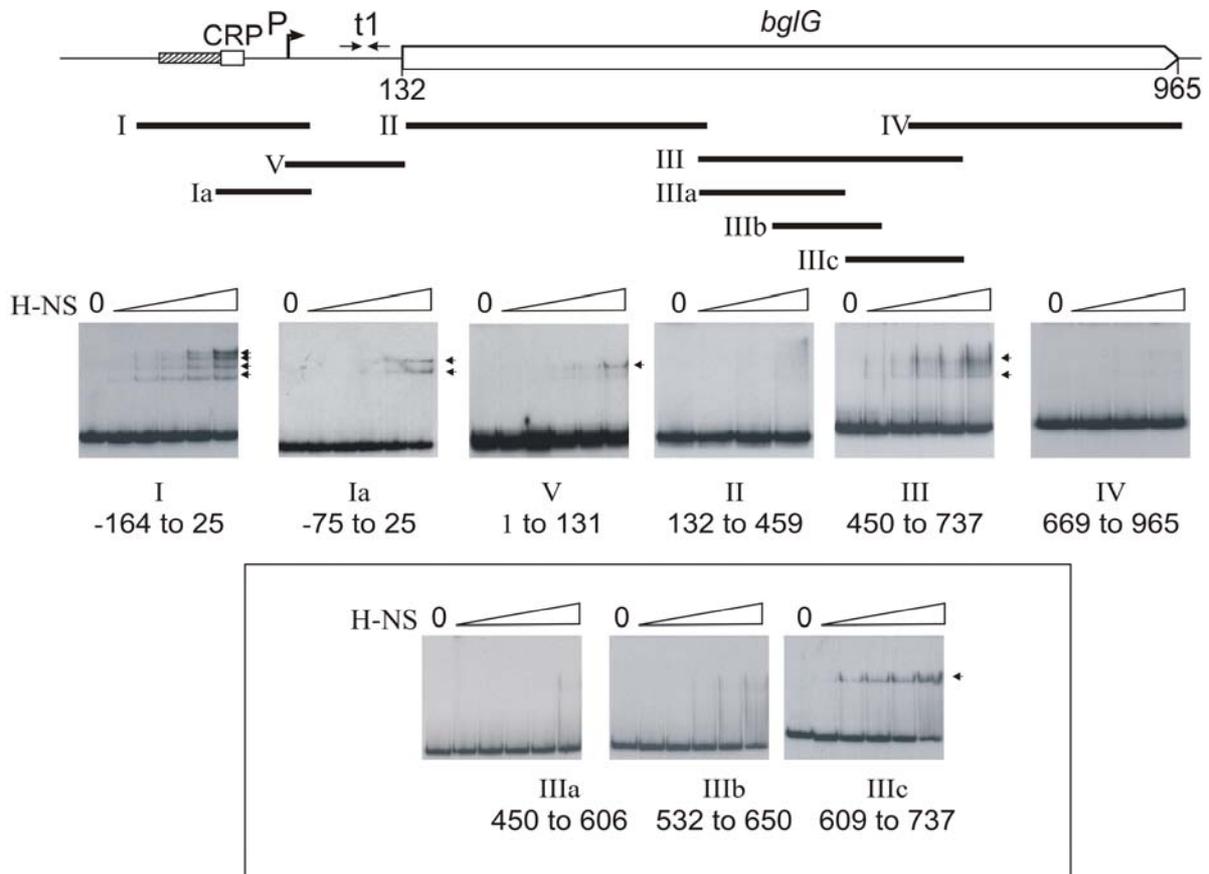
Fig. 4 Mapping of H-NS binding sites in *bgl*. The binding sites of H-NS within the downstream regulatory region were mapped by electrophoretic mobility shift experiments. In addition, binding of H-NS to the upstream regulatory element and the promoter was analyzed. Top: The structure of the *bgl* regulatory region including the upstream and the downstream regulatory region with terminator t1 and *bglG* is shown schematically. Fragments I, Ia, II, III, IIIa, IIIb, IIIc, IV and V used in the mobility shift experiments are represented by horizontal bars. Lower part and boxed area: The different [32P]-labeled 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 labeled fragment was 0.2 nM.

Since H-NS is known to bind to curved DNA sequences, computational analysis for DNA bending in *bgl* operon was carried out using the bend.it tool available at http://www.icgeb.trieste.it/dna/. Planar bends were predicted between regions -115 to -68 and + 615 to +700. These regions are encompassed on fragment I and fragment III, respectively, which were bound by H-NS in the DNA shift experiments (data not shown).

Taken together, these data show specific binding of H-NS to DNA upstream to the promoter, the promoter core, and ~600-700 bp downstream to the promoter. Both the upstream and the downstream binding sites show planar bends based on *in silico* analysis.

H-NS is known to bind RNA, although with weaker affinity than binding to DNA (Zhang et al., 1996; Sonnenfield et al., 2001; Brescia et al., 2004). To determine whether H-NS mediated repression through the downstream regulatory element involves binding to the *bgl*-RNA, EMSA experiments were performed using *in vitro* transcribed *bgl*-RNA fragments. The results and the RNA fragments used are shown in appendix, figure 19. Fragments I (position +132 to +459), II (position +367 to +737), III (position +637 to +965) which covers the entire *bglG* RNA were not shifted by H-NS eliminating the hypothesis that repression is mediated by binding of H-NS to the *bgl* RNA.

2   Cooperativity in repression of the *bgl* and *proU* operon by H-NS

The repression of the *bgl* and *proU* operons by H-NS is exceptionally high and in both operons the repression is achieved by specific binding of H-NS to upstream and downstream regulatory elements flanking the promoter (Dole et al., 2004b; Schnetz, 1995; Jordi and Higgins, 2000; Mellies et al., 1994a; Overdier and Csonka, 1992; Owen-Hughes et al., 1992).

2.1   Effective repression of the *bgl* operon requires both upstream and downstream regulatory elements

H-NS is the major component of the repression complex in the *bgl* operon. However *in vivo* and *in vitro* experiments indicated the efficiency of promoter repression is effective only when regulatory elements are present on both sides of the promoter (Schnetz, 1995; Schnetz and Wang, 1996; Dole et al., 2004b). To re-examine the contributions of the regulatory elements in repression, *lacZ* reporter fusions were constructed that carry the upstream or/and downstream regulatory elements. The reporter fusions were integrated into the chromosome of the *wt* and *hns* mutant and the expression level of β-galactosidase was determined.

A chromosomally encoded bgl_{URE}-Pbgl-$t1_{RAT}$-bgl_{DRE}-*lacZ* fusion, which carries the *bgl* promoter, flanked by the upstream (URE) and downstream regulatory elements (DRE) in the natural context (Figure 5a) was used for β-galactosidase activity measurements. In this construct, the terminator in the leader was inactivated to determine the expression independent of the antiterminator BglG. To this end, the leader sequence at position +67 to +68 relative to the transcription start site carries a mutation from AA to T resulting in stabilization of the secondary structure RAT and *BglG* RNA binding motif (the secondary

structure is shown in the appendix, Figure 20). As a result, the terminator t1 hairpin loop can not form (hereafter this mutation is referred as t1$_{RAT}$). In the *wt* background *bgl$_{URE}$-Pbgl-t1$_{RAT}$-bgl$_{DRE}$-lacZ* directed the expression of very low levels of β-galactosidase activity (16 units), whereas in the *hns* mutant the expression increases 33 fold to 533 units (figure 5a).

The expression directed by a *bgl$_{URE}$-Pbgl-lacZ* fusion consisting of the *bgl* promoter along with the upstream regulatory element alone was 128 units in *wt* and increased ~2-fold to 278 units in the *hns* mutant (figure 5b). This confirms that the downstream regulatory element is necessary for efficient repression.

To further address the role of the downstream regulatory element, the *bgl* promoter was replaced by a constitutive variant of the *lacUV5* promoter lacking the *lac* operator. The *lacUV5* promoter is not bound by H-NS, while the *bgl* core promoter is bound by H-NS (figure 4). In addition, the *lacUV5* promoter has the advantage that it is not catabolite regulated and is independent of CRP-cAMP. A chromosomally encoded *bgl$_{URE}$-PUV5-t1$_{RAT}$-bgl$_{DRE}$-lacZ*, which carries a replacement of the *bgl* promoter by the *lacUV5* promoter (PUV5) flanked by upstream and downstream regulatory elements of the *bgl* operon and derivatives of it were used for expression analysis in the wild-type and *hns* mutant (figure 5 c-g). In the *wt* the expression level directed by *bgl$_{URE}$-PUV5-t1$_{RAT}$-bgl$_{DRE}$-lacZ* was 39 units of β-galactosidase activity and increased ~20-fold to 763 units in the *hns* mutant (figure 5c). This shows that replacing the *bgl* promoter with the constitutive *lacUV5* promoter did not significantly affect the repression by H-NS. A *bgl$_{URE}$-PUV5-lacZ* fusion consisting of the *lacUV5* promoter along with the *bgl*-URE (upstream regulatory element) alone expressed 376 units in the *wt* and 405 units in the *hns* mutant (figure 5d). Thus the *bgl*-URE alone is not sufficient for repression of the *lacUV5* promoter by H-NS. The difference in repression of the *lacUV5* promoter by the URE and the *bgl* promoter, which is repressed 2-fold, maybe due to binding of H-NS to the *bgl* core promoter, but not to the *lacUV5* core promoter.

To determine the efficiency of repression by H-NS *via* the downstream regulatory element, a *PUV5- t1$_{RAT}$-bgl$_{DRE}$-lacZ* construct encompassing the *t1$_{RAT}$* and *bgl$_{DRE}$* inserted in between the constitutive *lacUV5* promoter (*PUV5*) and *lacZ* was used (figure 5e). In the *wt* background the *PUV5-t1$_{RAT}$-bgl$_{DRE}$-lacZ* construct directed 239 units, and the level of expression increased 3.6 fold to 860 units in the *hns* mutant (figure 5e).

Another reporter expressing *bgl$_{DRE}$* from position +95 to +972 fused to *lacZ* and driven by *PUV5* promoter was used for β-galactosidase activity measurement (*PUV5-bgl$_{DRE}$-lacZ*, figure 5f). The expression of this construct was 400 units and 961 units in *wt* and *hns* mutants respectively resulting in ~2.5fold repression by H-NS. A derivative of *PUV5-bgl$_{DRE}$-lacZ*,

which carries bgl_{DRE} with mutation of start codon and two additional AUG triplets at position 3 and 27 to GCG thereby eliminating translation of $bglG$ ($PUV5$-bgl_{DRE-NT}-$lacZ$, figure 5g) expressed 91 units in wt and 700 units in hns mutant resulting in ~7 fold repression by H-NS (Dole et al., 2004b)(figure 5g). This result shows that translation of bgl_{DRE} affects the efficiency of repression by H-NS. To rule out effects of H-NS on expression of $lacZ$, control experiments were done where the expression of $PUV5$-$lacZ$ was measured in wt and hns mutants (figure 5h). The level of expression of this construct in the wt was 4665 units (when grown in LB with 100 mM NaCl concentration) and 4340 units in the hns mutant. From this we can conclude that the repression of bgl by H-NS is specific.
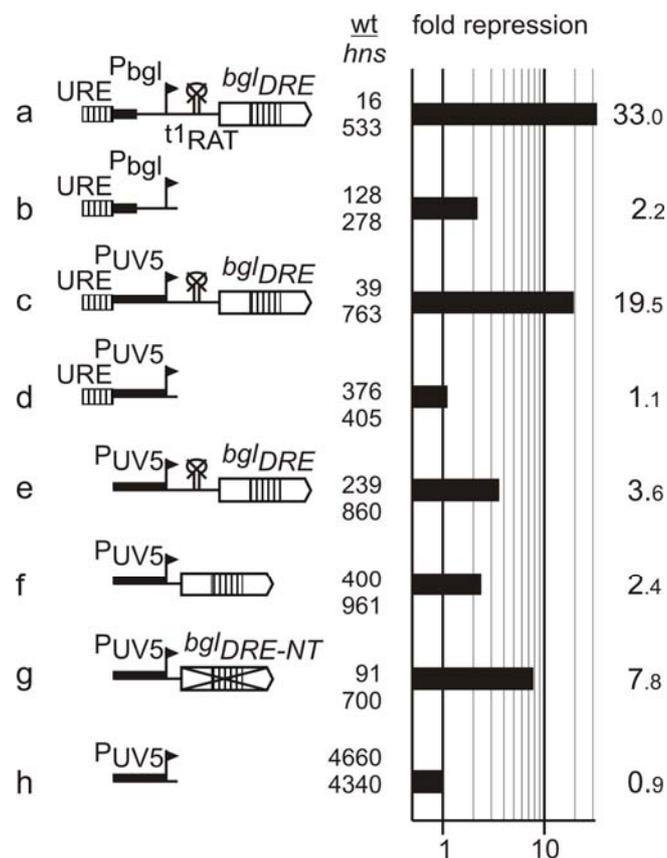
Figure 5. Cooperativity in repression by H-NS in the bgl operon. Chromosomal integrants having the bgl_{URE} and bgl_{DRE} in its natural context, the bgl_{URE} or the bgl_{DRE} alone expressed by $Pbgl$ or $PUV5$ are schematically shown from a to g. All the constructs have $lacZ$ fused transcriptionally to the 3' end for β-galactosidase activity measurement. Constructs from a to g were grown in LB media and construct (h) was grown in LB media with 0.1M NaCl LB for β-galactosidase assay. The numbers shown at the left of the bar chart represent the β-galactosidase values in units in wt and hns mutant. The values on the right show the fold repression by H-NS. The β-galactosidase values are average of at least 3 experiments and the standard deviation is less than 10%. The bar chart shows the fold repression by H-NS in black bars along the x-axis in logarithmic scale. Strains used are shown in the order wt, hns a) S3181, S3203 b) S1213, S3296 c) S3191, S3205 d) S3412, S3298 e) S1816, S3209 f) S1193, S3211 g) S1195, S3207 and h) S1907, S3122.

Taken together the data show that H-NS represses expression 1.5 to 3.5-fold when either the upstream or the downstream regulator element is present. However, repression is

significantly more efficient when both the upstream and downstream regulator elements are present together (20 to 33 fold repression). This enhancement suggests that repression by binding of H-NS to upstream and downstream regulatory elements is cooperative.

2.2   H-NS efficiently represses the *proU* operon through upstream and downstream regulatory elements.

The basal expression of *proU* operon is low, but it is rapidly induced when cells are grown in high osmolarity media (Overdier and Csonka, 1992; Jordi et al., 1997a; Dattananda et al., 1991). At low osmolality the *proU* operon is repressed by H-NS binding to regulatory elements that map upstream and downstream of the promoter. The binding site of H-NS downstream to the promoter maps within the first gene of the operon (*proV*) 150 to 300 bp downstream of the transcription start (Lucht et al., 1994a; Fletcher and Csonka, 1995b; Jordi et al., 1997b). It has been shown that the downstream site is required for effective repression of *proU* (Overdier and Csonka, 1992; Lucht et al., 1994a; Fletcher and Csonka, 1995a; Jordi et al., 1997a; Jordi and Higgins, 2000).

To determine whether repression of *proU* and *bgl* by H-NS binding to upstream and downstream regulatory element is similar, *lacZ* reporters were constructed that carry both the H-NS binding sites or the upstream or downstream regulatory elements only. The β-galactosidase activity directed by a chromosomally encoded *ProU$_{URE}$-PproU-proV'$_{DRE}$-lacZ* fusion, which carries the *proU* promoter flanked by upstream and downstream regulatory elements (-315 to +303 relative to the transcription start site) was determined from cultures grown in LB medium with various NaCl concentrations (figure 6a). When *wt* cells were grown in LB medium at low osmolarity (0.01M NaCl) the expression was low (54 units). The expression level gradually increased as the osmolarity was increased. In medium of high osmolarity (LB 0.3M NaCl) the expression increased 21 fold to 1150 units (Figure 6a). In the *hns* mutant grown in medium of low osmolarity the expression increased 16 fold to 861 units when compared to *wt*. In medium of high osmolarity the expression level in *hns* mutant reached 1280 units, and thus was 1.4 fold higher than at low osmolarity (figure 6a). These results show and confirm that H-NS represses the *proU* operon effectively under low osmolarity conditions (16 fold), and that the efficiency of repression decreases when the operon is activated by osmotic stress. Furthermore, the osmoregution of the *proU* operon is greatly reduced, from 21 fold in the *wt* to 1.4 fold in the *hns* mutant (figure 6a).

For the analysis of repression *via* the upstream regulatory element, the *proU* promoter region from position -315 to +20 relative to the transcription start site was transcriptionally fused to *lacZ*. This construct consist of the *proU* promoter along with the upstream regulatory element (figure 6b). The β-galactosidase activity was measured in the *wt* and *hns* mutant in cultures grown in LB medium with various salt concentrations. Under low osmolarity conditions in the *wt* the expression was 1095 units. Expression gradually increases to 3760 units (3.4 fold) in high osmolarity medium (Figure 6b). In the *hns* mutant the expression in low osmolarity medium was 1695 units and increased 1.9 fold to 3370 units in high osmolarity medium. These data confirm that repression by H-NS through the upstream regulatory element is very inefficient. It is only 1.5 fold at low osmolarity conditions. The data further show that the *proU* promoter is gradually activated by an increase in the osmolarity, which is in agreement with *in vitro* experiments reported before (Mellies et al., 1994b; Rajkumari et al., 1996a; Jordi and Higgins, 2000).
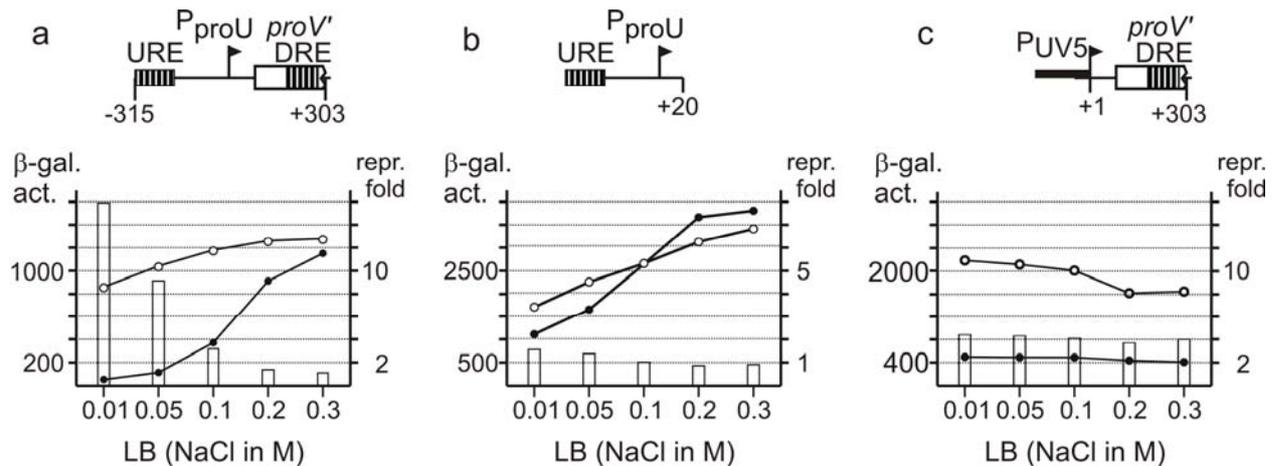
Figure 6. Cooperativity in repression by H-NS in the *proU* operon: The chromosomal integrants of *proU* operon containing *ProU_{URE}* and *proU_{DRE}* in the natural context (a), the *proU_{URE}* alone (b) and *proU_{DRE}* alone expressed from *PUV5* promoter (c) is shown schematically. The *lacZ* gene is fused transcriptionally to the 3' end for β-galactosidase measurement. The β-galactosidase activity was measured in LB media with 0.01 M, 0.05 M, 0.1 M, 0.2 M and 0.3 M NaCl respectively (shown in x-axis). The white bars indicate the fold repression by H-NS and the β-galactosidase activity in units is shown in line graph with white circles (*wt*) and filled circles (*hns*). The β-galactosidase activity and the fold repression by H-NS is shown in left and right y-axis respectively. Strains used are shown in the order *wt*, *hns* a) S2501, S3128 b) S2048, S3124 and c) S2137, S3126. The β-galactosidase activity expressed is in the order 0.01, 0.05, 0.1, 0.2, 0.3M NaCl LB in *wt* and *hns* mutant a) 51, 114, 370, 912, 1150 units and 861, 1041, 1178, 1260, 1278 units b) 1094, 1641, 2610, 3621, 3760 units and 1695, 2232, 2626, 3087, 3368 units c) 496, 489, 488, 435, 405 units and 2185, 2114, 2010, 1620, 1648 units. The β-galactosidase value indicated are the average of at least three experiments and the standard deviation is less than 10%.

