The Mechanism and Modulation of H-NS Mediated Repression in *Escherichia coli*

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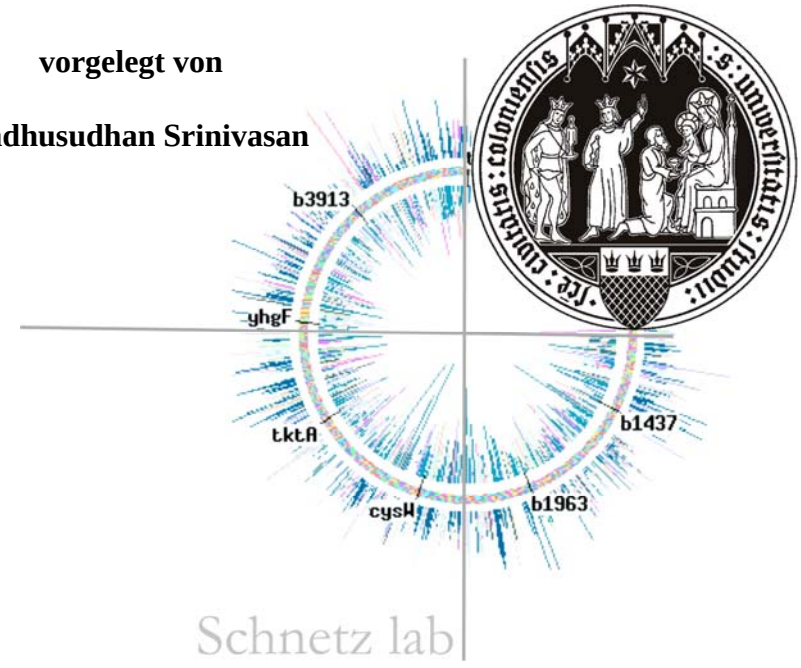

For the birds.....
Over the bridge

"I wonder why, I wonder why,
I wonder why I wonder;
I wonder why, I wonder why,
I wonder why I wonder."

-- Richard P Feynman.

Berichterstatter/in: Prof. Dr. Karin Schnetz

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Finally, amma, vasu, chcickappa, a zenouska moje, *ellam onakke*.

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Erklärung
Lebenslauf
Carriculum Vitae

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.
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
URE	upstream regulatory element
wt	wild type

I. Zusammenfassung

Das Histon-ähnliche Nucleoid-strukturierendes Protein H-NS agiert als globaler Regulator der Genexpression in Antwort auf Umwelteinflüsse und Streßkonditionen. Es wird angenommen, daß die Repression durch H-NS durch dessen Bindung an primäre "Nucleation sites" in der Nähe von Promotoren vermittelt wird. Ausgehend von diesen Nucleation sites bildet H-NS erweiterte Nucleoprotein-Komplexe, die die Transkriptionsinitiation inhibieren. Die Modulation der H-NS vermittelten Repression ist ein komplex, und erfolgt unter anderem durch spezifische Transkriptionsfaktoren und DNA-Strukturänderungen, die durch Änderung der zellulären Physiology induziert sind. . Das *bgl*- sowie das *proU*-Operon in *E. coli* werden mit hoher Spezifität von H-NS reprimiert. Beide Systeme verfügen über stromaufwärts (upstream) und stromabwärts (downstream) gelegene Regulationselemente (URE bzw. DRE), an die H-NS für eine effiziente Repression bindet. Die vorliegende Studie zeigt, daß H-NS die Initiation der Transkription ausgehend vom *bgl*-Promoter inhibiert, analog zur beschriebenen Repression des *proU*-Systems. Die Repression von *proU* und *bgl,* vermittelt durch die Bindung von H-NS an das stromaufwärts und stromabwärts gelegene Regulationselement, ist synergistisch. Zusätzlich beeinflussen sich im Falle des *bgl*-Operons die Repression durch Bindung von H-NS an das stromabwärts gelegene Regulationselement und die Transkription. Dies wird durch den Terminationsfaktor Rho, ko-transkriptionelle Translation, die Protease Lon und, wie hier gezeigt, ist das DnaKJ Chaperonsystem für die Repression über das *bgl*-DRE essentiell. Im Falle von *proU* ist die RNA Polymerase bei niedriger Osmolarität am Promoter gefangen (poising), während sie bei hoher Osmolarität den Promoter mit höherer Effizienz verlässt. Der Mechanismus sowie das Signal, das für ein effizientes Loslösen der RNA Polymerase vom *proU*-Promotor ("promoter clearence") bei hoher Osmolarität verantwortlich ist, sind unbekannt. Das *proU* Operon ist außerdem Gegenstand posttranskriptioneller Osmoregulation. Die *proU* mRNA wird innerhalb eines hochkonservierten Sequenzabschnittes durch die RNAse III prozessiert. Dies legt einen allgemeinen Regulationsmechanismus nahe, der wahrscheinlich innerhalb der Enterobakterien konserviert. Zusammenfassend zeigt die vorliegende Studie, daß der Mechanismus der H-NS vermittelten Repression des *bgl* -und *proU*-Operons sehr ähnlich ist. Die Modulation derselben ist ein komplexer Mechanismus, der eine Vielzahl von zusätzlichen Faktoren umschließt, die spezifisch für das jeweilige System sind, und erfolgt demnach in einer Kontext-spezifischen Art.

1. Summary

The histone-like nucleoid structuring protein H-NS acts as a global repressor of genes that are expressed in response to environmental stimuli and stress conditions. Repression by H-NS is presumably mediated by binding of H-NS to primary 'nucleation sites' close to promoters, and the formation of extended nucleoprotein complex from these nucleation sites to inhibit transcription initiation. Modulation of H-NS mediated repression is a complex process involving specific transcription factors and physiology dependent structural alterations. The *E. coli bgl* and *proU* operons are model systems that are repressed by H-NS with exceptional specificity. Both of these systems possess upstream and downstream regulatory elements (URE and DRE) bound by H-NS for efficient repression. The present study demonstrates that repression by H-NS binding upstream and downstream is synergistic in *proU* (as shown in a parallel study for *bgl*), and that H-NS when bound within the transcription unit represses transcription initiation at the *bgl* promoter, as reported before for *proU*. Repression by binding of H-NS downstream is known to be modulated. Common to both *proU* and *bgl* is that an increase in the promoter activity abrogates repression. For *bgl* it is known, that the H-NS mediated repression of the promoter is counteracted by transcription factors BglJ and LeuO. Further, termination factor Rho and the protease Lon are known to modulate repression by H-NS through the DRE, and as shown here the DnaKJ chaperone system is essential for this repression. In case of *proU*, the promoter is osmoregulated; the RNA polymerase is poised at the promoter at low osmolarity, while it clears the promoter with better efficiency at high osmolarity. Furthermore, the *proU* operon is subject to post-transcriptional osmoregulation. The *proU* mRNA is processed by RNAse III within a stretch of highly conserved sequence, suggesting a common mechanism of regulation among Enterobacteria. In summary, the present study demonstrates that the mechanism of H-NS mediated repression of the *bgl and proU* operons is very similar. However, its modulation is complex involving numerous additional factors specific to the two systems, and thus is achieved in a context specific manner.

2 Introduction

2.1 H-NS as a regulator of gene expression in Escherichia coli.

The bacterial histone-like nucleoid structuring protein (H-NS); initially described as a heat stable nucleoid associated protein (Falconi et al., 1988), is a small basic protein present at around 20,000 molecules per genome equivalent and is highly conserved in gram negative bacteria (Falconi et al., 1988). H-NS is implicated in maintaining the higher order structure of the nucleoid, compactation and partitioning of the chromosome (Kaidow et al., 1995). However, it is probably best characterized for its role in transcriptional regulation (Dorman, 2004) (Luijsterburg et al., 2006). Genomic and proteomic studies have 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., 1999) (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). Genome scale mapping of H-NS sites in the genomes of *Escherichia coli* and *Salomonelle enterica* (serovar Typhimurium) suggests that H-NS has a major role in silencing horizontally acquired genes. These include pathogenicity islands encoding important virulence factors (Lucchini et al., 2006a) (Navarre et al., 2006). In this work, aspects of repression by H-NS and its modulation were analyzed.