To study the efficiency of *proU* repression by H-NS *via* the downstream regulatory element, a *proU* operon fragment from position + 1 to + 303 relative to transcription start site encompassing the downstream H-NS binding region was fused transcriptionally to *lacZ*. In

this reporter expression is driven by a constitutive *lacUV5* promoter. The expression level directed by the *PUV5-proV'$_{DRE}$-lacZ* fusion under low osmolarity conditions was 496 units in the *wt*. Expression decreased gradually to 408 units at high osmolarity conditions. In the *hns* mutant the expression decreased from 2185 units at low osmolarity conditions to 1648 at high osmolarity conditions (figure 6c). The repression by binding of H-NS to the downstream regulatory element was ~4 fold irrespective of the osmotic conditions, indicating that this repression is unaffected by the osmolarity. In summary, the results indicate cooperativity in repression of *proU* by H-NS binding to upstream and downstream regulatory elements.

3.   Repression by binding of H-NS to the downstream regulatory element is affected by the transcription rate

Downstream repression by H-NS in *bgl* and *proU* operon could be the result of H-NS acting as a roadblock to the transcribing RNA polymerase thereby affecting transcription elongation. Studies on transcription through roadblocks and readblocks (intrinsic pause sites) suggested a general mechanism involved in overcoming the block (Epshtein and Nudler, 2003; Epshtein et al., 2003). The anti-road- and read block mechanism relies on the synergistic force of transcribing RNA polymerases. In other words, the efficiency of the antiroad- and read block mechanism depends on the transcription rate of the gene, wherein high rates of transcription result in effectively overcoming road- and readblocks.

To analyze whether repression of *proU* and *bgl* by H-NS binding downstream is influenced by the transcription rate, *lacZ* reporter fusions were constructed which carry the *bgl* or the *proU* downstream regulatory element inserted in between constitutive promoter of high, middle and low activity (*Ptac, PUV5, PlacI*), respectively, and the *lacZ* gene. The strongest among the promoters used is the tac promoter (*Ptac*) followed by the *lacUV5 (PUV5)* promoter, and the lacI promoter is the weakest (*PlacI*).

3.1   Transcription influences repression by binding of H-NS to the DRE in *bgl*.

Promoter-*bgl$_{DRE}$-lacZ* fusions carry a *bgl* fragment from position +95 to +972 encompassing the downstream regulatory element. The β-galactosidase activity of the chromosomally encoded *bgl$_{DRE}$-lacZ* fusion driven by various promoters was measured in the *wt* and in the *hns* mutant (Figure 7a). The expression level directed by the *PlacI-bgl$_{DRE}$-lacZ* fusion in the *wt* was 6 units of β-galactosidase activity, and it increased 12.5 fold to 76 units in the *hns* mutant (figure 7b). The *PUV5-bgl$_{DRE}$-lacZ* fusion, in which expression is directed

by the *lacUV5* promoter of medium activity, directed 400 units of β-galactosidase activity in the wild type. The activity increased 2.4-fold to 961 units in the *hns* mutant (figure 7b, 5f). The expression of the *Ptac-bgl_DRE-lacZ* fusion carrying the strong tac promoter directed 2302 units of β-galactosidase activity in the *wt* and 2787 units in the *hns* mutant (figure 7b). This construct is not repressed by H-NS (the difference is merely 1.2 fold). These results suggest that the repression by binding of H-NS downstream depends on the activity of the promoter and the transcription rate. Repression is effective only when transcription rates are low.
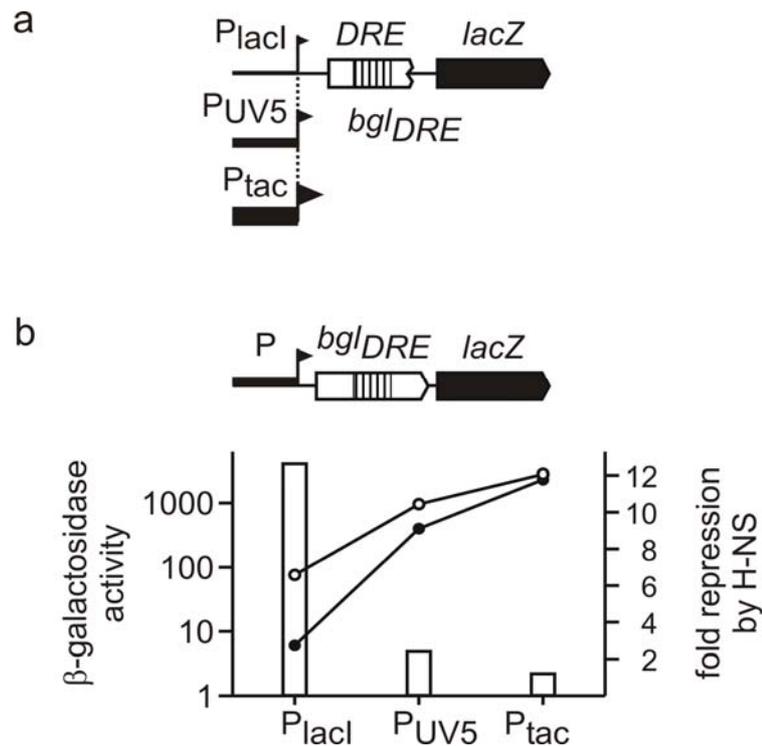
Figure 7. Transcription influences the repression by H-NS through *bgl_DRE*: Schematic representation of *bgl_DRE-lacZ* or *proU_DRE-lacZ* fusion expressed by promoters of different strength (PlacI, PUV5, Ptac) (a). The β-galactosidase activity expressed by *bgl_DRE-lacZ* fusion driven by various promoters measured in LB is shown in (b). The x-axis shows the different promoters used to express *bgl_DRE-lacZ* fusion. The white bars indicate the fold repression by H-NS and β-galactosidase activity is shown in line graph with white circles (*wt*) and filled circles (*hns*). The fold repression by H-NS and the β-galactosidase activity in Miller units is shown in right and left y-axis respectively. Stains used to express *bgl_DRE-lacZ* from *PlacI* [S3000 (*wt*) and S3300 (*hns*)], from *PUV5* [S1193 (*wt*) and S3211 (*hns*)], from *Ptac* [S2285 (*wt*) and S3299 (*hns*)]. The β-galactosidase activity expressed by *bgl_DRE-lacZ* in *wt* and *hns* mutants when expressed from PlacI is 6 and 76 units respectively, from PUV5 is 400 and 961 units respectively, when expressed from Ptac is 2302 and 2787 units respectively.

3.2 Transcription influences repression by binding of H-NS to the DRE in *proU*.

To determine whether repression by binding of H-NS to the DRE of *proU* is likewise affected by the transcription rate, the *proU* region from position +1 to +303 encompassing the downstream regulatory element (*proV'_DRE*) was expressed from promoters of different strength (*Ptac, PUV5, PlacI*). The level of expression directed by chromosomally encoded

constructs was measured in LB with various NaCl concentrations (LB with 0.01, 0.05, 0.1, 0.2, 0.3 M NaCl). In the *wt* the *PlacI-proV'$_{DRE}$-lacZ* fusion directed 8.7 units of β-galactosidase activity in medium of low osmolarity (LB with 0.01 M NaCl) (figure 8a). This construct is not osmoregulated, in LB medium of increased osmolarity (0.05 M NaCl, 0.1 M NaCl, 0.2 M NaCl, and 0.3M NaCl) similar levels of β-galactosidase activity was measured (7.5 units, 8.9 units, 9.0 units, and 9.4 units) In the *hns* mutant the expression level in medium of low osmolarity (0.01 M NaCl) was 97 units (figure 8a) the expression level gradually decreased with an increase of the osmolarity to 51 units of β-galactosidase (0.3 M NaCl). Thus the repression by H-NS varied between 11-fold in medium of low osmolarity to 5.4-fold in medium of high osmolarity The *PUV5-proV'$_{DRE}$-lacZ* fusion expressed 496 units of β-galactosidase activity when grown in LB with 0.01 M NaCl (Figure 8a). In the *hns* mutant the expression level was 2185 units of β-galactosidase activity when grown in LB with 0.01 M NaCl (figure 8a). At higher osmolarity the expression slightly decreased gradually to 1650 units (0.3 M NaCl) (data not shown). The *Ptac-proV'$_{DRE}$-lacZ* reporter construct, which carries the strong *tac* promoter, directed 3790 units of β-galactosidase activity at low osmolarity conditions (LB 0.01 M NaCl), while in the *hns* mutant 8065 units were measured at low osmolarity conditions (figure 8a). These data show that the repression of the strong *tac* promoter constructs by H-NS is only ~2-fold. Taken together these results demonstrate that the repression of *proU* by binding of H-NS to the downstream regulatory element is influenced by the transcription rate and parallels repression of *bgl* by H-NS.

To rule out effects of H-NS on the promoters and on expression of *lacZ*, control experiments were done, in which the expression level directed by chromosomally encoded *Ptac*, *PUV5* and *PlacI lacZ* fusions was measured in LB media of different osmolarity. The *Ptac-lacZ* reporter directed the expression of 11590 units in the wild-type and 13405 units in the *hns* mutant (figure 8b) in low osmolarity medium. Similar results were obtained in media of higher osmolarity (data not shown). These results suggest that H-NS does not affect the activity of the *tac* promoter and the expression of *lacZ* under the experimental conditions used. Similar results were also observed for *PUV5-lacZ* fusion and *PlacI-lacZ* fusion tested under different osmolarity condition. The expression of *PUV5-lacZ* was 5285 units in the *wt* (0.01 M NaCl) and 5100 units in the *hns* mutant (figure 8b). The expression directed by the *PlacI-lacZ* was 183 units in the *wt* (0.01 M NaCl), and 163 units in the *hns* mutant (figure 8b). From these (Figure 8) and further results (data not shown) it can be concluded that the expression of *lacZ* directed by *Ptac*, *PUV5* and *PlacI* is not repressed by H-NS under different osmolarity conditions.
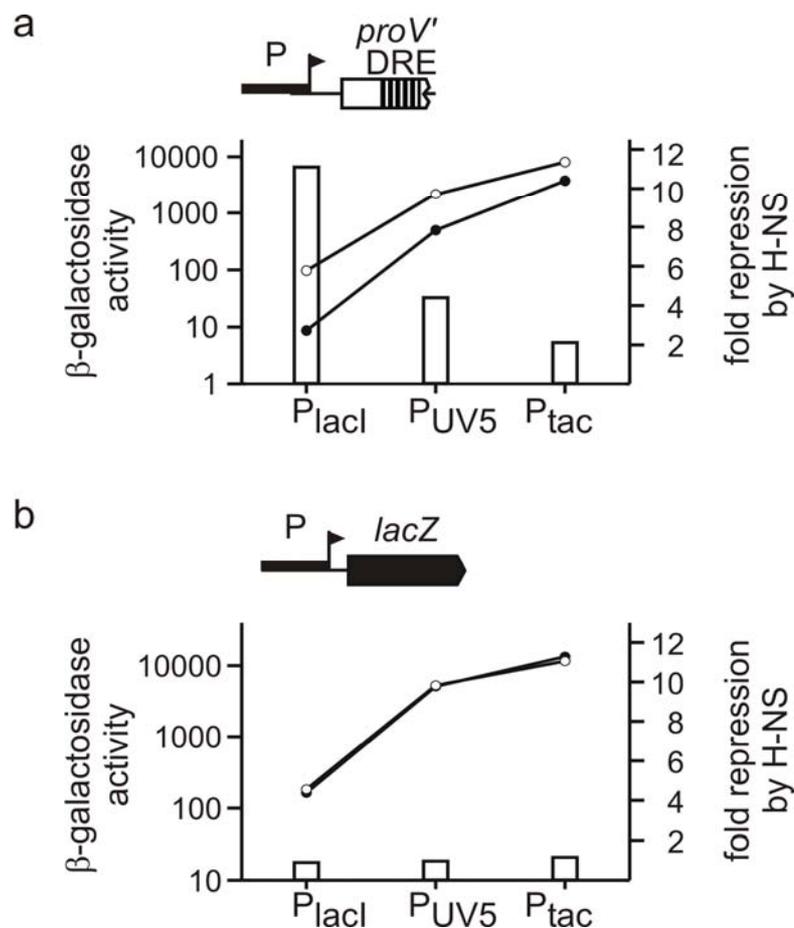
**Figure 8. Transcription influences the repression by H-NS through the proU$_{DRE}$**: Schematic representation of *proU$_{DRE}$-lacZ* fusion used to express from promoters of different strength (*PlacI, PUV5, PlacI*) (a) and the *PUV5-lacZ* control construct (b). The β-galactosidase expression of *proU$_{DRE}$-lacZ* and *lacZ* driven by promoters of different strength is measured in LB media with 0.01M NaCl (shown in x-axis). The β-galactosidase values measured in *wt* (circle) and *hns* mutant (filled circle) is shown in line graph. The white bars in the figure represent fold repression by H-NS. The β-galactosidase activity in Miller units and the fold repression by H-NS is shown in left and right y-axis respectively. Stains used to express *proU$_{DRE}$-lacZ* from *PlacI* [S3034 (*wt*) and S3134 (*hns*)], from *PUV5* [S2137 (*wt*) and S3126 (*hns*)], from *Ptac* [S3058 (*wt*) and S3175 (*hns*)]. The β-galactosidase activity expressed by *proU$_{DRE}$-lacZ* in *wt* and *hns* mutants when expressed from PlacI is 8.7 and 97 units respectively, from PUV5 is 496 and 2185 units respectively, when expressed from Ptac is 3792 and 8063 units respectively. Strains used for expressing *lacZ* from PlacI is S3005 (*wt*) and S3171 (*hns*), from PUV5 is S1906 (*wt*) and S3122 (*hns*) and from Ptac is S2287 (*wt*) and S3169 (*hns*) respectively. The β-galactosidase activity in Miller units expressed by *lacZ* gene when expressed from *PlacI* is 183 units and 163 units in *wt* and *hns* mutant respectively. When expressed from PUV5 is 5286 units and 5101 units in *wt* and *hns* mutant respectively. When expressed from Ptac is 11588 units and 13406 units in *wt* and *hns* mutant respectively.

3.3   Termination factor Rho is required for efficient downstream repression by H-NS in the *bgl* operon

As shown by DNA shift experiments H-NS bind specifically 600-700bp downstream to the promoter. H-NS binding downstream to the promoter could repress the operon by acting as a roadblock to the transcribing RNA polymerase and thereby causing polarity in gene expression (Dole et al., 2004b).

In general polarity is the result of a pause in transcription allowing termination factor Rho to catch up with RNA polymerase at the paused site and to terminate transcription elongation.

To address whether repression by binding of H-NS downstream can be the result of H-NS acting as a roadblock to the elongating RNA polymerase and subsequent termination of transcription by Rho, the *lacZ* reporter which carries a *bgl* operon fragment from position +95 to +972 encompassing the downstream H-NS site, inserted in between the constitutive *lacUV5* promoter and the *lacZ* gene was used. In this reporter translation of *bglG* was eliminated (Dole et al., 2004b). This non-translatable downstream regulatory element was used because translation of *bglG* affects the repression of H-NS (Dole et al., 2004b) (figure 5 f and g). The expression of this $PUV5$-bgl_{DRE-NT}-$lacZ$ was measured in a temperature sensitive *rho-702(ts)* mutant, *hns rho-702(ts)* double mutant, and in the wild-type and *hns* mutant as control (Figure 9b). The assays were carried out at permissive (28°C) and non-permissive temperature (42°C). For comparison, the expression level in the *wt* and *hns* mutant was determined when grown at identical temperature conditions. In the *wt* background the $UV5$-bgl_{DRE-NT}-$lacZ$ reporter directed 130units of β-galactosidase at 28°C and 100 units at 42°C (figure 9b). In the *rho-ts* mutant the expression level increased to 260 units at 28°C and 390 units, i.e. two fold at the permissive and four fold at non-permissive temperature of 42°C. However, in the *hns* background the *rho-ts* mutant had no effect. In both the *hns* and the *hns rho* double mutant similar β-galactosidase levels were detected (1015 and 1025 units) (figure 9b). These results demonstrate that Rho is important for H-NS to repress *bgl via* the downstream regulatory element. These data support the model that H-NS acts as a road block to the elongating RNA polymerase as a prerequisite for Rho-mediated termination.

To further confirm the involvement of Rho in H-NS mediated repression by binding downstream the expression level directed by the downstream reporter ($UV5$-bgl_{DRE-NT}-$lacZ$) was measured in LB with and without sub-lethal concentrations of bicyclomyin (20μg/ml) at 37°C. Bicyclomycin is a specific inhibitor of transcription termination factor Rho (Nishida et al., 1972; Zwiefka et al., 1993; Magyar et al., 1996). The downstream reporter expressed 91 units of β-galactosidase activity in media without bicyclomycin at 37°C, whereas the expression increased ~2 fold to 171 units with the addition of bicyclomycin (20μg/ml) at 37°C (figure 9c). In the *hns* mutant the expression did not vary with and without bicyclomycin (804 and 811 units) demonstrating that the effect of Rho depends on the presence of H-NS. This further confirms the involvement of termination factor Rho in repression by H-NS *via* the downstream regulatory element and suggests that H-NS acts as a roadblock in the *bgl* operon.
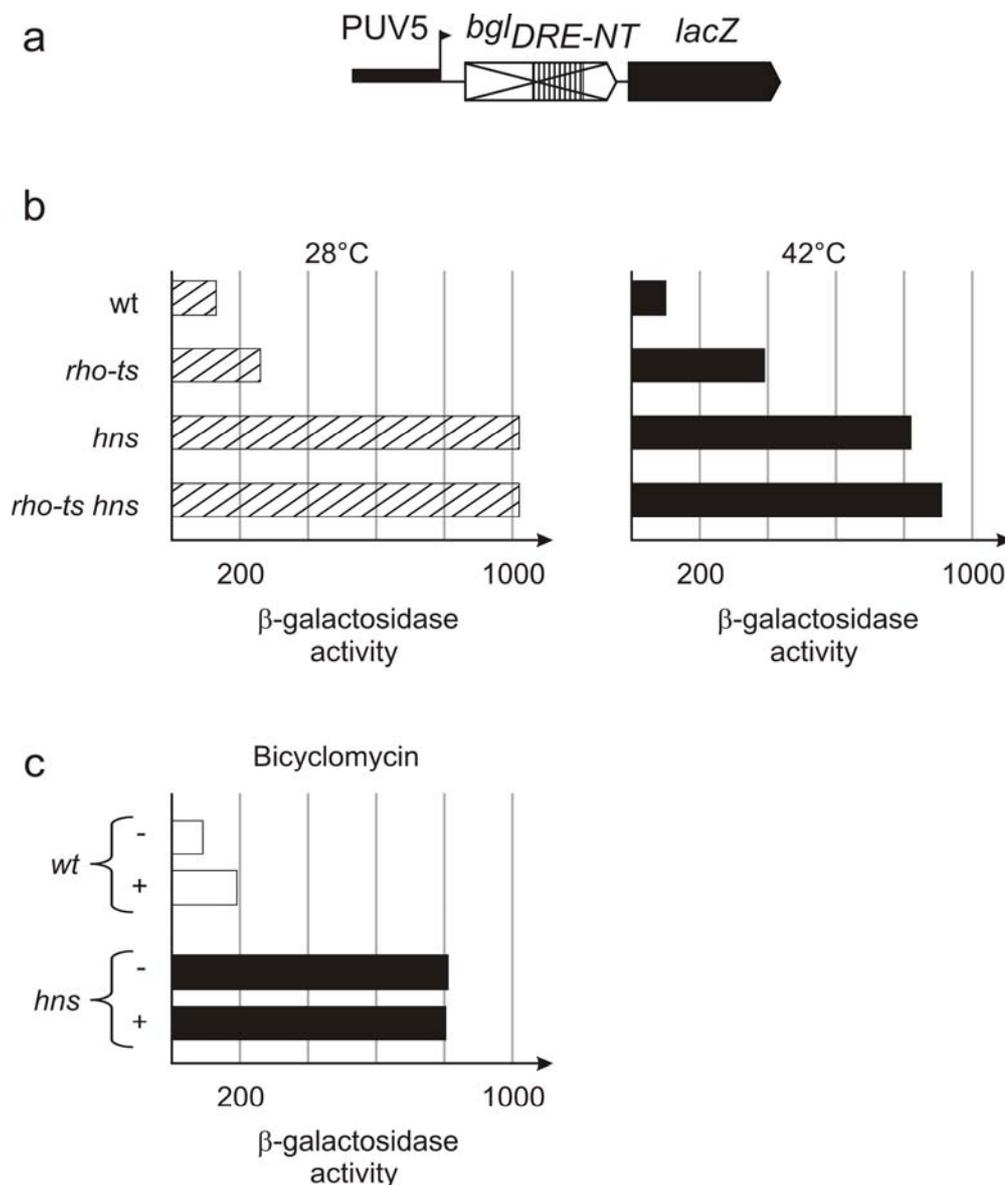
Figure 9. Termination factor Rho is required for the H-NS mediated repression *via* the downstream regulatory element. The expression level directed by *PUV5-bgl$_{DRE-NT}$-lacZ* (a) was determined in the wild-type, *rho-ts*(702) mutant, *hns* mutant, and the *rho-ts*(702) *hns* double mutant of cells grown at 28°C (b) and at 42°C (b) 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. The expression of *PUV5-bgl$_{DRE-NT}$-lacZ* (a) was determined in LB medium with and without bicyclomycin (20μg/ml) at 37°C(c). At 37°C the expression of *PUV5-bgl$_{DRE-NT}$-lacZ* in wild type was 91 units without bicyclomycin and 171 units with bicyclomycin (c). In *hns* mutant the expression was 804 and 811 units with and without bicyclomycin. Strains used for the β-galactosidase assays are S1195 (*wt*), S1258 (*hns*), S1995 (*rho-ts*) and S1997 (*rho-ts hns*).