2.2 H-NS structure and mechanism of repression.

The H-NS protein (136 amino acids, 15.6 KDa) can be divided into three structural domains. The N-terminal domain is required for dimerization of H-NS and extends up to the 65th amino acid residue (Fig. 1a). The carboxy-terminal DNA-binding domain extends from amino acid residue 90 until the end of the protein. The N- and C-terminal domains are connected by a highly flexible linker domain. This unstructured linker is involved in formation of higher order oligomers of the protein (Badaut et al., 2002; Bloch et al., 2003; Dorman et al., 1999; Esposito et al., 2002). H-NS is believed to be a dimer in solution, although oligomers were observed at high protein

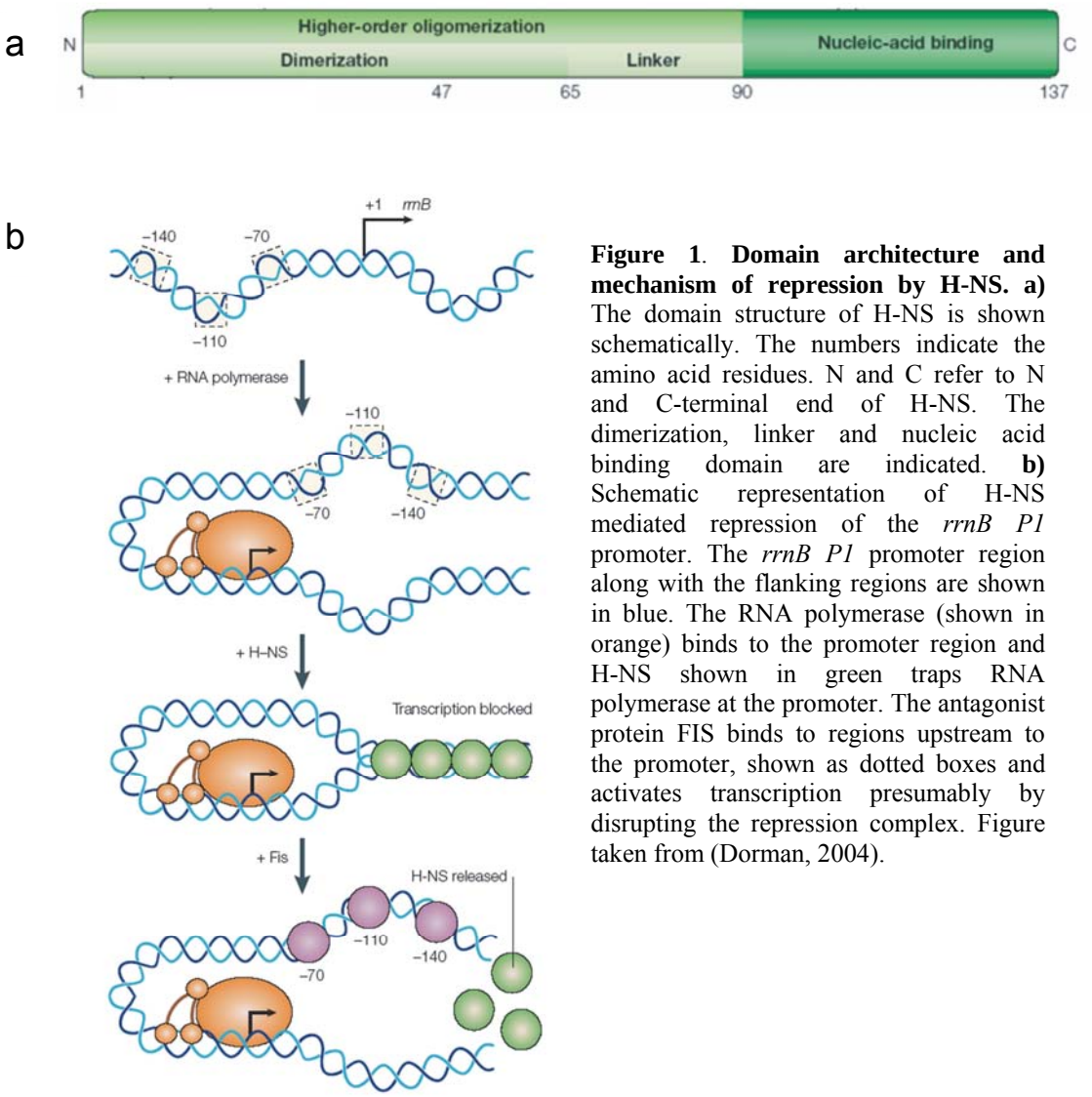


Figure 1. **Domain architecture and mechanism of repression by H-NS. a)** The domain structure of H-NS is shown schematically. The numbers indicate the amino acid residues. N and C refer to N and C-terminal end of H-NS. The dimerization, linker and nucleic acid binding domain are indicated. **b)** Schematic 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. Figure taken from (Dorman, 2004).

concentrations (Falconi et al., 1988; Smyth et al., 2000). H-NS binds preferentially to AT-rich and intrinsically curved DNA sequences (Dame et al., 2001; Rimsky et al., 2001; Schroder and Wagner, 2002). Upon binding to such high affinity 'nucleation sites' lateral interaction of H-NS dimers allows the binding to flanking low affinity sequences, to form extended nucleoprotein complexes. (Rimsky et al., 2001; Bouffartigues et al., 2007). Thus, when binding close to a promoter, H-NS represses transcription by trapping the RNA polymerase at the promoter or by excluding the binding of RNA polymerase (Rimsky, 2004) (Dorman, 2004) (Fig. 1b). Trapping of RNA polymerase has been shown in case of the ribosomal *rrnB P1* promoter (Dame et al., 2005; Dame et al., 2000; Dame et al., 2002) and the *hdeAB* promoter (Shin et al., 2005). In these cases, 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 and to zip the two double strands that flank the promoter together. DNA loop formation presumably is mediated by DNA-H-NS-DNA bridge formation, since a biophysical analysis demonstrated that one dimer of H-NS can bind to two DNA double strands (Dame et al., 2006)

H-NS controls gene expression under specific environmental conditions in response to pH, osmolarity and temperature (Atlung and Ingmer, 1997) (Dorman, 2007) (Amit et al., 2003). A function of thermosensing is attributed to H-NS with the discovery that more than 75% of the 531 genes showing altered expression due to a temperature up shift were dependent on H-NS (Ono et al., 2005). Such models have been bolstered by the observation that the structure of H-NS changes in response to temperature and osmolarity (Amit et al., 2003). However, several loci including the *E.coli proU* operon and the *eltAB* system are repressed with equal efficiency by H-NS at low and high temperatures (Lucht et al., 1994a) (Yang et al., 2005) (Umanski et al., 2002). Further, repression of the temperature regulated *Shigella virF* gene is subject to a temperature dependent DNA bend (Prosseda et al., 2004) Also, many H-NS regulated genes are unaffected by changes in osmolarity (Atlung and Ingmer, 1997). This questions the role of H-NS as a global osmo- and thermosensor. Considering this, a recent review calls for a model where the H-NS mediated repression is regulated context specific, by specific transcription factors and by environemental parameters like temperature and osmolarity that change to DNA structure or activity of a protein at a specific locus (Navarre et al., 2007).

H-NS regulates gene expression by acting as a transcriptional repressor. However, repression by H-NS is different from that by standard transcription factors, due to the low binding specificity and the sensitivity of nucleoprotein complex formation to structural changes (that may be intrinsic to the DNA or based on protein binding). Further, it is becoming evident that RNA polymerase itself at the step of transcription initiation and elongation can modulate the repression by H-NS (Shin et al., 2005; Dole et al., 2004a, Nagarajavel et al., 2007; Navarre et al., 2007). Therefore, the essential features of the bacterial transcription cycle are presented below.