3.4   H-NS affects transcription elongation in repression *via* the *bgl* DRE

There is a possibility that H-NS, when binding downstream in the *bgl* and *proU* operon, acts as a barrier to the transcribing RNA polymerase, since termination factor Rho and translation affect repression (Figures 5f, 5g and 9c, see also (Dole et al., 2004b)). To address this possibility a dual reporter assay was employed, where a *bgl* operon fragment

from position +95 to +972 encompassing the downstream regulatory element was placed between two reporters, *uidA* and *lacZ* (figure 10a). Expression of this dual reporter is directed by the constitutive *lacUV5* promoter (*PUV5*) (figure 10a). If H-NS affected transcription elongation, then the expression of β-glucuronidase encoded by the first reporter (*uidA*) should not be altered in the *hns* mutant as compared to the *wt*. However, the expression of β-galactosidase encoded by the second reporter (*lacZ*), should be increased in the *hns* mutant compared to the *wt*. To this end, the expression of a chromosomally encoded *PUV5-uidA-bgl$_{DRE-NT}$-lacZ* was measured in the *wt* and *hns* mutant. It should be noted that the *bgl$_{DRE}$* was placed downstream to the *uidA* gene and thus separated from the promoter by ~1800bp. In the *wt* 584 units of β-galactosidase was measured and in the *hns* mutant 781 units were measured, demonstrating that repression of the dual reporter by H-NS is marginal 1.3-fold (figure 11a). This result contradicts the assumption that H-NS affects transcription elongation. However, as shown above (figure 7b, section 3.1) repression by binding of H-NS to the DRE is affected by the transcription rate. It therefore was possible that the loss of repression is caused by a high transcription rate, since the expression of dual reporter is high (584 units) when compared to the single reporter *PUV5-bgl$_{DRE-NT}$-lacZ* (90 units). In order to reduce the transcription rate a Rho independent terminator (*rrnBT1*) was placed between the *uidA* gene and the *bgl$_{DRE}$* . The level of *uidA* and *lacZ* expression of this modified dual reporter (*PUV5-uidA-rrnBT1-bgl$_{DRE-NT}$-lacZ*) was measured in the *wt* and the *hns* mutant. The expression level of β-glucuronidase, encoded by *uidA*, did not vary between the *wt* and the *hns* mutant (149 and 151 units respectively) (Figure 10b). In contrast the expression of the second reporter, *lacZ,* increased 2.2-fold from 175 units in the *wt* to 395 units in *hns* mutant (Figure 10b). These data suggest that H-NS indeed affects the process of transcription elongation by binding to the *bgl* downstream regulatory element.

To rule out effects of H-NS on expression of *uidA* or *lacZ*, control experiments were done, in which the expression of a dual reporter lacking any downstream regulatory fragment was measured in the *wt* and *hns* mutant (figure 10e). The level of β-galactosidase directed by this control dual reporter that carries the *rrnBT1* terminator was determined. This *UV5-uidA-rrnBT1-lacZ* directed similar levels of β-glucuronidase and β-galactosidase in the *wt* and *hns* mutant (163 units versus 151 units β-glucuronidase activity, and 1522 units versus 1625 units β-galactosidase activity) (figure 10e). These experiments show that the expression of *uidA* and *lacZ* are not influenced by H-NS.
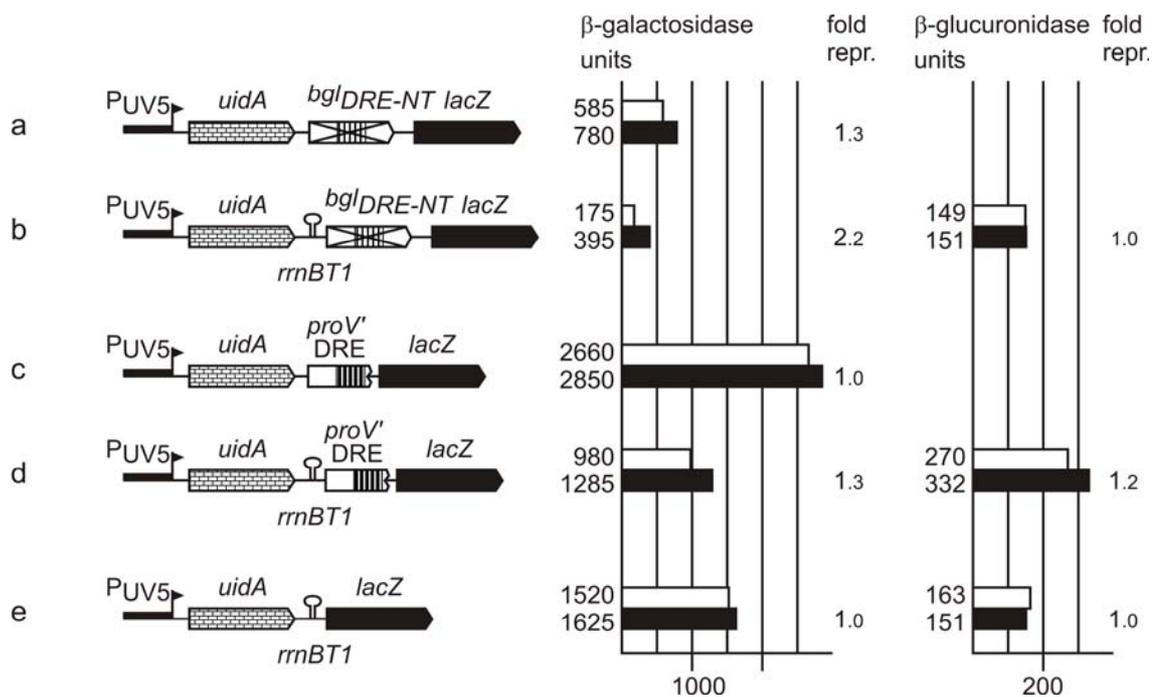
Figure 10. H-NS repression *via* the downstream regulatory region affects transcription elongation. Schematic representation of the *bgl_DRE* or *proU_DRE* placed between *uidA* and the *lacZ* is shown from a to d. The control construct without DRE is shown in (e). The bar graph at the extreme right shows the β-glucuronidase assay values and the one in the middle shows β-galactosidase activity. The numbers on the left side of the bar chart shows the β-galactosidase activity and β-glucuronidase units. The numbers to the right side of the graph indicate the fold repression by H-NS. Strains used were in the order *wt, hns* a) S2368, S3270 b) S2692, S3272 c) S2327, S3290 d) S2704, S3292 e) S2696, S3283.

3.5   H-NS does not affect transcription elongation in the *proU* operon

To determine whether the process of transcription elongation is also affected by binding of H-NS to the downstream regulatory element in *proU*, likewise a dual reporter assays was performed. The *proU* downstream fragment (+1 to +303) was placed in between the *uidA* gene and *lacZ*. Expression of the reporter is directed by the *lacUV5* promoter (figure 10c). The expression of *lacZ* directed by the *PUV5-uidA-proV'_DRE-lacZ* fusion was not significantly different between the *wt* and the *hns* mutant (2660 units in *wt* and 2850 units in the *hns* mutant, figure 12a) and the levels of β-galactosidase activity were rather high. This is similar to what was observed for the *PUV5-uidA-bgl_DRE-lacZ* fusion. To reduce the transcription rate, the Rho independent terminator rrnBT1 was placed in between *uidA* and the downstream *proV'_DRE* fragment (figure 10d). The expression of β-glucuronidase activity directed by this dual reporter was 270 units in the *wt* and 332 units in the *hns* mutant, respectively when the assay was carried out in LB with 0.01M NaCl. The expression of β-galactosidase activity was also not significantly changed between *wt* (979 units) and *hns* mutant (1286 units, figure 10d) under the same assay conditions. Unlike the role for H-NS in transcription elongation in the *bgl* operon, H-NS does not have an effect on transcription

elongation in the *proU* operon. However, the transcription rate of this construct is still rather high, which could possibly affect the formation of H-NS repression complex.

4.   *In situ* RNA polymerase footprinting in the *bgl* and *proU* operons

4.1   RNA polymerase pauses at an intrinsic pause site in *bgl*

This section of work involving CAA footprinting was done in collaboration with Dr.Rachid Rahmouni, Centre de Biophysique Moleculaire, CNRS, France. The probing experiments was carried out by Madhusudan.S.

H-NS binds within the transcription unit of *proU* (Lucht et al., 1994b; Fletcher and Csonka, 1995a; Jordi et al., 1997a) and in *bgl* (Figure 4) (Dole et al., 2004b). The genetic analysis suggests that H-NS acts as a roadblock to the transcribing RNA polymerase in case of the *bgl*$_{DRE}$. Thus, one would expect that H-NS acts as a physical barrier obstructing the movement of RNA polymerase and therefore causing pausing of transcription elongation just upstream of the H-NS binding site. Such pausing events can be detected by *in situ* footprinting using the single strand specific probe chloracetaldehyde (CAA) (Epshtein et al., 2003; Schwartz et al., 2003; Krasilnikov et al., 1999)}. CAA modifies residues C and A to a lesser extent G in the transcription bubble where DNA is unwound and single stranded. During the active transcription process the translocation kinetics of RNA polymerase engaged in transcription elongation is high and thus prevents CAA to modify the nucleotides in the transcription bubble whereas, when RNA polymerase becomes paused at a particular site the kinetics is altered and that gives enough time for the CAA probe to gain access to the transcription bubble and modify the nucleotide. These CAA induced modifications are subsequently visualized by primer extensions to precisely map the location of the paused RNA polymerase complex.

To investigate whether H-NS mediated repression by binding to downstream regulatory element is mediated by acting as roadblock to the transcribing RNA polymerase, two plasmids were constructed. Pausing in *bgl* was analyzed using plasmid pKENV63, which carries the *lacUV5* promoter, followed by the *bgl* downstream fragment *bgl*$_{DRE-NT}$ and terminators rrnB-T1T2 (schematically shown in figure 11a). In addition, high copy plasmid pKEM53 was used, which carries lacUV5 promoter expressing *bglG*$_{DRE-NT}$ fused to *lacZ*. For analysis of pausing of transcription in the *proU* system, plasmid pKENV73 was used, which carries the *proU* promoter flanked by upstream and downstream regulatory elements of *proU* followed by terminators rrnB-T1 and T2 (data not shown). CAA footpinting was performed of transformants of the *wt* strain (S541) and the *hns* mutant strain (S614). As control, a high

copy plasmid carrying the *bgl* downstream regulatory element without promoter (pKEM54) was used to determine whether the CAA footprints are dependent on transcription or reflect the DNA structure. A mock reaction without addition of CAA was included to differentiate the reactivity induced by CAA and non-specific primer extension stops. The primer extension products were run along a sequencing ladder generated with the same primer used for the probing experiments. A schematic overview of the constructs used and the primer used for CAA footprinting is shown in figure 11a.
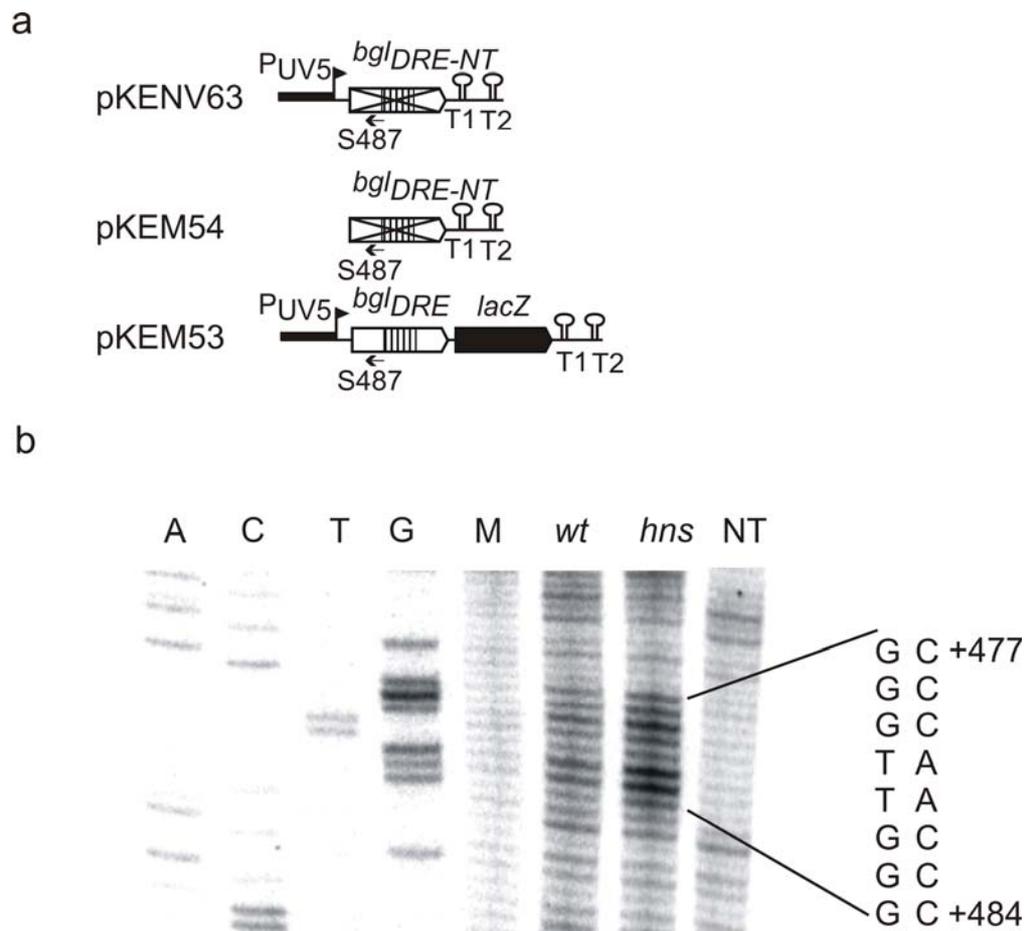
Figure 11. RNA polymerase pauses within the *bglG*$_{DRE}$: Schematic representation of high copy plasmids used for *in situ* CAA probing. T1 and T2 refer to rrnB-T1 and T2 terminators. The primer (S487) used for primer extension and the position to which it matches in the *bgl*$_{DRE}$ is shown in (a). Bottom part (b): Representative gel showing the CAA induced pause signal at position +477 to +484 relative to the transcription start site. The first four lanes show the sequencing ladder generated from pKEM54 using primer S487, M-refers to the mock reaction without CAA treatment, NT-refers no transcription control (CAA probing done on pKEM54 which has the *bglG*$_{DRE}$ without the promoter). For *wt* and *hns* lane, pKEM54 plasmid was transformed to S541 (*wt*) and S614 (*hns*) and was probed with CAA and primer extension done with S487.

The CAA footprinting of the *bgl* downstream regulatory element revealed a clear reactivity at position +477 to +484 relative to the transcription start site (Figure11b, compare *wt*, mock). This reactivity is transcription dependent because no reactivity was observed when the control plasmid pKEM54 lacking a promoter was probed. Surprisingly this reactivity was also seen in the *hns* mutant, where it was more pronounced than in the *wt* (figure 11b). These data demonstrate that pausing of RNA polymerase occurs at positions +477 to +484 relative to the transcription start of *bgl*. This pausing is intrinsic and independent of the presence or absence of H-NS. The enhanced reactivity in the *hns* mutant presumably reflects a higher rate of transcription in the *hns* mutant. No H-NS dependent pausing was observed immediately upstream of the H-NS binding region (+600 to +700) in *bgl* using primer S487 (data not shown). Thus it can be concluded that H-NS does not act as physical barrier (roadblock) to RNA polymerase.

Similar *in situ* CAA probing experiments were performed for *proU* using the high copy plasmid *proU$_{URE}$-PproU-proV'$_{DRE}$* (pKENV73) in the *wt* and *hns* mutant (S3346) grown in LB medium with 0.01M Nacl. As a control for transcription dependent CAA reactivity plasmid pKENV56 carrying *PlacI-proV'$_{DRE}$-lacZ* was used. Expression of *proV'$_{DRE}$-lacZ* from *PlacI* is very weak so it could be used as negative control. The primer extension products were separated next to a sequence ladder, which was generated with the same primer, S420 (data not shown). The *in situ* CAA footprint on *proU* revealed no pause site in the *wt* and *hns* mutant (data not shown).

Taken together the CAA footprint on the *bgl* and *proU* downstream regulatory regions demonstrated that H-NS does not form roadblock to the RNA polymerase. Interestingly, the probing of *bgl* operon revealed an intrinsic pause site at position +477 to +484 relative to the transcription start site.

5.   **Northern analysis of *bglG* mRNA shows truncated products**

In addition to the paradigm of gene regulation at the level of transcription initiation, transcription elongation and termination serve as important targets for regulation. The transcription elongation complex stalled at a pause site undergoes modulations, which ultimately decide the outcome of pausing (Landick, 2006; Neuman et al., 2003; Artsimovitch and Landick, 2000). Pausing can lead to termination, in case a weak RNA:DNA hybrid in the transcription elongation complex is preceded by a stable hairpin structure (Artsimovitch and Landick, 2000; Landick et al., 1996). Pausing also is used as a mechanism of RNA

surveillance for defective mRNAs by slowing down transcription and eventually released by Rho termination factor (Landick et al., 1985; Landick, 2006).