2.3 The transcription cycle in bacteria.

The transcription cycle is composed of three steps namely initiation, elongation and termination. Each of these processes involve complex mechanisms and are regulated by various factors (reviewed in (Mooney and Landick, 1999)). Upon promoter engagement by the RNA polymerase associated with a sigma factor, the DNA duplex of ~12bp at the promoter is melted. The melted region extends from -10 to +2 relative to the transcription start (Dehaseth and Helmann, 1995). This process may be accompanied by a change in the conformation of the RNA polymerase and is termed 'open complex' formation (Browning et al., 2000) (Fig. 2). Once the open complex has formed, transcription is initiated with the synthesis of RNA of about 9 to 11 bp. In this 'initial transcription complex' RNA polymerase remains strongly associated with the promoter. The initial transcription may result in the release of the short RNA transcripts, which is termed 'abortive transcription' (Vo et al., 2003), or it may result in clearing of the promoter and transition of the RNA polymerase into the transcription elongation phase. This requires overcoming of the interaction of RNA polymerase to the promoter DNA (Tadigotla et al., 2006). The regulation and kinetics of these steps determine the efficiency of promoter clearance, and thus the 'strength' of a promoter (Mooney and Landick, 1999). 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 of 8 to 9bp until it reaches the termination signal. Transcription initiation and elongation complexes serve as important targets for regulatory factors (Borukhov et al., 2005) (Browning et al., 2004).

Transcription elongation is not a uniform process; it is marked by various punctuations that cause RNA polymerase to pause briefly or for an extended time. Pausing involves RNA polymerase isomerizing from the rapidly translocating complex to an alternative state, where RNA chain extension becomes reversibly inhibited (Dalal et al., 2006) (Herbert et al., 2006) (Tadigotla et al., 2006) (Landick, 2006). Pausing is also a prerequisite for transcription termination and occurs by at least two mechanisms. Firstly, pausing can be induced by sequence dependent destabilization of the elongation complex, including for example an AT-rich stretch of DNA followed by a GC-rich sequence or RNA secondary structures formation at the

exit channel of RNA polymerase (Artsimovitch and Landick, 2000; Landick, 2006). Secondly, pausing occurs by elements which act as physical barriers to RNA polymerase translocation. These include roadblocks by DNA-binding proteins, misincorporated substrates, DNA lesions and special DNA sequences (Fish et al., 2002). Such pausing, at instances leads to the backward movement of the RNA polymerase (Artsimovitch and Landick, 2000; Toulme et al., 1999). Repeated backtracking and re-extension is thought to help the polymerase to eventually overcome the sequence or the physical barrier (Epshtein et al., 2003; Mosrin-Huaman et al., 2004). Alternatively, the stalled RNA polymerase might get into a state of arrest with neither forward nor backward movement (Davenport et al., 2000).The pausing