As shown by *in situ* CAA footprinting experiments (Figure 11b), RNA polymerase pauses in *bgl* at position +477 to +484 relative to the transcription start site. To determine whether this pausing event results in termination of transcription, Northern analysis was performed using a probe complementary to the 5' end of the *bgl* RNA from position +132 to +268 relative to the transcription start site (figure 12a). If pausing leads to transcription termination, a truncated RNA should be detected by the probe complementary to the 5' end of *bgl* mRNA. Furthermore, this approach allows quantifying and comparing the ratios of full length and truncated mRNA products in the *wt* and the *hns* mutant.
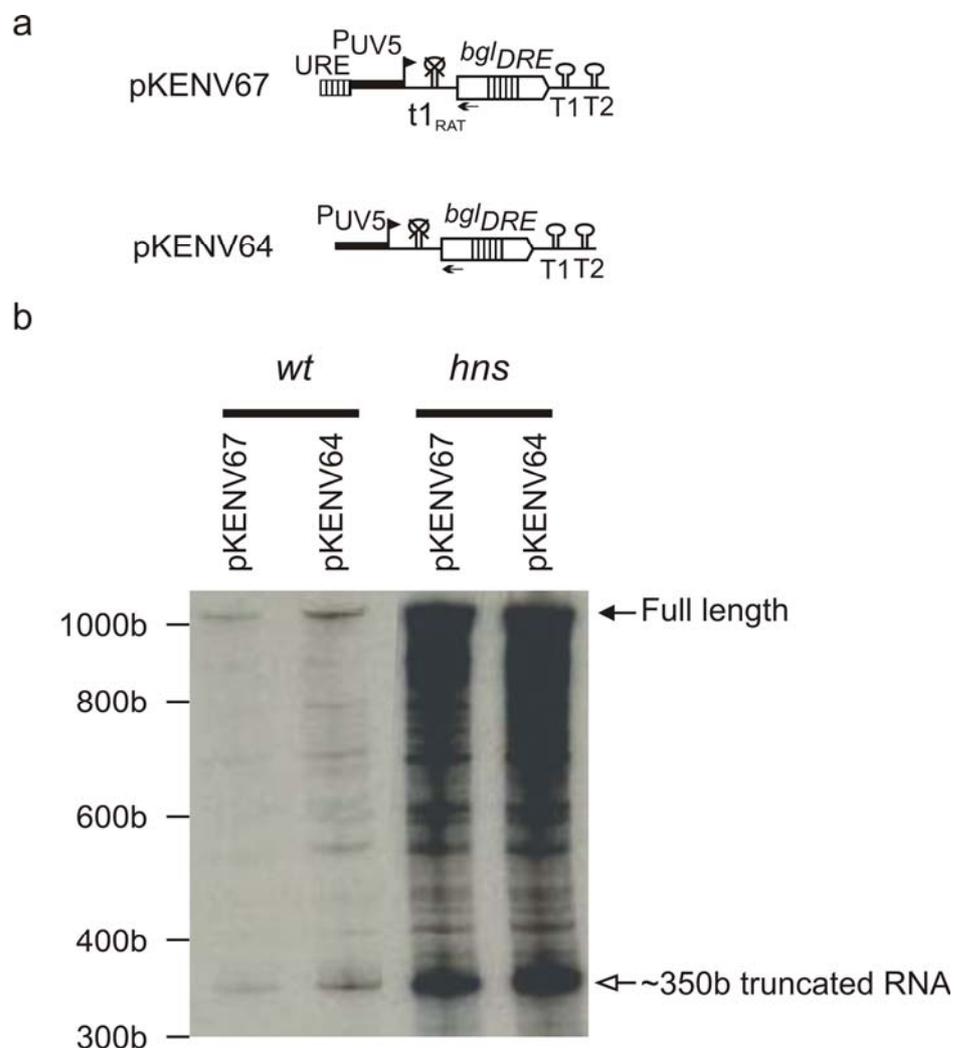
Figure 12. Northern analysis shows truncation in *bglG*: Constructs used for northern analysis is shown schematically (a), pKENV67 has *PUV5* flanked by *bgl_{URE} and t1_{RAT} bgl_{DRE}* followed by strong rrnB-T1 and T2 terminators, pKENV64 has *t1_{RAT}-bgl_{DRE}* expressed by *PUV5*. The position in *bgl_{DRE}* to which RNA probes match is shown by arrows. b) A representative gel showing the results of northern analysis, the plasmids used for the RNA isolation (from *wt* and *hns* mutant) are indicated above each lane. The full length (filled arrow) and ~350bp RNA product (open arrow) is indicted to the right of the gel. The RNA ladder positions are shown to the left of the gel.

For the Northern analysis, high copy plasmids carrying a promoter, the *bgl* regulatory elements followed by the efficient ribosomal terminator *rrnB*-T1 and T2 were constructed. Plasmid pKENV67 (*bgl$_{URE}$-PUV5-t1RAT-bgl$_{DRE}$*) carries the *lacUV5* promoter (*PUV5*) flanked by the upstream and downstream regulatory elements of *bgl* (figure 12a). Expression of this reporter is independent of BglG due to the mutation t1$_{RAT}$ described above (Fig 20). Plasmid (pKENV67) was transformed into the *wt* (S541) and Δ*hns* (S3346) mutant and total cellular RNA was isolated. Of this RNA preparations 7.5μg was separated on a 5% denaturing polyacrylamide gel (7M UREA, 0.5xTBE) and hybridized overnight at 65°C (for detailed protocol see materials and methods). The positions to which the RNA probe matches in *bgl* and the results are shown in figure 12.

The Northern blot of RNA isolated from transformants of the *wt* revealed two prominent bands, one corresponding to full length RNA and another to a truncated RNA product of ~350 base length (figure 12b, pKENV67 in *wt* and *hns*). Quantification of these signals demonstrated that the full length RNA and the truncated RNA are present in equal amounts (~1:1 ratio, figure 12b). The Northern blot of RNA isolated from transformants of the *hns* mutant (S3346/ pKENV67) demonstrated that the amount of RNA was ~30 fold higher for the full length product and ~15 fold higher for the ~350 bp truncated product than in the *wt*. These data correlate well with the fold repression observed using *lacZ* reporter assays (figure 5c). Furthermore in the *hns* mutant the ratio of full length RNA to truncated RNA is 2 fold higher when compared to *wt* (figure12b) suggesting that in the *hns* mutant the formation of the full length product is favored over ~350bp truncated RNA. Although, in both the *wt* and the *hns* mutant numerous truncated RNA were detected the ~350bp RNA product was the most prominent (figure 12b).

A derivative of pKENV67, lacking the upstream regulatory element *PUV5-t1$_{RAT}$-bgl$_{DRE}$-lacZ* (pKENV64) was also used for northern analysis. The Northern blot of RNA isolated from transformants of *wt* (S541) and Δ*hns* (S3364) mutant likewise revealed two prominent bands, one corresponding to full length RNA and another to a truncated RNA product of ~350 base length (figure 12b). The amount of RNA in *wt* was higher when compared to RNA from pKENV67 which has both upstream and downstream regulatory elements whereas in *hns* mutant the levels of RNA from pKENV64 and pKENV67 are similar.

This suggests that the formation of truncated product does not require the presence of upstream regulatory element. Another high copy plasmid pKENV65 which has the *pbgl* flanked by upstream and downstream regulatory elements (*bgl$_{URE}$-Pbgl-t1$_{RAT}$-bgl$_{DRE}$*) was also

used for northern analysis. The expression of this construct was very weak in *wt* to be detected by northern (data not shown).

Taken together these results suggest that transcription of *bgl* leads to the formation of full length and truncated RNAs with a prominent ~350 base RNA, suggesting that *bgl* for some reason is difficult to transcribe or that the RNA is processed by RNases. Interestingly, the quantification of RNA isolated from pKENV67 in *wt* and *hns* mutants revealed that the fold difference in RNA (~30 fold) corresponds well with the genetic data shown in figure 5c. The ~350 base major truncation product does not correspond to the size of an RNA that was expected if transcription termination occurred at the +477 to +484 bp pause site.

5.1.   Deletion of the +480 pause does not affect repression by H-NS

In situ RNA polymerase footprinting of *bgl* revealed an intrinsic pause site at position +477 to +484 relative to the transcription start site. To investigate the biological function of the +480 pause in *bgl*, RNA secondary structure predictions was performed using mfold tool available at http://www.bioinfo.rpi.edu/applications/mfold/. This analysis suggests the presence of a stable hairpin at positions +459 to +476 preceding the pause site (+477 to +484) (figure 13a). In order to understand the biological significance of the pause in *bgl*, deletions encompassing the RNA secondary structure alone (Δ+459 to +476) or the secondary structure and the pause site (Δ+459 to +485) were constructed (figure 13b), and the effect of this deletions on expression was determined in the *wt* and the *hns* mutant. The deletions were introduced into plasmid pKENV68 (*bgl_{URE}-PUV5-t1_{RAT}-bgl_{DRE}-lacZ*), which carries the *lacUV5* promoter (*PUV5*) flanked by the upstream and downstream regulatory regions along fused to *lacZ* (figure 13b, pKENV68). The parent plasmid pKENV68 directed the expression of 928 units of β-galactosidase activity in the *wt* and the expression increased 16-fold to 15320 units in the *hns* mutant (figure 13b, pKENV68). Plasmid pKENV77 (*bgl_{URE}-PUV5-t1_{RAT}-bgl_{DRE}Δ+459-+476-lacZ)*, with a deletion from +459 to +476 relative to the transcription start site, eliminating the RNA secondary structure that precedes the pause site) directed the expression of 940 units of β-galactosidase activity in the *wt* and 16-fold higher levels (15320 units) in the *hns* mutant (figure 13b, pKENV77). This demonstrated that deleting the RNA secondary structure preceding the pause site does not affect H-NS mediated repression (Compare figure13b, pKENV68 and pKENV77). The expression of second pause site deletion derivative (deletion +459 to +485), which lacks DNA coding for the RNA secondary structure and the pause site (pKENV76, *bgl_{URE}-PUV5-t1_{RAT}-bgl_{DRE}Δ+459-+485-lacZ*) was measured in the *wt* and *hns* mutant. The expression level was 540 units in the *wt*

and increased 14-fold to 7600 units in the *hns* mutant (figure 13b, pKENV76). Thus the regulation by H-NS was unchanged although the expression level was lower. Taken together these results suggest that mutating the RNA secondary structure preceding the pause site or along with the pause site does not influence repression by H-NS, when both the URE and the DRE are present.
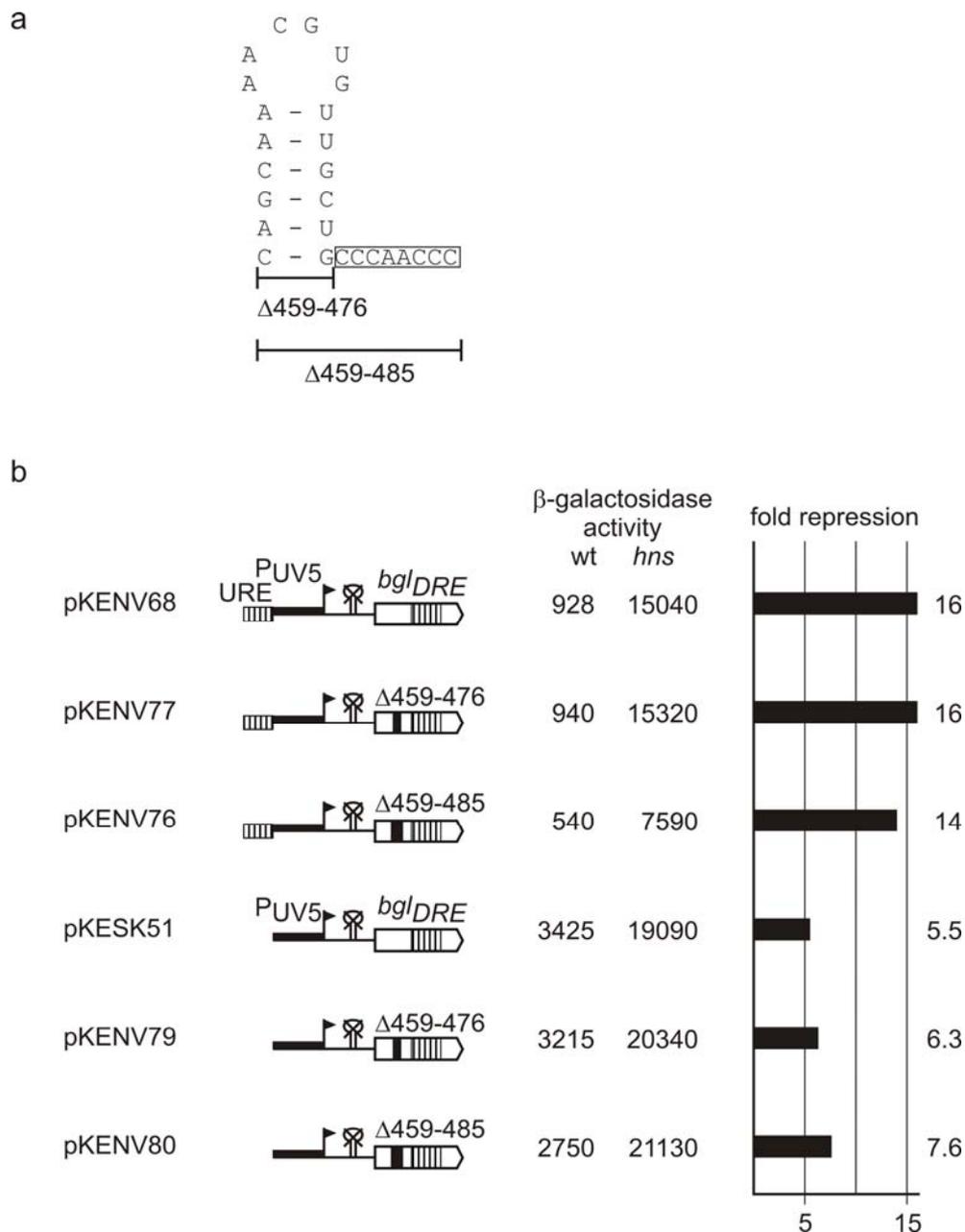
Figure 13. H-NS repression is not affected by deletion of pause site *in bgl$_{DRE}$*: The predicted secondary structure based on mfold program available at http://www.bioinfo.rpi.edu/applications/mfold/ (a). The pause site sequence detected by *in situ* CAA probing is shown within box. Deletions were made from Δ459-476 (which deletes the secondary structure preceding the pause) and Δ459-485 deletes the secondary structure and the pause site). (b) Plasmids name used for the experiments is shown in the left. Schematic representation of the constructs used for β-galactosidase activity measurement is shown to the immediate right of plasmid names. The bar chart shows the fold repression by H-NS (shown in black bars). The β-galactosidase activity in Miller units is given to the left of the bar chart. The numbers to the right of the graph indicates the fold repression by H-NS

To determine the significance of RNA polymerase pausing at the +480 pause, when only the downstream regulatory element is present, $PUV5$-$t1_{RAT}$-bgl_{DRE}-$lacZ$ (pKESK51) and its pause site deletion derivatives pKENV79 ($PUV5$-$bgl_{DRE}\Delta+459$-$+476$-$lacZ$) and pKENV80 ($PUV5$-$t1RAT$-bgl_{DRE} $\Delta+459$-$+485$-$lacZ$) were used for β-galactosidase activity measurement in the *wt* and *hns* mutant (figure 13b). The β-galactosidase activity directed by pKESK51 in the *wt* was 3430 units and it increased 5.5-fold to 19100 units in the *hns* mutant (figure 13b, pKESK51). Plasmid pKENV79, which carries a deletion from position +459 to + 476 relative to the transcriptional start site, directed 3210 units in the *wt* and 6.3 fold higher levels (20350 units) in the *hns* mutant (figure 113b, pKENV79). The expression directed by pKENV80, which does not have sequence encoding the RNA secondary structure and the pause site was 2750 units of β-galactosidase in the *wt* and 21130 units in *hns* mutant, resulting in 7.6 fold repression by H-NS (figure 113b, pKENV80).

Based on these genetic analysis results, the deletion of RNA secondary structure preceding the pause site alone or with the pause site in *bgl* does not affect the repression by H-NS.

6   Deletion analysis to find the minimum sequence requirement in bgl_{DRE} for H-NS repression

Mapping of the binding site for H-NS within the bgl_{DRE} by electrophoretic mobility shift assays (EMSA) demonstrated that H-NS binds to position +600 to +700bp relative to the transcription start site (Figure 4), (Dole et al., 2004b). To investigate the minimum sequence required for repression by H-NS, various deletions in bgl_{DRE} were constructed in the $PUV5$-bgl_{DRE-NT}-$lacZ$ reporter (figure 5g), and the expression was analyzed in the *wt* and *hns* mutant, and compared to the reporter constructs that carries the complete bgl_{DRE-NT} ($PUV5$-bgl_{DRE-NT}-$lacZ$). This regulation of this reporter is ~8 fold as shown above (see Figure 5g and Figure 14a).

A bgl_{DRE} construct that caries the DRE fragment from +95 to +550 ($PUV5$-bgl_{DRE-NT} (+95 to 550)-$lacZ$) directed the expression of 870 in *wt* and 1685 units in *hns* mutants, demonstrating a marginal 1.9 fold repression by H-NS (figure 14b). Similarly $PUV5$-$bglG_{DRE-NT}$ (+661 to 968)-$lacZ$ expressed 800 units and 1190 units in *wt* and *hns* mutant respectively (figure 14c, 1.4 fold repression by H-NS). A bgl_{DRE} construct that carries a DRE fragment from position +561 to +737 ($PUV5$-bgl_{DRE-NT} (+561 to 737)-$lacZ$) directed the expression of 1130 units of β-galactosidase in *wt* and 1895 units in the *hns* mutant, demonstrating that repression by H-NS is a marginal 1.4 fold (Figure 14d). This was surprising because the DNA

fragment from position +600 to +700 bp relative to the transcription start site showed efficient binding to H-NS. There are several explanations possible for the loss of H-NS repression in this construct, first, the rate of transcription in this construct is high (1132 units in wt, whereas only 91 units of β-galactosidase activity for *PUV5-+95bgl$_{DRE-NT}$-lacZ* in *wt*) which may prevent efficient formation of the H-NS repression complex. Second, it may be possible that H-NS binding alone may not be sufficient for repression by H-NS; it may require flanking DNA sequences, which could contribute structurally or through specific sequence for repression by H-NS. To check weather the region 5' of the H-NS binding site contribute to the repression by H-NS, a *bgl$_{DRE}$* construct that carries a DRE fragment from position +95 to +737 relative to the transcription start site was analyzed. To abolish the translation of *bgl$_{DRE}$+95-+737* the start codon and two further AUG at position 3 and 27 were mutated (*PUV5-bgl$_{DRE-NT}$(+95 to+737)-lacZ*). The expression level was 250 units in the *wt* and it increased 3.5-fold to 882 units in the *hns* mutant. This suggests that the 5' flanking sequence of the H-NS binding site is required for repression by H-NS (figure 14e). The 5' sequence could contribute to reduce the rate of transcription, which may favor efficient H-NS repression complex.

A reporter that carries a *bgl$_{DRE}$* fragment from position +561 to +968 encompassing the H-NS binding region along with 3' flanking DNA directed 1770 units in the *wt* and 3110 units in the *hns* mutant, which resembles an only 1.7-fold repression by H-NS (figure 14f). This result suggests that the DNA flanking the H-NS binding site downstream contributes only marginally to the repression by H-NS.

Taken together these results signify the requirement of 5' region of *bglG* (+95 to +561) for efficient repression by H-NS by binding 600-700 bp downstream to the promoter. The region between +95 to +561 could contribute structurally for efficient formation of repression complex or it could serve as region to reduce transcription elongation rates, which in turn would favor H-NS repression. The latter possibility is supported by the fact that the expression of the construct carrying the *bgl*$_{DRE}$ from +95 to +737 is 4.5 fold lower then that of the construct lacking the DNA flanking the H-NS binding site upstream (*bgl$_{DRE}$* +561 to +737 (figure 14, compare d and e).

As a further control the H-NS binding region was deleted from position +600 to +700. The expression level directed by this *bgl$_{DRE}$*(Δ+600 to +700) construct was 618 units in the *wt* and it increased 3.7-fold to 2285 units in the *hns* mutant (figure 14g). The result was surprising. The repression by H-NS in the absence of the downstream H-NS binding site may

suggest that H-NS has weak affinity to bgl_{DRE} DNA other than the fragment from +600 to +700, which was not detected by the EMSA.
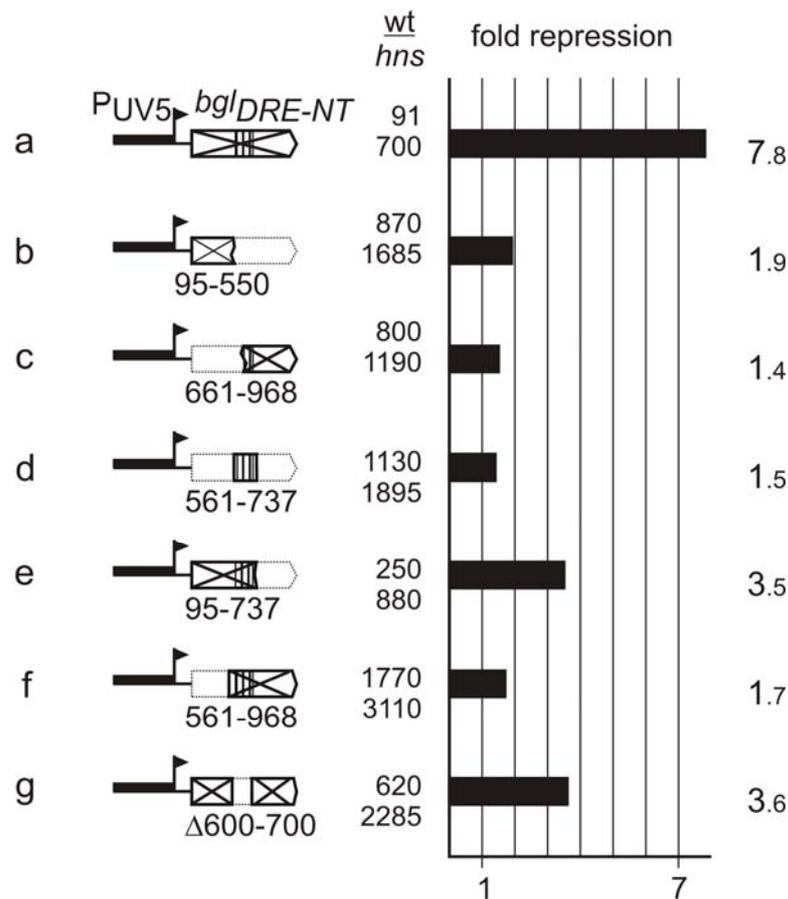
Figure 14. Deletion analysis of bglDRE to determine the minimum sequence requirement for H-NS repression. Schematic representation of chromosomal integrant having bgl_{DRE}-*lacZ* fusion driven by PUV5 promoter (a) and its deletion derivatives from b to g are shown at the extreme left. The bar diagram shows fold repression by H-NS (black bars). The β-galactosidase values expressed by the constructs in wt and hns mutant is shown to the left of the bar diagram. The numbers to the right of the bar chart shows the fold repression by H-NS. Strains used were shown in the order *wt* and *hns* a) S1195 and S3207 b) S2329 and S3258 c) 2298 and S3262 d) S2131 and S3256 e) S2462 and S3266 f) S2464 and S3303 g) S2168 and S3416.

IV. Discussion

Specificity of repression by H-NS is exceptionally high in the *E.coli bgl* and *proU* operons (Schnetz, 1995; Jordi and Higgins, 2000; Jordi et al., 1997a; Fletcher and Csonka, 1995a; Mellies et al., 1994a; Higgins et al., 1988; Owen-Hughes et al., 1992; Overdier and Csonka, 1992). In both systems repression requires binding of H-NS to upstream and downstream regulatory elements flanking the promoter. In this study the mechanism of repression by H-NS in the *bgl* and *proU* operons was analyzed. The high rate of repression of these operons by H-NS in the presence of both the upstream and downstream regulatory elements as compared to the repression obtained in the presence of the URE or the DRE alone suggests cooperativity. Furthermore, the repression by binding of H-NS to the downstream regulatory element is dependent on the transcription rate, and the repression is effective at low transcription rates only. However, this study shows that H-NS does not act as roadblock to the transcribing RNA polymerase, but affects transcription initiation at the promoter by binding downstream. In *bgl* H-NS bound to the downstream regulatory element also affects transcription elongation. These results are integrated into a model (figure 15). How highly specific repression by H-NS is mediated and how a small enhancement of the promoter activity may results in effective induction of *bgl* and *proU,* i.e. a model for how a protein that binds with low specificity to DNA (less than 4-fold) can specifically repress a gene more than 20 fold.

1. Cooperative repression by H-NS in the *bgl* and *proU* operon in *E.coli*

Here, H-NS has been shown to bind to upstream and downstream regulatory elements flanking the *bgl* promoter (Dole et al., 2004b) (Results, Section 1). Similarly, binding of H-NS to the flanking regions of the *proU* promoter has been shown previously (Lucht et al., 1994a). The downstream H-NS binding element in the *bgl* operon is located ~600 to 700 bp downstream to the promoter and the DRE in *proU* is located 150 to 300bp downstream (Dole et al., 2004b; Lucht et al., 1994a). The repression rate by binding of H-NS to either the upstream or downstream regulatory element alone is rather low in both systems. It is 1.5 to 2-fold in the presence of the URE, and ~3-fold in the presence of the DRE. In contrast, when both regulatory elements are present the repression is ~20 to 30 fold suggesting cooperativity (Results, Section 2). This result is consistent with previous reports implying the importance of both the upstream and downstream regulatory elements in effective repression of the *bgl* and *proU* promoter (Fletcher and Csonka, 1995a; Lucht et al., 1994a; Dole et al., 2004b; Dole, 2001; Schnetz, 1995). Cooperative repression is not a new phenomenon, it has been studied

extensively in the *lac, gal* and the *ara* operons in *E. coli* (Lyubchenko et al., 1997; Mandal et al., 1990; Muller et al., 1998). How may this cooperativity in repression by H-NS be achieved in the *bgl* and *proU* operons? Repression by H-NS bound to the URE and the DRE, respectively, presumably both repress transcription initiation. Repression of the promoter exerted by the URE may facilitate repression of transcription initiation *via* the DRE, and *vice versa*. It is also possible that this involves a DNA loop. H-NS is known to zip two DNA strands together (Dorman, 2004; Dame et al., 2002). Recent studies have shown that this DNA bridging (looping) by H-NS does not occur when both DNA strands were bound by H-NS, i.e. it does not occur by protein-protein interaction (Dame et al., 2006). Rather H-NS bound to one double strand can trap a second DNA double strand into the complex, whereby singly H-NS dimers bind to two double strands and form bridge. Therefore, in *bgl* and *proU*, H-NS bound to the URE may trap a DNA segment downstream and H-NS bound to the DRE may bridge to an upstream DNA segment. In the presence of both the upstream and downstream regulatory elements DNA bridging may be mutually enhanced resulting in the formation of a stable repressing complex.

2.   Transcription affects repression by H-NS bound to the downstream regulatory element

The repression of H-NS through the downstream regulatory region in both *bgl* and *proU* is affected by the transcription rate, wherein low transcription rate favors effective repression (Results, Section 3 and Figures 7 and 8). It has been shown that the biological function of H-NS depends on its ability to bind DNA and oligomerize along the DNA (Rimsky, 2004; Badaut et al., 2002; Smyth et al., 2000; Dorman et al., 1999). So it is conceivable that a low transcription rate would allow the effective formation of a stable H-NS repression complex as follows. Unlike H-NS bound to the upstream regulatory element, H-NS bound to the downstream regulatory element will encounter transcription by RNA polymerase. Data shown in this work suggests that RNA polymerase engaged in elongation reduces repression *via* the DRE. In transcription elongation RNA polymerase frequently encounters pause sites, at which transcription may terminate or may be resumed. This process is affected by termination factor Rho (which enhances termination) (result section 3.3), co-transcriptional translation of the RNA (which counteracts termination) (result section 3.2), and the transcription rate (result section 3.1 and 3.2). Termination factor Rho releases stalled RNA polymerase leading to termination of transcription (Magyar et al., 1996; Chan and Landick, 1993; Stewart et al., 1986; Richardson, 2003; Burns and Richardson, 1995; Modrak and Richardson, 1994). At a high transcription rate, with a high number of RNA polymerases

engaged in transcription, pausing is reduced due to the force applied by trailing RNA polymerases to a paused RNA polymerase. Repression of *bgl* and *proU via* the DRE is affected by the transcription rate and termination factor Rho. These results suggest that transcribing RNA polymerases remove H-NS from the downstream regulatory element.

In repression by H-NS *via* the DRE, H-NS could act as a road block to the transcribing RNA polymerase and induce pausing of RNA polymerase. Secondly, binding of H-NS to the downstream regulatory element may repress transcription initiation at the promoter from a distance. The finding that the effect of Rho in repression of *bgl* strictly depends on the presence on H-NS supports the model of H-NS acting as a road block to RNA polymerase. Roadblock formation was further addressed using dual reporter assays, which showed that H-NS affects transcription elongation 2-fold in *bgl* (result section 3.4). However, while repression of transcription elongation in *bgl* is only 2 fold in the dual reporter assay, it is 7 fold in single reporter assays suggesting that H-NS bound to the DRE represses transcription initiation at the promoter.

3.   H-NS does not repress the *bgl* and *proU* operons by acting as roadblock to RNA polymerase

In situ RNA polymerase footprinting using CAA in the *bgl* and *proU* operons did not reveal an H-NS dependent transcription pause site upstream of the DRE, which suggests that H-NS does not form a roadblock to the transcribing RNA polymerase (result section 4). This result is in good agreement with recent studies showing that the H-NS mediated DNA bridge, which resists a force of 9 pN, could be effectively removed by the force (20 pN) generated by an RNA polymerase engaged in transcription elongation (Dame et al., 2006). So, how does H-NS repress *bgl* and *proU* by binding to downstream regulatory element? Ongoing studies suggest that H-NS specifically affects open complex formation in *bgl* and *proU* promoters (Madhusudan S. and Schnetz, unpublished data). These experiments include *in situ* CAA footprinting of RNA polymerase at the *bgl* and *proU* promoters. Transcription initiation is a complex process, that involves a multitude of steps, including binding of RNA polymerase to the promoter (closed complex formation), melting of the DNA double helix (open complex formation), initiation of transcription and synthesis of the first di-ribonucleotide bond (initiating complex), transition to a still promoter bound initial transcribing complex and the synthesis of a 7 to 9 base long RNA, followed by either abortive transcription or promoter clearance (Kapanidis et al., 2006; Revyakin et al., 2006). Repression of open complex formation by H-NS has been shown before for *proU* (Jordi and Higgins, 2000). Trapping of RNA polymerase at the promoter by H-NS has been shown for the *rrnB*-P1 promoter and the

promoter of another operon *hdeAB* (Shin et al., 2005; Dame et al., 2002). However, at the *hdeAB* promoter RNA polymerase is trapped in the open complex. Current work is focused on determining which step of transcription initiation is specifically affected in *bgl* and *proU*. Possibly RNA polymerase is trapped in the closed complex or the formation of closed complex is affected by H-NS. Preliminary results indicate that RNA polymerase is poised at the *bgl* and the *proU* promoter, i.e. the promoter clearance rate is low (Madhusudan and Schnetz, unpublished data). Poising of RNA polymerase at the promoter and concomitant DNA bending may contribute to DNA bridging by H-NS bound to the downstream regulatory element. It further remains to be examined whether H-NS binding to the upstream regulatory element also affects the same step of transcription initiation as does H-NS bound to downstream regulatory element. This would help understanding the cooperativity of repression by H-NS in the *bgl* and *proU* operons.

4.   Complexity of the *bgl* downstream regulatory element

In situ CAA footprinting revealed an intrinsic pause site (+477 to +484 relative to the transcription start site) in *bgl,* which is located well upstream of the H-NS binding site in DRE. RNA secondary structure prediction in this region revealed a stable hairpin loop structure preceding the pause (result section 5.1). Such a hairpin loop structure is reminiscent of RNA polymerase pause sites, and RNA polymerase pausing plays a significant role in gene regulation (Landick, 2006). Northern analysis performed in this study shows a prominent full length RNA and truncated RNA of ~350bases. The full length to truncated RNA ratio in the *wt* and *hns* mutant indicates that H-NS prevents full length RNA synthesis by a factor of 2 (which is in agreement with the result of the dual reporter system, suggesting a 2-fold repression of elongation). It has been shown before that expression of the *bgl* operon is limited by the levels of an antiterminator encoded by *bglG,* which encompasses the *bgl* DRE (Dole et al., 2002; Dole, 2001). BglG mediates antitermination in the leader of the operon. Therefore, the full length to truncated RNA bias would result in less BglG, reduced antitermination in the leader and lower transcription elongation rates across the *bgl* DRE. The ~350base truncation product detected by Northern analysis does not correspond to the size of RNA that would be expected if termination occurred at the RNA polymerase pause site (which would be ~480 base). It could be speculated that the truncation observed is the result of RNA processing by an RNase, but further analysis needs to be done to validate this claim. Further genetic analysis demonstrated that deleting the pause and the secondary structure preceding the pause does not affect H-NS repression of *bgl via* the DRE. It still needs to be confirmed experimentally that no pausing occurs in the pause site deletion mutants.

Genetic analysis to find the minimum downstream regulatory element required for repression by H-NS suggest the significance of DNA sequence preceding the H-NS binding site in *bgl* DRE. The role of this region between the promoter and the H-NS binding region is unknown. This region could either attribute structural contribution or somehow act to regulate the rate of transcription elongation resulting in effective binding of H-NS to the DRE and formation of a repressing complex by DNA bridging.

4.   **Model**

Our current working model for repression of *bgl* and *proU* by H-NS relies on mechanistic parallels including the result that small modulation of transcription affects the repression by H-NS in both systems. In this model it is assumed that H-NS bound to the URE interacts with a downstream DNA segment resulting in DNA loop formation. Likewise H-NS bound to the DRE would form a bridge to an upstream DNA segment. In the presence of both regulatory elements (DRE and URE) a stable repressing complex is formed that traps RNA polymerase at the promoter, which is in agreement with the current view of H-NS mediated repression (Dorman, 2004).
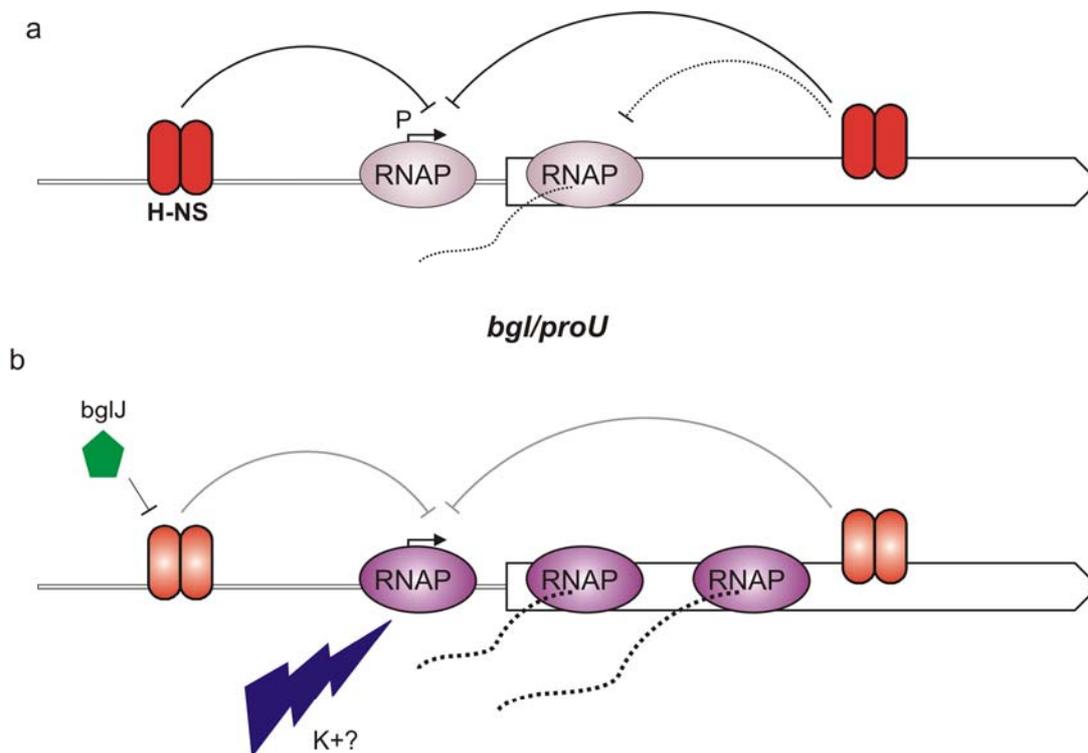
Figure 15. Model for H-NS mediated repression in the *bgl* and *proU* operons: a) simplified schematic showing the repression of *bgl* and *proU* by H-NS. H-NS binds to upstream and downstream regulatory elements (shown in red oval). The binding of H-NS upstream to the promoter (P) presumably affects transcription initiation. The binding of H-NS downstream to the promoter affects transcription initiation and also elongation. B) The repression by H-NS is overcome by slight activation of transcription. In case of *bgl* the transcription factor bglJ activates the promoter by ~3fold resulting in enhanced transcription, in *proU* upon osmotic shift the transcription is activated presumably by potassium glutamate. This activation at the promoter enhances the RNA polymerase (purple ovals) read-through, which affects the downstream binding of H-NS.

The *proU* operon is activated at high osmolarity. At high osmolarity the *proU* promoter activity is increased ~2-fold. The *bgl* promoter can be activated roughly 3-fold by the transcription factor BglJ, which presumably interferes with binding of H-NS to the URE (Madhusudan et al., 2005). A moderate, 2 to 3-fold, increase of the promoter activity could completely relieve repression as follows. With a stronger promoter more RNA polymerases will escape trapping at the promoter and engage in transcription elongation. The transcribing RNA polymerase will counteract DNA bridging by H-NS by simply removing H-NS from the DNA. This will result in a further enhancement of the promoter activity, a further increase of the transcription elongation rate, and cause complete removal of the repressing DNA-bridging complex formed by H-NS.

V. Materials and methods

1. Chemicals, enzymes and other materials

Chemicals and enzymes were purchased from commercial sources unless otherwise specified. Oligonucleotides were purchased from Invitrogen life technologies. The antibiotic Bicyclomcin is a gift from Fujisawa Pharmaceutical Co., Ltd. Osaka, Japan.

2. Media and agar plates

LB medium (1 l)	10g Bacto Tryptone (Difco)
	5g Yeast-extract (Difco)
	5g NaCl
	(for plates 15g Bacto Agar, Difco)
NB medium (1 l)	8g Bacto NB broth, dehydrated (Difco)
	(3g Bacto Beef extract, 5g Bacto peptone)
SOB medium (1 l)	prepare SOB in 970ml H_2O
	20g Bacto Tryptone (Difco)
	5g Yeast-extract (Difco)
	0.5g NaCl
	1.25ml 2M KCl
	adjust pH 7.0 with NaOH
	autoclave, add 10ml 1M $MgCl_2$
	for SOC add 19.8ml 20% glucose

3. Antibiotics

Antibiotics	stock	storage	final conc.
Ampicillin	50mg/ml in 50% ethanol	-20°C	50μg/ml
Chloramphenicol	30mg/ml in ethanol	-20°C	15μg/ml
Kanamycin	10mg/ml in H_2O	+4°C	25μg/ml
Spectinomycin	50mg/ml in 30% ethanol	-20°C	50μg/ml
Tetracyclin	5mg/ml in 70% ethanol	-20°C	12μg/ml
Bicyclomycin	1mg/ml in H_2O	Room temperature	20μg/ml
Rifampicin	100mg/ml in methanol	-20°C	200μg/ml

4. General methods

Methods of molecular biology like restriction enzyme digestions, ligations and other enzymatic reactions, PCR, plasmid purification, auto-radiography were performed as described (Sambrook et al., 1989; Sambrook and Russell, 2001) or according to the manufacturer instructions, unless otherwise stated.