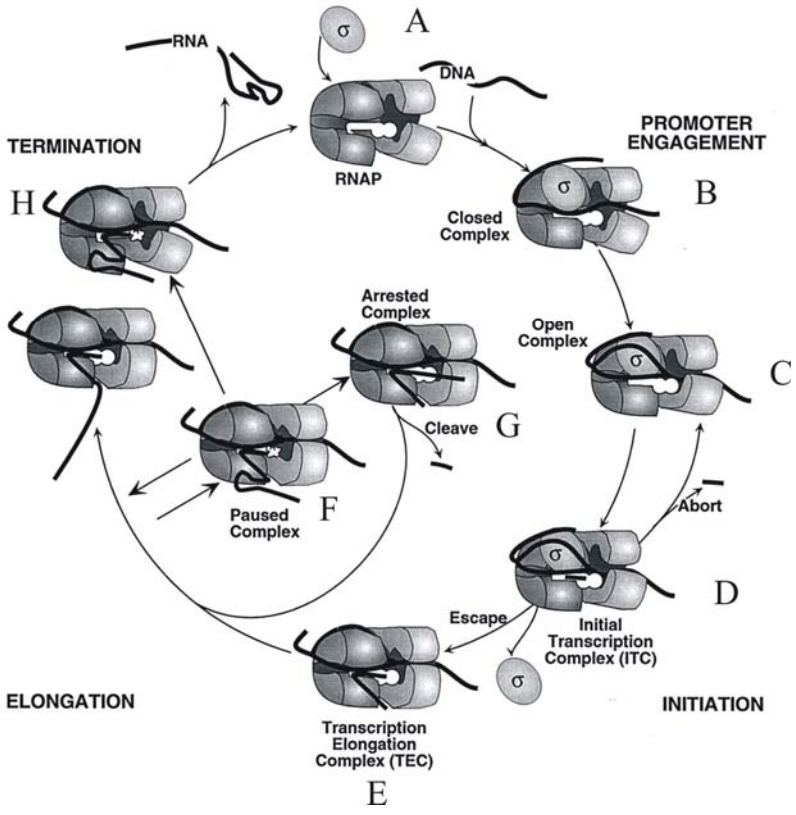


Figure 2. The transcription cycle in *E. coli*. A) The preinitation complex with RNA polymerase (RNAP), DNA, σ factor. B) The promoter engagement where the RNA polymerase-σ factor holoenzyme binds to the promoter DNA. C) The open complex formation where the duplex DNA melts in the promoter region. D) 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 reversibly move into an arrested complex (G) or can lead to termination (H). 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 (Mooney and Landick, 1999).

events, depending on the formation of a stable RNA hairpin, followed by a uridine-rich (U-rich) tract and consequently, a weak RNA:DNA hybrid (Yarnell et al., 1999) or the action of Rho protein, which translocates along the nascent RNA until it reaches the polymerase, whereupon it induces transcript dissociation (Richardson, 2002) can lead to transcription termination. Pausing of the transcription elongation complex 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). RNA degradation counterbalances transcription, and therefore plays an important regulatory role in determining the steady-state level of a given mRNA.The mRNA generated from transcription is in many cases subject to post-transcriptional modifications. A brief introduction to post-transcriptional regulation in bacteria is outlined in the following section.

2.4 Post-transcriptional regulation of gene expression in Bacteria.

Unlike stable ribosomal and transfer RNAs, many mRNAs are susceptable to degradation by ribonucleases (Kushner, 2002). The average half-life of bacterial mRNAs is about 6.8 minutes (Selinger et al., 2003). Ineffiecient translation of mRNAs caused by poor binding of the ribosome to the leader has been shown to decrease their stability (Arnold et al, 1998). Thus, the 5' untranslated regions of many bacterial mRNAs serve as elements controlling the fate of transcripts. Additionally, regulatory factors such as RNA-binding proteins like StpA, Hfq and even H-NS (Brescia et al., 2004) (Mayer et al., 2007) (Valentin-Hansen et al., 2004), noncoding regulatory RNAs (ncRNAs) that usually basepair with the 5'-UTR harbouring the translation initiation region of their target mRNAs, which results in inhibition of translation leads to degradation of the ribosome-free mRNAs (Gottesman, 2004; Majdalani et al., 2005), low molecular weight (LMW) effectors such as amino acids, coenzymes or vitamins have been recently found to bind to the 5'-UTR of many mRNAs to regulate their function (Winkler et al., 2002; Nahvi et al, 2002) (Vitreschak et al., 2002), and physical parameters like temperature (Morita et al.,

1999) (Narberhaus et al., 2006), play important roles in deciding the stability of a given mRNA (Fig. 3).