5. Plasmids and DNA fragments

Large scale preparations of plasmid DNAs were performed using the plasmid maxiprep/midiprep kit (Qiagen/Promega) according to manufacturer's instructions from a culture volume of 250ml. A brief description of the plasmid constructions can be found in the

appendix, table 2. Details of the plasmid constructions are documented in the lab records and sequences are compiled in Vector NTI (Invitrogen).

A series of plasmid starting from pKENV01 were derived from pKEM02 (Figure 16), which has a pACYC (p15A) origin of replication. These plasmids carry the λ phage attachment site, *attP*, to allow λ integrase mediated recombination insertion into the *attB* site of the *E.coli* chromosome (Diederich et al., 1992). The plasmids also have a Ω cassette which contains the spectinomycin resistance gene, *aadA*, and strong transcriptional terminators at its 3' end. Plasmid pKEM02 has convenient restriction enzyme sites (SalI, EcoRI, XbaI), which allows easy cloning of DNA fragment before the *lacZ* gene. For integration of the reporter into the chromosome, the plamids were digested with BamHI or BglII and the origin less fragment containing the spectinomycin resistance gene and the gene of interest fused to the *lacZ* reporter was inserted into the *attB* site of the chromosome as described (Diederich et al., 1992). Plasmids pKENV63 to 67, 69, 71 and 73 were derivative of pKK177-3 (Figure 17) (Brosius and Holy, 1984), which has a pBR322 based origin of replication, ampicillin resistance gene (*bla*) and two strong Rho independent transcriptional terminators rrnBT1 and T2.
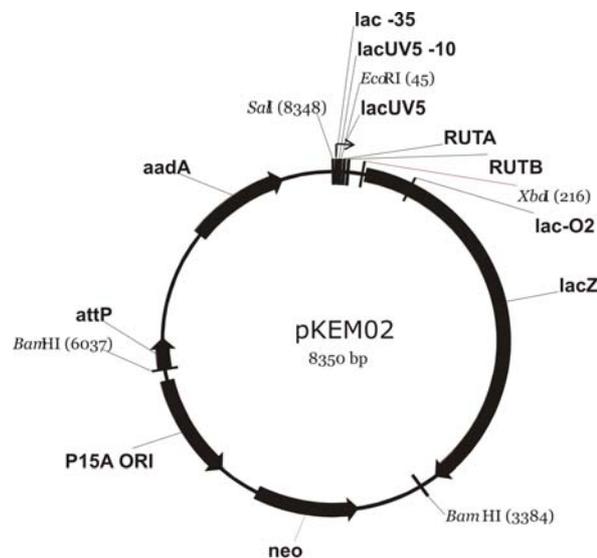
Figure 16: Schematic representation of pKEM02. Plasmid pKEM02 and a series of plasmid starting from pKENV01 have similar features for integration into the *attB* site of the *E.coli* chromosome. All these plasmids carry the *attP* site for integration, resistance markers for spectinomycin (*aadA*) and kanamycin (*neo*).
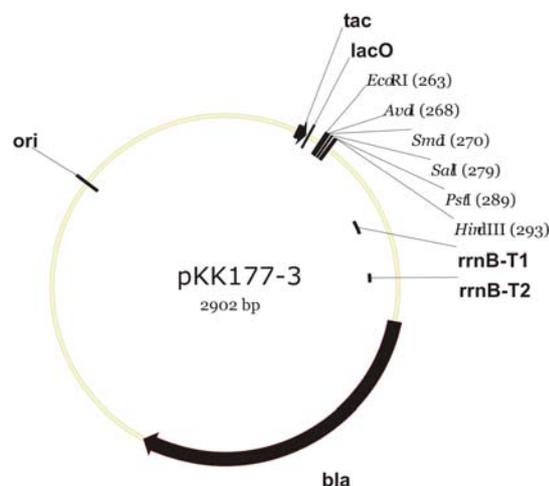
Figure 17: Schematic representation of pKK177-3. Plasmid pKENV63 to 67, 69, 71 and 73 were derived from pKK177-3, which has a pBR322 origin of replication, and carries the ampicillin resistance gene (*bla*) and strong Rho independent terminators *rrnB* T1 and T2. Convenient restriction sites for cloning are located between the *tac* promoter and the terminator as indicated in the figure.

6.   DNA sequencing

DNA sequencing was done with the Big dye terminator cycle sequencing kit (version 1.1 or version 3.1, ABI prism) according to manufactures instruction and using automated DNA sequencer. For sequencing the reaction was carried out in a total volume of 10μl with 1μl of Big dye sequencing mix. Nucleotide sequence alignments were performed using the Vector NTI program (Invitrogen).

7.   Preparation of competent cells and transformation

CaCl₂ method

TEN buffer: 20mM Tris-Hcl pH 7.5, 1mM EDTA, 50mM NaCl

Cells were grown in 25ml LB to an OD_{600} =0.3 and centrifuged at 3000rpm for 10 minutes at 4°C. The pellets were resuspended in 12.5ml of ice cold 0.1M $CaCl_2$ and spun for 10 minutes at 3000 rpm. The resulting pellet was resuspended in 1ml of 0.1M $CaCl_2.$ For transformation 10-20ng of plasmid DNA or 10μl of ligations in 50μl of TEN buffer was mixed with 100μl of competent cells. The cells were incubated for 20 minutes followed by heat shock at 42°C for 2 minutes and incubated for additional 10 minutes on ice. The competent cells were transferred to 1ml of LB medium and incubated for 1 hour at 37°C. 100μl of the culture was plated on suitable selection plates.

Electrocompetant cells and electroporation

Cells were grown overnight in 3ml SOB medium with appropriate antibiotics and at appropriate temperature. Of this culture 200μl were inoculated to 50ml of SOB media with appropriate antibiotics and grown to an OD_{600} of 0.7. The culture was transferred to prechilled tubes and centrifuged at 4°C for 15 minutes. The pellet was resuspended in 50 ml of ice-cold H_2O and spun at 4°C for 15 minutes at 3000rpm. The pellet was again resupended in 25 ml of prechilled H_2O and centrifuged at 4°C for 15 minutes at 3000rpm. Then the cells were resuspended in 2ml of ice-cold 10% glycerol and pelleted by centrifugation (3000 rpm for 15 minutes). Finally, cells were resuspended in 200μl of ice-cold 10% glycerol. The cells were either used immediately for electroporation or for long term storage, further incubated for 1 hour on ice and stored in 40μl aliquots at -80°C. For transformation 40μl of competent cells were mixed with plasmid DNA or a DNA fragment and incubated for 10 minutes on ice. The mix was transferred to prechilled electroporation cuvette (Biorad). The cuvettes were placed in the electroporator and the electric shock was given for 3 seconds at 1.8kV. 1ml of SOC medium was immediately added to the cuvettes. Then the cells were transferred to glass tubes and incubated at 37°C for 1 hour. 100μl of the culture was plated on appropriate selection plates.

8.   Integration of plasmids into the *attB* site of the *E.coli* chromosome

Integration of plasmids into the chromosome was done as described (Diederich et al., 1992). This technique is used to integrate DNA fragment in the λ *attB* site on *E.coli* chromosome. Briefly, strain S541 or its derivatives were first transformed with a temperature sensitive plasmid (pLDR8) expressing integrase, and the transformants were selected at 28°C on LB kanamycin plates. Plasmids having the λ *attP* site, the gene *lacZ* fusion of interest and the spectinomycin resistance cassette were digested with BamHI (or BglII). The origin less fragment was gel purified and eluted using the Qiagen gel extraction kit. 10ng of the origin less fragment was religated and half of the relegation was used to transform competent cells of S541/pLDR8. The transformants were selected at 42°C on LB spectinomycin plates. At 42°C the integrase gene is expressed and the integrase promotes recombination between the λ *attB*

and *attP* sites resulting in integration of the DNA fragment. In addition, replication of the temperature sensitive plasmid pLDR8 stops. The colonies were analyzed for kanamycin sensitivity (loss of pLDR8) and the integration was verified by PCR using the primers described below. Two independent integrants were selected for use in further experiments.

> S93/S164: to test the *attB/P`*-side
> S95/S96: to test the *attP/B`*-side
> S95/S164: to see integrations of dimers
> suitable primers to test the fragment

9.   Deletion of *hns* gene according to (Datsenko and Wanner, 2000)

Deletion of the *hns* gene was done according to (Datsenko and Wanner, 2000). This system is based on the λ Red based recombination between linear DNA fragment and the chromosomal gene. The basic strategy is to replace the chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR and by using primers with 20 to 50 nt homology extensions of the gene to be deleted. Briefly, the cells were transformed with temperature sensitive plasmid (pKD46) which has λ red system under the control of inducible arabinose promoter. The PCR product for deletion of *hns* was generated using primers S655/S672 and plasmid pKD4 as template. This PCR generates a fragment carrying the kanamycin resistance gene flanked by a short homology to upstream and downstream sequences of the *hns* gene. In addition, the kanamycin resistance gene is flanked by FRT site, which allows the deletion of the resistance gene by the Flp recombinase after gene replacement. 100ng of the gel purified PCR products were used to electro-transform cells harboring the helper plasmid (pKD46) expressing λ red recombinase. The recombinants were selected at 42°C on LB kanamycin plates. The loss of the helper plasmid was confirmed by sensitivity to ampicillin and the deletion of *hns* was confirmed by PCR using primers S2/S602. Two independent colonies were stored and used in further experiments.

10.   Transduction with phage T4GT7 (Wilson et al., 1979; Dole et al., 2002)

> T4-Topagar
> 6g Bacto-Agar (Difco)
> 10g Bacto-Tryptone (Difco)
> 8g NaCl
> 2g Tri-Natriumcitrate-Dihydrate
> 3g Glucose
> add 1l H_2O

The technique is based on generalized transduction, which makes use of the bacteriophage T4*GT7* to transfer DNA between bacteria. Briefly, 100µl of the overnight culture to be transduced was incubated with 10µl, 5µl, and 2µl of T4GT7 lysate prepared from the cells which had the DNA of interest (Donor strain). The incubation was carried out for 20 minutes at room temperature and 100µl was plated on respective selection plates. The tranductants were restreaked at least twice to get rid of the contaminating phages and the transfer of the gene was verified by PCR.

11.   β-galactosidase assay (Miller, 1972)

> Z buffer (100mM Na-phosphate pH 7.0, 10mM KCl, 1mM $MgSO_4$,
>   100µg/ml chloramphenicol)

The β-galactosidase activity measurement was carried out essentially as described (Miller, 1972), with only minor modifications. Briefly, strains were grown in 3ml LB or LB medium with various NaCl concentrations overnight. Subcultures were made in fresh LB or LB with various NaCl concentrations to an OD_{600} of 0.15-0.2. The cultures were grown to an

OD$_{600}$ of ~0.5 before harvesting. Three different dilutions of culture were made in duplicates in Z-buffer in a final volume of 1ml. The cells were permeablized by addition of 10μl of 0.1%SDS and 20μl of chloroform. The dilutions were preincubated at 28°C for 10 minutes followed by addition of 200μl of ONPG (4mg/ml in 0.1M phosphate buffer pH 7.0). The assay was stopped by the addition of 0.5ml 1M Na$_2$CO$_3$. The OD$_{420}$ was measured and the β-galactosidase activity was calculated as described below. The enzyme activities were performed at least three times from independent strains or transformants and the standard deviations were less than 10%.

$$\text{Miller units} = \frac{\text{OD}_{420} \text{ x dilution factor x 1000}}{\text{OD}_{600} \text{ x time (minutes)}}$$

12. β-glucuronidase assay (modified from (Sean R.Gallagher, 1992))

GUS assay buffer: 50mM NaPO$_4$ pH7.0, 1mM EDTA, 5mM DTT, 1.25mM PNPG
GUS buffer stock: 50mM NaPO$_4$ pH7, 1mM EDTA, 1M DTT
Stop solution: 0.4 M Na$_2$CO$_3$

For the β-glucuronidase assay cells were grown to an OD$_{600}$ ~0.5 in LB or LB with various salt concentrations. 1ml of the culture was harvested and centrifuged at 13,000rpm for 1 minute. The pellet was resuspended in 175μl of B-PER (Pierce), 25μl of protease and phosphatase cocktail (Sigma) and 0.5μl of chloramphenicol. Then the samples were vortexed for 1 minute and incubated on ice for 5 minutes. Dilutions of the lysate were made in prewarmed GUS assay buffer (37°C) to a final volume of 200μl. At least two different dilutions were made in duplicate. The tubes were incubated for 30 minutes at 37°C. The reaction was stopped by addition of 1ml of 0.4 M Na$_2$CO$_3$. The absorbance at OD$_{405}$ was measured. The enzyme activities were performed at least three times and the standard deviation were less than 10%. The rate of the reaction can be calculated by the following formula.

R= 50 x S/(V x OD$_{600}$)
S is the slope (OD$_{405}$ / time)
V is the volume used for the assay

13. RNA analysis by Northern blotting

(modified from Current protocols in molecular biology available at http://www3.interscience.wiley.com/cgi-bin/mrwhome/104554809/HOME)

20x SSPE: 3M NaCl, 100mM NaH2PO4·H2O, 10mM EDTA
100x Denhardt solution: 10g Ficoll 400, 10g polyvinylpyrrolidone,
        10 g BSA (pentax fraction V), H$_2$O to 500 ml
20×SSC: 3 M NaCl, 0.3 M Na$_3$citrate×2H$_2$O, Adjust pH to 7.0 with 1 M HCl
10xTBE: 890mM Tris base, 890mM Boric acid, 10 mM EDTA pH 8.0

Total cellular RNA was isolated from cells grown to OD$_{600}$ ~0.5, and 1ml was used for RNA isolation using the Promega SV total RNA isolation system (Promega) according to manufacturer's instruction. 7.5μg of total RNA in 5μl of DEPC H$_2$O was mixed with 5μl of 2x RNA loading dye (98% (v/v) deionized formamide, 10 mM EDTA pH 8.0, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromphenol blue), heat denatured at 95°C for 5 minutes, and cooled on ice. Samples were resolved on denaturing acrylamid gels (5% polyacrylamide 19:1 acrylamide:bisacrylamide, 7M Urea, 0.5xTBE run at 300V) and transferred to Hybond N+ (GE Healthcare, Germany) positively charged nylon membrane using a Trans-blot SD semi

dry apparatus (Biorad.) at 15V for 1 hour. The RNA transfer to the blot was verified by staining with 0.2% methylene blue in 0.3M Na-Acetate pH5.5. The position of an RNA marker ladder (Fermentas) were marked with Indian ink. The blots were baked at 80°C for two hours and prehybridized for 3 hours in prehybridization solution (5x SSPE, 5x Denhardt solution, 50% formamide, 0.5%(w/v) SDS and 72μg/ml denatured herring sperm DNA) at 65°C.

After prehybridization the blot was placed in fresh hybridization solution, 400μl of the eluted radioactive probe (see below) was added and hybridized overnight at 65°C. After hybridization the blots were washed twice with 2x SSC/0.1% SDS for 5 minutes at 37°C (low stringency washes). A medium stringency wash was done twice for 15 minutes at 42°C with 0.2xSSC/0.1%SDS. Two more additional washes were carried out using 0.1xSSC/0.1%SDS for 15 minutes at 68°C (high stringency washes). Finally the membrane was washed in 2xSSC and exposed to phosphorimager plates (Fuji film, BAS-MP 2040)/X-ray film (Kodak biomax film MS-1). The signals were quantified using ImageQuant TL software (GE Healthcare, Germany).

For preparation of the RNA probe, 0.2pmol of PCR product (primers S303/S314, PCR template pFDX733) containing the T7 RNA polymerase promoter sequence was *in vitro* transcribed in a 20μl reaction containing the following

> 0.2pmol of PCR product
> 1μl 10mM ATP, GTP, CTP stock
> 1μl 100μM UTP
> 2.5μl α-32P UTP (800ci/mmol, 20mCi/ml) [GE Healthcare, Germany]
> 4μl 5xTranscription buffer (Fermentas, Germany)
> DEPC H$_2$O to 20μl
> 1μl T7RNA polymerase 20U/μl (Fermentas, Germany).

The unincorporated nucleotides were removed by passing through a Nick Sephadex^{TM} G50 columns (GE Healthcare, Germany) and eluted in 400μl of 10mM Tris-Cl pH8.0.

14.   Electrophoretic mobility shift experiments (EMSA) with H-NS

DNA mobility shift experiments

DNA gel mobility shift experiments were essentially carried out as described (Dole et al., 2004b). For shift experiments, fragments were generated by PCR and agarose gel purified. Approximately 5 pmol of each fragment was labelled at the 5' end with T4 polynucleotide kinase (Fermantas) in the presence of adenosine 5'-[γ^{32}P]-triphosphate (5000 Ci/mmol, 10mi/ml). Nonincorporated nucleotides were removed using a Nick Sephadex^{TM} G50 column (GE Healthcare, Germany). In the binding assays ~2 fmol of the labelled fragment (15000–30000 cpm, 0.2 nM final concentration) were incubated for 15 min at 30°C in 10μl (20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, 10% glycerol) with various amounts of H-NS (50 nM, 75 nM, 100 nM, 150 nM, and 200 nM), and separated on a 7.5% acrylamide, bis-acrylamide (29.2 : 0.8) gel in 0.5 xTBE (i.e. 45 mM Tris-borate pH 8.3, 1 mM EDTA), 2.5% glycerol, which was run in the cold-room.

For competitive shift experiments ~2 fmol of DNA was incubated with 200nM of H-NS and titrated with unlabelled specific or non-specific competitor DNA at 5, 10, 20 and 50 fold excess of labeled DNA. The binding and the gel running conditions were similar as described above.

RNA mobility shift experiments

For RNA shift experiments, ~0.2 pmol of gel purified PCR fragments having T7-RNA polymerase promoter sequence were *in vitro* transcribed and internally labeled with α-^{32}P UTP (800ci/mmol, 20mCi/ml) using T7 RNA polymerase (Fermentas, Germany).

Nonincorporated nucleotides were removed using a Nick Sephadex^{TM} G50 column (GE Healthcare, Germany). The labeled RNA was heat denatured at 95°C for 5 minutes and cooled on ice immediately before the binding assay. In the binding assay ~ 2fmol of labeled RNA were incubated for 15 minutes at 30°C in 10μl (20mM Tris-HCl pH 7.5, 100mM KCl, 1mM DTT, 10% Glycerol) with various amounts of H-NS (50 nM, 75 nM, 100 nM, 150 nM, and 200 nM). The samples were separated on a 5% acrylamide, bis-acrylamide (19:1) gel in 0.5x 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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Table 1: *E.coli* K-12 strains used in the present work

Strain	Relevant genotype or structure[a]	construction[b]/reference
CAG1843	*F⁻, λ⁻, rph-1, ilvD500::Tn10*	CGSC#7462
CSH50	*bgl° Δ(lac-pro) ara thi (=S49)*	(Miller, 1972)
HD152	*F⁻,thr-33,λ⁻,trpE9829(Am), serU126(ts, AS),his-2130,*	
	tyrA15(Am),thyA707,IN(rrnD-rrnE)1, rho-702(ts)	CGSC#6106
PD32	MC4100 *hns-206*::Ap^R str^R (=S102)	(Dersch et al., 1993)
S541	S539 *Δbgl-AC11 ΔlacZ-Y217*	(Dole et al., 2004b)
S1193	S541 *attB*::[SpecR *PUV5 bgl*$_{DRE}$ *lacZ*]	x pKESD48, (Dole et al., 2004b)
S1195	S541 *attB*::[SpecR *PUV5 bgl*$_{DRE-NT}$ *lacZ*]	x pKESD49, (Dole et al., 2004b)
S1213	S541 *attB*::[Spec^R *bgl*$_{URE}$ *Pbgl +25 lacZ*]	x pKEKB30, (Dole et al., 2004b)
S1258	S1195 *hns::amp*^R	xT4GT7(PD32), (Dole et al., 2004b)
S1816	S541*attB*:: [spec^R *PUV5 t1*$_{RAT}$ *bgl*$_{DRE}$ *lacZ*]	x pKESK51
S1906	S541 *attB*::[SpecR *PUV5lacZ*]	x pKES99
S1946	S541 *ilvD::Tn10*	x T4GT7(CAG 18431)
S1956	S1946 *rho-702(ts) ilvD+*	x T4GT7(HD152)
S1995	S1956 *attB*::[SpecR *PUV5 bgl*$_{DRE-NT}$ *lacZ*]	x pKESD49
S1997	S1995 *hns::amp*^R	xT4GT7(PD32)
S2048	S541 *attB*::[SpecR *ProU*$_{URE}$*PproU (-315 to +1) lacZ*]	x pKES109
S2056	S541 *uidA::miniTn10Cm*^R	x mutagenesis screen
S2131	S541 *attB*::[specR *PUV5 bgl*$_{DRE}$ *(+561 to +737) lacZ*]	x pKEN02
S2135	S541 *attB*::[specR *PUV5 proV'*$_{DRE-NT}$*lacZ*]	x pKENV04
S2137	S541 *attB*::[specR *PUV5 proV'*$_{DRE}$ *lacZ*]	x pKENV03
S2285	S541 *attB*::[SpecR *Ptac bglG*$_{DRE}$ *lacZ*]	x pKENV11
S2287	S541 *attB*::[SpecR *Ptac lacZ*]	x pKENV13
S2298	S541 *attB*::[specR *PUV5 bgl*$_{DRE-NT}$ *(+660 to+970) lacZ*]	x pKENV21
S2327	S2056 *attB*::[specR *PUV5 uidA proV'*$_{DRE}$ *lacZ*]	x pKENV10
S2329	S541 *attB*::[specR *PUV5 bgl*$_{DRE}$ *(+95 to +550) lacZ*]	x pKENV19
S2368	S2056 *attB*::[specR *PUV5 uidA bgl*$_{DRE-NT}$ *lacZ*]	x pKENV18
S2462	S541 *attB*::[specR *PUV5 bgl*$_{DRE-NT}$ *(+95 to +737) lacZ*]	x pKENV30
S2464	S541 *attB*::[specR *PUV5 bgl*$_{DRE-NT}$ *(661 to 698) lacZ*]	x pKENV31
S2468	S541 *attB*::[specR *PUV5 bgl*$_{DRE-NT}$ *(Δ+601 to +700) lacZ*]	x pKENV33
S2501	S541 *attB*::[SpecR *proU*$_{URE}$ *PproU proV'*$_{DRE(}$*-315 to +303) lacZ*]	x pKENV34
S2692	S2056 *attB*::[SpecR *PUV5 uidA rrnBT1 bgl*$_{DRE-NT}$ *lacZ*]	x pKENV42
S2696	S2056 *attB*::[SpecR *PUV5 uidA rrnBT1 lacZ*]	x pKENV44
S2704	S2056 *attB*::[SpecR *PUV5 uidA rrnTt1 proV'*$_{DRE}$ *lacZ*]	x pKENV48
S3000	S541 *attB*::[SpecR *PlacI bgl*$_{DRE}$ *lacZ*]	x pKENV56
S3005	S541 *attB*::[SpecR *placI lacZ*]	x pKENV58
S3010	S541 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3034	S541 *attB*::[SpecR *PlacI proV'*$_{DRE}$ *lacZ*]	x pKENV56
S3058	S541 *attB*::[SpecR *Ptac proV'*$_{DRE}$ *lacZ*]	x pKENV59
S3122	S1906 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3124	S2048 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3126	S2137 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3128	S2501 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3134	S3034 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3165	S2135 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3169	S2287 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3171	S3005 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3175	S3058 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3181	S541 *attB*::[SpecR *bgl*$_{URE}$*Pbgl t1*$_{RAT}$ *bgl*$_{DRE}$ *lacZ*]	x pKENV61
S3191	S541 *attB*::[SpecR *bgl*$_{URE}$ *PUV5 t1*$_{RAT}$ *bgl*$_{DRE}$ *lacZ*]	x pKENV68
S3203	S3181 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3205	S3191 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3207	S1195 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3209	S1816 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3211	S1193 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3256	S2131 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4
S3258	S2329 *Δhns::kan*$_{KD4}$	x S665/S672,pKD4

S3262	S2298 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3266	S2462 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3270	S2368 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3272	S2692 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3283	S2696 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3290	S2327 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3292	S2704 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3296	S1213 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3299	S2285 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3300	S3000 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3303	S2464 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3346	S541 Δhns::Kan $_{KD4FRT}$	x S665/S672,pKD4
S3412	S541 attB::[SpecR bgl$_{URE}$PUV5+25 lacZ]	x pKENV78
S3416	S2468 Δhns::kan$_{KD4}$	x S665/S672,pKD4
S3420	S3412 Δhns::kan$_{KD4}$	x S665/S672,pKD4

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a: The relevant genotype of the strains (which are all CSH50 derivatives) refers to the *bgl, lac, hns, proU* and *uidA* loci. The r*ho(ts)* allele was sequenced and found to have three mutation: an A to G exchange of the first base of the codon 158 resulting in an amino acid change from threonine to alanine, an G to A exchange of the first base of the codon 224 causing an amino acid shange from glutamate to isoleucine, and a G to A exchange of the first base of the codon 304 causing alanine to threonine exchange. Thus, *rho(ts)* encodes a Rho-T158A-E224I-A304T mutant protein. Deletions made in *bgl$_{DRE}$* were indicated by the positions of the deleted regions within brackets (eg. Δ+601 to +700 refers to deletion of sequence from 601 to 700 bp relative to the transcription start site, +95 to +737 refers to the presence of sequence from +95 to +737 relative to the transcription start site). *bgl$_{DRE-NT}$* refers to mutation in the start codon and two additional ATG codons at position 3 and 27 to CGC, thereby rendering the *bgl$_{DRE}$* non-translatable. *t1$_{RAT}$* indicates a mutation in the leader region of *bgl* operon at position +67 and +68 from AA to T making the construct independent of BglG mediated anti-termination. *proU$_{DRE}$* refers to the *proU* fragment from postion +1 to +303 relative to the transcription start site. CGSC#6106 and CGSC#7462 were strains obtained from E.coli genetic stock center (maintained by molecular, cellular and development biology department, Yale University, New Haven, Connecticut).

b: Construction of strains by transduction using T4GT7 is explained in materials and methods. Integration of plasmids into the *attB* site of chromosome was done as decribed (Diederich et al., 1992) (see materials and methods). The deletion of *hns* allele was constructed according to (Datsenko and Wanner, 2000) and is explained in detail in material and methods. Δ*hns::kan$_{KD4}$* refers to the replacement of the chromosomal *hns* gene by a kanamycin resistance gene cassette, which was amplified from plasmid pKD4. Strain S2056 was constructed by random transposon (miniTn10) mediated mutagenesis as described in (Dole et al., 2004a; Dole, 2001)of S541 and selected on LB plates containing 4-nitrophenyl-β-D-glucuronide. The *uidA::Tn10cm^R* gene was sequenced to find the orientation and precise location of the transposon insertion.

Table 2: Plasmids used in the current work

Name	Relevant structure/description[a] and replicon/resistance[b]	Source/construction[c]/ reference
pCP20	for induction of FLP synthesis, temperature sensitive, amp^R, cam^R	(Datsenko and Wanner, 2000)
pKD4	template plasmid for generating $\Delta hns::KD4$ fragment, kan^R	(Datsenko and Wanner, 2000)
pLDR8	λ repressor, temperature sensitive allele cI-857; int under control of λP_R, pSC101 rep-ts, Kan^R	(Diederich et al., 1992)
pFDX733	*wt bgl* operon, kan^R	Lab collection (Schnetz et al., 1987)
pFDX840	*galK rrnBT1 T2* terminators, ampR, *ori-pBR*	Lab collection
pKEKB30	*PUV5 bgl$_{URE}$ Pbgl +25 lacZ*	Lab collection
pKEM02	*PUV5 λ cro tR1 lacZ*	Lab collection
pKEM03	*PUV5 λ cro tR1 lacZ*	Lab collection
pKES15	*attP bgl$_{URE}$ Pbgl +54 lacZ*	Lab collection
pKES99	*attP PUV5 lacZ*	Lab collection
pKESD08	*attP bgl$_{URE}$ Pbgl t1 bgl$_{DRE}$ lacZ*	Lab collection (Dole et al., 2002)
pKESK23	*LacIq lacO3 Ptac lacO1 MCS*	Lab collection
pKESD20	*attP PUV5 t1 bgl$_{DRE}$ lacZ*	Lab collection (Dole et al., 2002)
pKESD48	*attP PUV5 bgl$_{DRE}$ lacZ*	Lab collection (Dole et al., 2004b)
pKESD49	*attP PUV5 bglG$_{DRE-NT}$ lacZ*	Lab collection (Dole et al., 2004b)
pKESK51	*attP PUV5 t1$_{RAT}$ bgl$_{DRE}$lacZ*	Lab collection
pKENV01	*attP PUV5 RBS$_{lacZ}$ bgl$_{DRE}$ lacZ*	V: 1. pKEM02 EcoRI/XhoI, phosphatase V: 2. pKESD08 AflII/XhoI F: PCR template pFDX733 S419/S92
pKENV02	*attP PUV5 bgl$_{DRE}$ (+561to+737) lacZ*	V: pKEM02, EcoRI/XbaI, phosphatase F: PCR template pFDX733 S383/S384 EcoRI/XbaI
pKENV03	*attP PUV5 proV'$_{DRE}$ lacZ*	V: pKEM02 EcoRI/XbaI, phosphatase F: PCR template S527 S372/S420, EcoRI/XbaI
pKENV04	*attP PUV5 proV'$_{DRE-NT}$ lacZ*	V: pKEM02 EcoRI/XbaI, phosphatase F: 1 .PCR template S527 S372/S421 F 2. PCR template S527 S422/S420, PCR template products 1+ 2 PCR S372/S420 EcoRI/XbaI
pKENV05	*attP PUV5 RBS$_{lac}$ proV'$_{DRE}$ lacZ*	V: pKEM02 EcoRI/XbaI, phosphatase F: PCR template S527 S424/S420, EcoRI/XbaI
pKENV06	*attP PUV5 uidA bglG(+561 to +737) lacZ*	V: pKEM02 EcoRI/XbaI, phosphatase F: 1. PCR template S527 S425/S381 F: 2. PCR template pFDX733 S415/S384 PCR template products 1+2 S425/S384 EcoRI/XbaI
pKENV07	*attP PUV5 uidA proV' lacZ*	V: pKEM02 EcoRI/XbaI, phosphatase F: 1. PCR template S527 S425/S381 F: 2. PCR template S527 S423/S420 PCR template products 1+2, PCR S425/S420 EcoRI/XbaI
pKENV08	*attP PUV5 uidA lacZ*	V: pKEM02 EcoRI/XbaI, phosphatase F: PCR template S527 S425/S382, EcoRI/XbaI
pKENV09	*attP PUV5 uidA bglG(+561 to +737) lacZ*	V: pKEM03 EcoRI/XbaI, phosphatase F: pKENV06 EcoRI/XbaI
pKENV10	*attP PUV5 uidA proV' lacZ*	V: pKEM03 EcoRI/XbaI, phosphatase F: pKENV07 EcoRI/XbaI
pKENV11	*attP Ptac bgl$_{DRE}$ lacZ*	V: pKESD08 SalI/ AflII, phosphatase F: 1. PCR template pKESK23 S283/S284 F: 2 PCR template pKESD48 S448/S92 PCR template products 1+2 S283/S92 SalI/ AflII

pKENV12	*attP Ptac bgl $_{DRE-NT}$ lacZ*	V: pKESD08 SalI/ AflII, phosphatase F: 1. PCR template pKESK23, PCR S283/S284 F: 2 PCR template pKESD48, S448/S92 PCR template products 1+2, PCR S283/S92 SalI/ AflII
pKENV13	*attP Ptac lacZ*	V: pKES15 SalI/Eco81I, phosphatase F: PCR template pKES99 S447/S100, SalI/Eco81I
pKENV14	*attP Phis bgl$_{DRE}$ lacZ*	V: pKESD08 SalI/AflII, phosphatase F: 1. PCR template S527 S444/S445 F: 2. PCR template pKESD48 S446/S92 PCR template 1+2, PCR S444/S92 SalI/AflII
pKENV15	*Phis bgl $_{DRE-NT}$ lacZ*	V: pKESD08 SalI/AflII, phosphatase F: 1. PCR template S527 S444/S445 F: 2. PCR template pKESD49 S446/S92 PCR template 1+2, PCR S444/S92 SalI/AflII
pKENV16	*Phis lacZ*	V: pKES15 SalI/XbaI, phosphatase F: PCR template S527 S444/S445 SalI/XbaI
pKENV17	*attP PUV5 uidA bgl $_{DRE-NT}$ lacZ*	V: pKENV01 EcoRI/AflII, phosphatase F: 1. PCR template S527 S425/S381 F: 2. PCR template pFDX733 S416/S92 PCR template products 1+2 S425/S92 EcoRI/AflII
pKENV18	*attP PUV5 uidA bgl $_{DRE-NT}$ lacZ*	F: pKEMS03 salI/Eco81I, phosphatase V: pKENV17 salI/Eco81I
pKENV19	*attP PUV5 bgl $_{DRE}$ (+95 to +550) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: 1. PCR template pKESD49 S451 / S452
pKENV20	*attP PUV5 bgl $_{DRE}$ (+120 to +550) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: pKESD49 S453/S452 EcoRI/XbaI
pKENV21	*attP PUV5 bgl $_{DRE-NT}$ (+661 to +972) lacZ*	F: pKEMS02 EcoRI/XbaI, phosphatase V: PCR template pKESD49 S454/S455 EcoRI/XbaI
pKENV22	*attP PUV5 uidA bgl $_{DRE-NT}$ (+95 to +550) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: 1. PCR template S527 S425/S381 F: 2. PCR template pKESD49 S451/S452 PCR template products 1+2, S425/S452 EcoRI/XbaI
pKENV23	*attP PUV5 uidA bgl $_{DRE-NT}$ (+95 to +550) lacZ*	V: pKEMS03 EcoRI/XbaI, phosphatase F: pKENV22 EcoRI/XbaI
pKENV24	*attP PUV5 uidA bgl $_{DRE-NT}$ (+120 to +550) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: 1. PCR template S527 S425/S381 F: 2. PCR tempate pKESD49 S453/S452 PCR template products 1+2, S425/S452 EcoRI/XbaI
pKENV25	*attP PUV5 uidA bgl $_{DRE-NT}$ (+120 to +550) lacZ*	V: pKEMS03 EcoRI/XbaI, phosphatase F: pKENV24 EcoRI/XbaI
pKENV26	*attP PUV5 uidA bgl$_{DRE}$ (+661 to+972) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: 1. PCR template S527 S425/ S381 F: 2. PCR template pKESD49 S454/455 PCR template products 1+2 S425/S455 EcoRI/XbaI
pKENV27	*attP PUV5 uidA bgl$_{DRE}$ (+661 to +972) lacZ*	V: pKEM03 EcoRI/XbaI, phosphatase F: pKEVN26 EcoRI/XbaI
pKENV28	*attP PUV5 uidA lacZ*	V: pKEMS03 SalI/Eco81I, phosphatase F: pKENV08 SalI/Eco81I
pKENV29	*attP PUV5 bgl$_{DRE}$ (+95 to +737) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: PCR template pFDX733 S451/S486, EcoRI/XbaI
pKENV30	*attP PUV5 bgl$_{DRE-NT}$ (+955 to +737) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase

		F: PCR template pKESD49 S451/S384, EcoRI/XbaI
pKENV31	*attP PUV5 bgl*$_{DRE-NT}$ *(+561 to+972) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: PCR template pFDX733 S383/S455, EcoRI/XbaI
pKENV32	*attP PUV5 bglDRE (Δ+601 to +700) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: 1. PCR template pFDX733 S451/S487 F: 2. PCR tempate pFDX733 S488/S455 PCR template products 1+2, S451/S455 EcoRI/XbaI
pKENV33	*attP PUV5 bgl*$_{DRE-NT}$*(Δ601 to 700) lacZ*	V: pKEMS02 EcoRI/XbaI, phosphatase F: 1. PCR template pKESD49 S451/S487 F: 2. PCR tempate pFDX733 S488/S455 PCR template products 1+2, S451/S455 EcoRI/XbaI
pKENV34	*attP proU*$_{URE}$ *PproU proV'*$_{DRE}$ *lacZ*	V: pKENV03 SalI/MscI, phosphatase F: pKES108 SalI/MscI
pKENV35	*attP Ptac op bgl*$_{DRE}$ *lacZ*	V: pKESK23 EcoRI/XhoI, phosphatase F: pKESD08 AflII/XhoI
pKENV36	*attP Ptac op bglG*$_{DRE-NT}$ *lacZ*	V: pKESK23 EcoRI/XhoI, phosphatase F: 1. pKESD08 AflII/XhoI F: 2. PCR template pKESD49 S451/S92 EcoRI/AflII Three fragment ligation
pKENV37	*attP Ptac op proV'*$_{DRE}$ *lacZ*	V: pKESK23 EcoRI/XhoI, phosphatase F: pKENV03 EcoRI/XhoI
pKENV38	*attP Ptac op proV'*$_{DRE-NT}$ *lacZ*	V: pKESK23 EcoRI/XhoI, phosphatase F: pKENV04 EcoRI/XhoI
pKENV39	*attP PUV5 uidA λTR2 proV'*$_{DRE}$ *lacZ*	V: pKENV28 XbaI/XhoI, phosphatase F: pKENV03 Xho1/EcoR1 F: PCR tempate λ lysate S550/S551 XbaI/EcoRI Three fragment ligation
pKENV40	*attP PUV5 uidA λTR2 bgl*$_{DRE-NT}$ *lacZ*	V: pKENV39 MunI/EcoRI, phosphatase F: pKENV18 MunI/AflII F: PCR tempate pKESD49 S541/S92 EcoRI/AflII Three fragment ligation
pKENV41	*attP PUV5uidA rrnBT1 proV'*$_{DRE}$ *lacZ*	V: pKENV28 XbaI/XhoI, phosphatase F: pKENV03 XhoI/EcoRI F: PCR tempate pFDX840 S555/S554 XbaI/EcoRI Three fragment ligation
pKENV42	*attP PUV5 uidA rrnBT1 bgl*$_{DRE-NT}$ *lacZ*	V: pKENV41 MunI/EcoRI, phosphatase F: pKENV18 MunI/AflII F: PCR tempate pKESD49 S541/S92 EcoRI/AflII
pKENV43	*attP PUV5 uidA λtR2 lacZ*	V: pKENV28 XbaI, phosphatase F: PCR tempate λlysate S550/S552 XbaI
pKENV44	*attP PUV5 uidA rrnBt1 lacZ*	V: pKENV28 XbaI, phosphatase F: PCR tempate pFDX840 S554/S556 XbaI
pKENV45	*attP PUV5 λtR2 lacZ*	V: pKEM02 EcoR1/XbaI, phosphatase F: PCR template λ lysate S553/S552 EcoRI/XbaI
pKENV46	*attP PUV5 rrnBT1 lacZ*	V: pKEM02 EcoR1/XbaI, phosphatase F: PCR template pFDX840 S557/S556 EcoRI/XbaI
pKENV47	*attP PUV5 uidA λTR2 proV'*$_{DRE}$ *lacZ*	V: pKENV39 Eco81I/XhoI, phosphatase F: pKEGN28 Eco81I/XhoI
pKENV48	*attP PUV5 uidA rrnBT1 proV'*$_{DRE}$ *lacZ*	V: pKENV41 Eco81I/XhoI, phosphatase F: pKEGN28 Eco81I/XhoI

pKENV49	*attP bgl$_{DRE}$ lacZ*	V: pKEM04 EcoRI/Eco81I, phosphatase
		F: pKENV36 EcoRI/Eco81I
pKENV50	*attP lacI PUV5 op bgl$_{DRE-NT}$ lacZ*	V: pKENV36 SalI/EcoRI, phosphatase
		F: pKESD10 SalI/EcoRI
pKENV51	*attP PUV5 bgl$_{DRE}$ (+420 to+737) lacZ*	V: pKEM02 EcoRI/XbaI, phosphatase
		F: PCR template pFDX733 S646/S384
		EcoRI/XbaI
pKENV52	*attP bgl$_{URE}$Pbgl t1 bgl$_{DRE-NT}$(Δ601 to 700) lacZ*	V: pKENV32 SalI/AflII, phosphatase
		F: pFDX733 SalI/AflII
pKENV53	*attP PUV5 t1 bgl$_{DRE-NT}$(Δ601 to 700) lacZ*	V: pKESD20 AflII/XhoI, phosphatase
		F: pKENV32 AflII/XhoI
pKENV54	*attP PlacI bgl$_{DRE}$ lacZ*	V: pKENV14 SalI/AflII, phosphatase
		F: 1. PCR template S541, S668/S670
		F: 2. PCR template pFDX733, S671/S92
		PCR template 1+2 S668/S92 SalI/AflII
pKENV55	*attP PlacI bgl$_{DRE-NT}$ lacZ*	V: pKENV14 SalI/AflII, phosphatase
		F: 1. PCR template S541 S668/S670
		F: 2. PCR template pKESD49 S671/S49
		PCR template 1+2 S668/S92 SalI/AflII
pKENV56	*attP PlacI proV'$_{DRE}$ lacZ*	V: pKENV54 EcoRI/XhoI, phosphatase
		F: pKENV03 EoRI/XhoI
pKENV57	*attP PlacI proV'$_{DRE'-NT}$ lacZ*	V: pKENV54 EcoRI/XhoI, phosphatase
		F: pKENV04 EcoRI/XhoI
pKENV58	*attP PlacI lacZ*	V: pKENV16 SalI/XbaI, phosphatase
		F: PCR template S541 S668/S669
pKENV59	*attP Ptac proV'$_{DRE}$ lacZ*	V: pKEM02 SalI/XbaI, phosphatase
		F: 1. PCR template pKESK23 S283/284
		F: 2. PCR template pKENV03
		S568/S420
		PCR template 1+2 S283/S420 SalI/XbaI
pKENV60	*attP Ptac proV'$_{DRE'-NT}$ lacZ*	V: pKEM02 SalI/XbaI, phosphatase
		F: 1. PCR template pKESK23 S283/S284
		F: 2. PCR template pKENV04 S568/S420
		PCR template 1+2 S283/S420 SalI/XbaI
pKENV61	*bgl$_{URE}$ Pbgl t1$_{RAT}$ bgl$_{DRE}$ lacZ* , PBR, amp	V: pKESD08 SalI/AflII, phosphatase
		F: 1. PCR template pKESK51 S330/S92
		F: 2. PCR template pKEKB30+PCR 1
		S145/S92 SalI/AflII
pKENV62	*attP bgl$_{URE}$CRP+ Pbgl t1$_{RAT}$ bgl$_{DRE}$ lacZ*	V: pKESD08 SalI/AflII, phosphatase
		F: 1. PCR template pKESK51 S330/S92
		F: 2. PCR template pKEYK1+PCR 1 S145/S92
		SalI/AflII
pKENV63	*UV5 bgl$_{DRE}$*,PBR, amp	V: pKEM53 AflII/PstI, phosphatase
		F: PCR template pFDX733 S330/S726
		AflII/PstI
pKENV64	*UV5 t1RAT bgl$_{DRE}$*,PBR, amp	V: pKENV63 SalI/AflII, phosphatase
		F: pKESK51 SalI/AflII
pKENV65	*bgl$_{URE}$Pbgl t1$_{RAT}$ bgl$_{DRE}$*, PBR, amp	V: pKENV63 SalI/AflII, phosphatase
		F: pKENV61 SalI/AflII
pKENV66	*bgl$_{URE}$ CRP+ Pbgl t1$_{RAT}$ bgl$_{DRE}$*, PBR, amp	V: pKENV63 SalI/AflII, phosphatase
		F: pKENV62 SalI/AflII
pKENV67	*bgl$_{URE}$ PUV5 t1$_{RAT}$ bgl$_{DRE}$* PBR, amp	V: pKENV64 SalI/AflII, phosphatase
		F: 1. PCR template pFDY241 S145/S196
		F: 2. PCR template pKENV64 S117/S92
		PCR template 1+2 S145/S92 SalI/AflII
pKENV68	*attP bgl$_{URE}$ PUV5 t1$_{RAT}$ bgl$_{DRE}$ lacZ*	V: pKESK51 SalI/AflII, phosphatase
		F: 1. PCR template S145/S196
		F: 2. PCR template S117/S92
		PCR template 1+2 S145/S92 SalI/AflII
pKENV69	*bgl$_{URE}$ Pbgl t1$_{RAT}$ bgl$_{DRE}$ (323CtoA, 330CtoA)* PBR, amp	V: pKENV63 SalI/XbaI, phosphatase
		F: 1. PCR template pKESD08 S791/S726
		F: 2. PCR template pKENV61 S145/S790

		PCR template 1+2 SalI/XbaI
pKENV70	*attP bgl$_{URE}$ Pbgl t1$_{RAT}$ bgl$_{DRE}$ (323CtoA, 330CtoA) lacZ*	V: pKES15 SalI/Eco81I, phosphatase F: 1. PCR template pKESD08 S791/S100 F: 2. PCR template pKENV61 S145/S790 PCR template 1+2 S145/S100 SalI/Eco81I
pKENV71	*bgl$_{URE}$ PUV5 t1$_{RAT}$ bgl$_{DRE}$ (323CtoA,330CtoA) PBR, amp*	V: pKENV63 SalI/XbaI, phosphatase F: 1. PCR template pKESD08 S791/S726 F: 2. PCR template pKENV68 S145/S790 PCR template 1+2 S145/S726 SalI/XbaI
pKENV72	*attP bgl$_{URE}$ PUV5 t1$_{RAT}$ bgl$_{DRE}$ (323CtoA,330CtoA) lacZ*	V: pKES15 SalI/Eco81I, phosphatase F: 1. PCR template pKESD08 S791/S100 F: 2. PCR template pKENV68 S145/S790 PCR template 1+2 S145/S100 SalI/Eco81I
pKENV73	*proU$_{URE}$PproU proV'$_{DRE}$ rrnBT1T2*, PBR, amp	V: pKENV63 SalI/XbaI, phosphatase F: pKENV34 SalI/XbaI
pKENV74	*attP bgl$_{URE}$ Pbgl t1$_{RAT}$ bgl$_{DRE}$ (+459 to +476) lacZ*	V: pKENV61 AflII/Eco81I, phosphatase F: 1. PCR template pFDX733 S12/S823 F: 2. PCR template pKESD08 S821/S100 PCR template 1+2 S12/S100 AflII/Eco81I
pKENV75	*attP bgl$_{URE}$ Pbgl t1 bgl$_{DRE}$ (+459 to +485) lacZ*	V: pKENV61 AflII/Eco81I, phosphatase F: 1. PCR template pFDX733 S12/S823 F: 2. PCR template pKESD08 S822/S100 PCR template 1+2 AflII/Eco81I
pKENV76	*attP bgl$_{URE}$ PUV5 t1 bgl$_{DRE}$ (+459 to +485) lacZ*	V: pKENV75 AflII/XhoI, phosphatase F: pKENV68 AflII/XhoI
pKENV77	*attP bgl$_{URE}$ PUV5 RAT t1 bgl$_{DRE}$ (+459 to +476) lacZ*	V: pKENV68 AflII/Eco81I, phosphatase F: 1. PCR template pFDX733 S12/S823 F: 2. PCR template pKESD08 S821/S100 PCR template 1+2 AflII/Exo81I
pKENV78	*attP bgl$_{URE}$ PUV5 +25 lacZ*	V: pKES15 SalI/XbaI, phosphatase F: PCR template pKENV68 S145/S212 SalI/XbaI
pKENV79	*attP PUV5 t1$_{RAT}$ bgl$_{DRE}$ (+459 to +476) lacZ*	V: pKESK51 AflII/XhoI, phosphatase F: pKENV74 AflII/XhoI
pKENV80	*attP PUV5 t1$_{RAT}$bgl$_{DRE}$ (+459 to +485) lacZ*	V: pKENV75 AflII/XhoI, phosphatase F: pKESK51 AflII/XhoI

a: The relevant structure of the plasmids is schematically shown. *bgl$_{DRE}$* refers to *bgl* operon from position +95 to +972 relative to the transcription start site. Deletions made in *bgl$_{DRE}$* were indicated by the positions of the deleted regions within brackets (eg. Δ+601 to +700 refers to deletion of sequence from 601 to 700 bp relative to the transcription start site, +95 to +737 refers to the presence of sequence from +95 to +737 relative to the transcription start site). *bgl$_{DRE-NT}$* refers to mutation in the start codon and two additional ATG codons at position 3 and 27 to CGC, thereby rendering the *bgl$_{DRE}$* non-translatable. *t1$_{RAT}$* indicates a mutation in the leader region of *bgl* operon at position +67 and +68 from AA to T making the construct independent of BglG mediated anti-termination. *proU$_{DRE}$* refers the *proU* operon from postion +1 to +303 relative to the transcription start site. CRP+ refers to C to T exchange at postion -66 relative to the transcription site).

b: All plasmids when not stated differently carry a pACYC (pA15) origin of replication (Chang and Cohen, 1978) and kanamycin and spectinomycin resistance markers. Plasmids carrying pBR origin (Bolivar, 1978) of replication carry an ampicillin resistance marker.

c: Short description of the plamid construction. The first line (V:) refers to the vector fragment. The second line (F:) indicates the insert fragment. PCR reactions are indicted in the order; PCR template DNA and the primers used. The restriction enzyme used for cloning are also indicated. For all PCR based clonings the DNA sequence of the cloned fragment was confirmed by sequencing. Detailed description of the plasmid construction is documented in lab records and the sequences are compiled in the lab Vector NTI (Invitrogen) database.
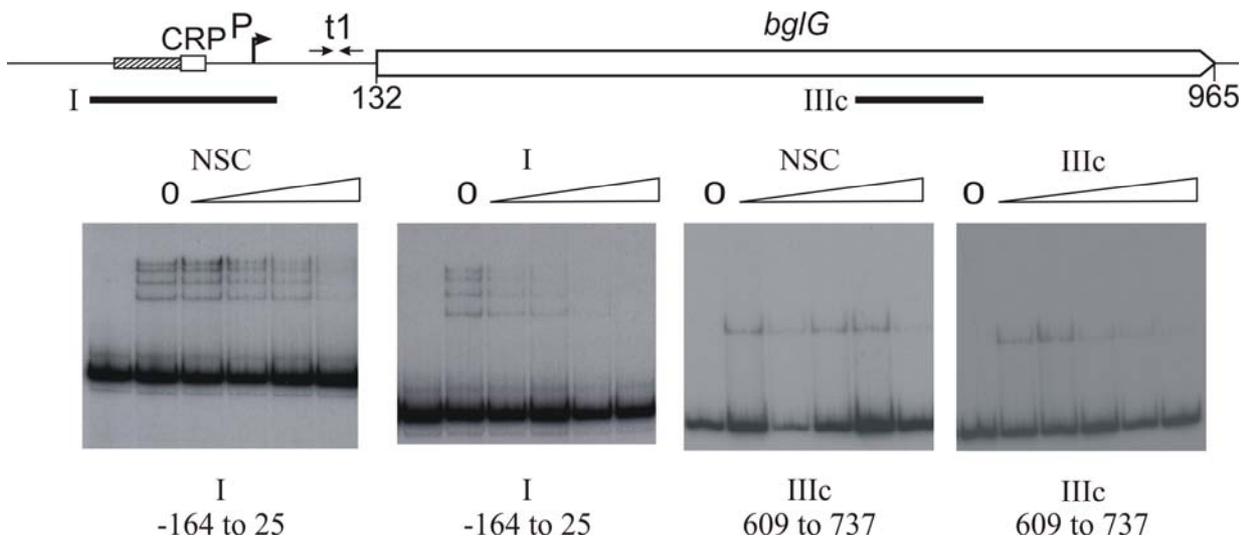
Figure 18. **Competitive shift experiments showing specific binding of H-NS to the upstream and downstream regulatory region:** The specificity of H-NS binding to fragment I and fragment IIIc is confirmed by competitive shift assay. Top: The structure of the *bgl* regulatory region including the upstream and the downstream regulatory region with terminator t1 and *bglG* is shown schematically. Fragments I andIIIc in the competitive mobility shift experiments are represented by horizontal bars. Lower part: 0.2nM of [32P]-labeled fragments (I and IIIc), whose map positions are given relative to the *bgl* transcription start site were incubated with 200nM of H-NS and competed with increasing concentration of specific unlabelled competitor (fragment I and fragment IIIc) and non-specific competitor (NSC, *bgl* operon from position -460 to -160 relative to the transcription start site to which H-NS does not bind, data not shown). The specific and non-specific competitor used are 5, 10, 20 and 40 fold excess to labeled fragment I and IIIc. The DNA-protein complexes were separated on acrylamide gels which were run at 4°C. The labeled fragment I and IIIc were effectively competed for H-NS binding with unlabelled I and IIIc but not with NSC.
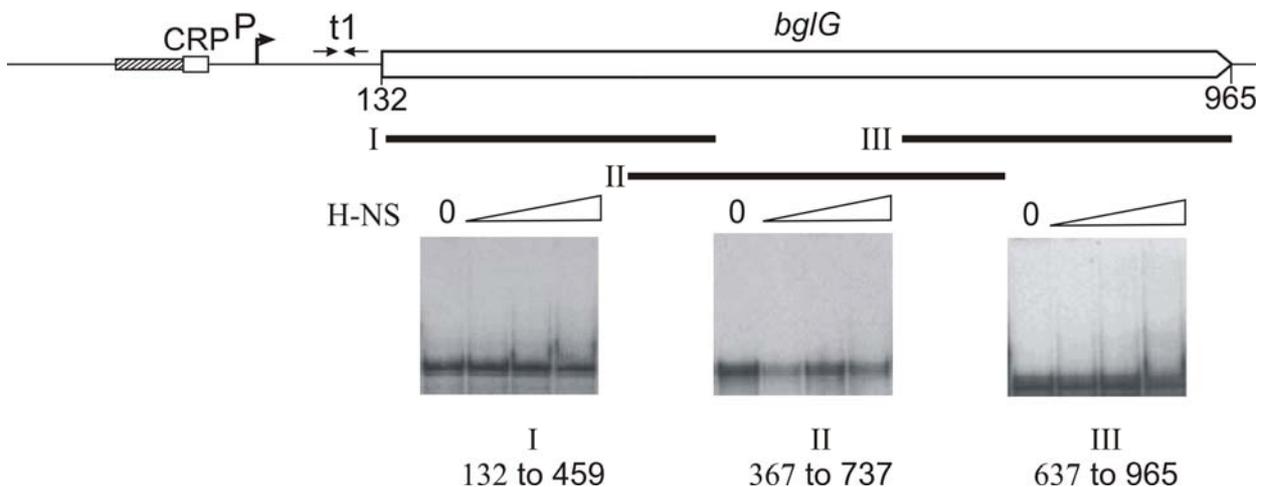

Figure 19. H-NS does not bind to *bgl* downstream regulatory region: The possibility of H-NS regulation by binding to *bglG* mRNA was examined by RNA mobility shift experiments. Top: The structure of the *bgl* regulatory region including the upstream and the downstream regulatory region with terminator t1 and *bglG* is shown schematically. RNA fragment I, II and III used for RNA mobility shift experiments and their respective map positions are given relative to the transcription start site (shown in horizontal bars). Lower part: 0.2nM of *in vitro* transcribed [32P]-labeled fragments I, II and III were incubated with increasing concentrations of H-NS (50 nM, 100 nM and 200 nM) and separated on acrylamide gels which were run at 4°C.
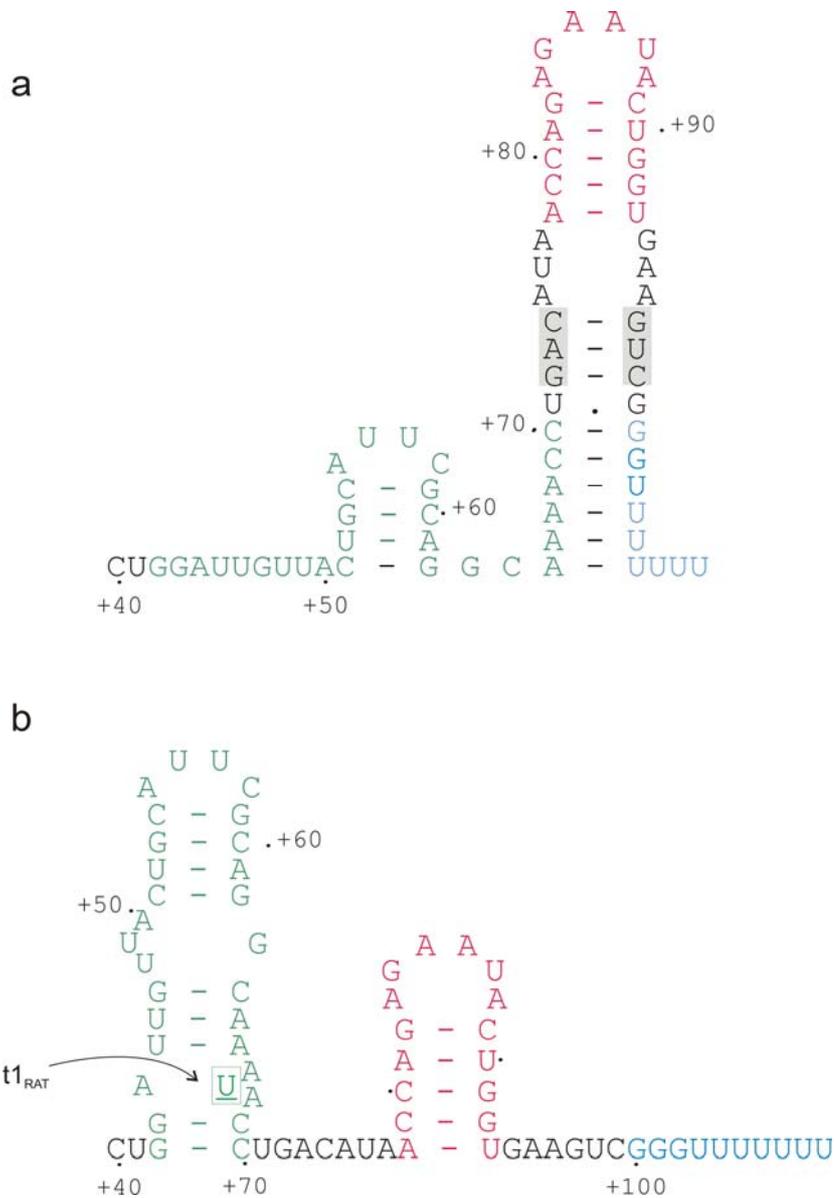
Figure 20. Secondary structure of t1_RAT leader RNA: The predicted secondary structure of stem loop formed in the leader sequence of the *bgl* operon is shown schematically (a). The position from +40 to + 109 relative to the transcription start site reveals two hairpin loops, the first loop and the sequence shown in green is the bglG binding region and the second loop is the Rho independent terminator t1. (b) Leader sequence at position +67 to +68 relative to the transcription start site carries a mutation from AA to T (shown by arrow) thereby altering the secondary structure of the leader RNA and making it independent of bglG mediated anti-termination.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Karin Schnetz betreut worden.

Teilpublikationen:

Dole,S., Klingen,Y., Nagarajavel,V., and Schnetz,K. (2004). The protease Lon and the RNA-binding protein Hfq reduce silencing of the Escherichia coli *bgl* operon by H-NS. J. Bacteriol. *186*, 2708-2716.

Dole,S., Nagarajavel,V., and Schnetz,K. (2004). The histone-like nucleoid structuring protein H-NS represses the Escherichia coli *bgl* operon downstream of the promoter. Mol. Microbiol. *52*, 589-600.

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