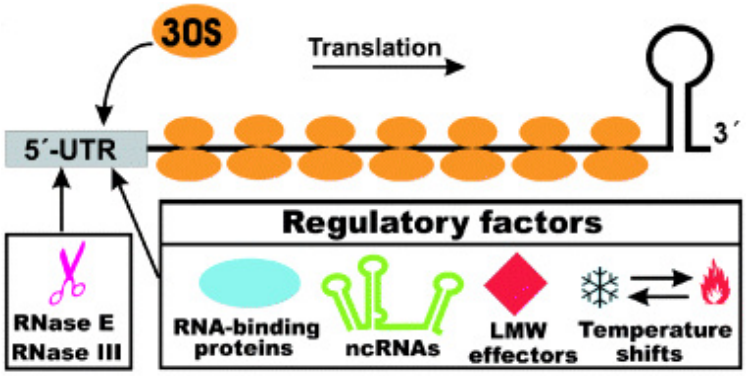


Figure 3. Post transcriptional regulation in prokaryotes. The fate of the transcript of many bacterial mRNAs is controlled by the 5' untranslated regions. Factors influencing the fate of bacterial mRNAs include RNA-binding proteins, noncoding regulatory RNAs (ncRNAs), low molecular weight (LMW) effectors, temperature, endoribonucleases (RNase E and RNase III), and binding of ribosomes. Figure modified from (Kaberdin et al., 2006).

2.5 Escherichia coli bgl and proU operons as reporters of repression by H-NS.

The *bgl* and *proU* operons 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 (termed *bgl*-URE and *bgl*-DRE respectively) is required for effective repression (Schnetz, 1995; Druger-Liotta et al., 1987; Overdier and Csonka, 1992) (Fig. 4).

The *bgl* operon encodes gene products necessary for the uptake and fermentation of aryl-β,D-glucosides. Two Rho-independent transcriptional terminators, *t1* and *t2*, flank the first gene of the operon *bglG,* which encodes an antiterminator (Schnetz et al., 1987; Schnetz and Rak, 1988a; Schnetz, 1995). Although the operon is highly conserved in *E.coli*, no condition that allows the expression of the operon is known (Neelakanta, 2005). The operon is repressed ~100-fold by H-NS (Schnetz, 1995). 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 is thought to be formed by H-NS in *bgl* (Schnetz, 1995) (Caramel and Schnetz, 1998). Silencing by

H-NS in *bgl* 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. These mutations disrupt the repressing nucleoprotein complex formed by H-NS and thus activete the operon (Mukerji and Mahadevan, 1997) and references therein (Schnetz and Rak, 1992). The activated operon is regulated substrate-specifically. The activity of the antiterminator BglG is modulated by reversible phosphorylation in response to the availability of the specific substrate, β-glucosides. In the absence of β-glucosides, BglG is inactivated by phosphorylation catalyzed by the by the sugar-specific permease enzymeII^{Bgl}, encoded by *bglF* (Schnetz and Rak, 1988b) (Amster-Choder, 2005), while in presence of the β-glucosides the phosphorylation and activation of BglG is mediated by Hpr, a major component of the phospho-*enol*-pyruvate-dependent phosphotransfer system (Gorke and Rak, 1999).

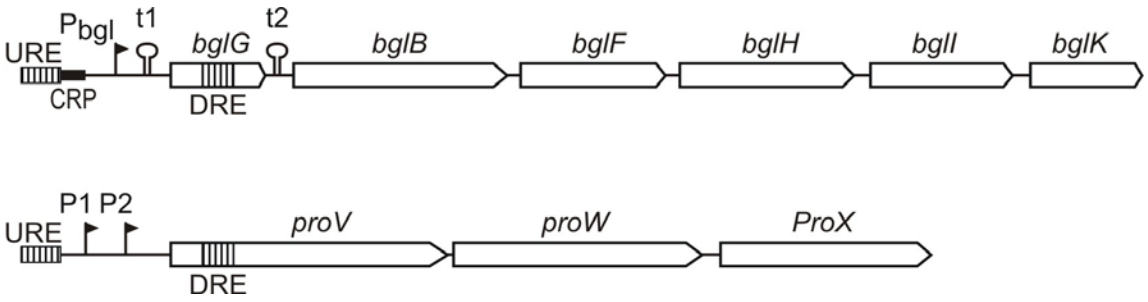


Figure 4. The *E. coli bgl* and *proU* operons. Scheme showing the *bgl* and *proU* operons with the promoter (Pbgl), the CRP binding site (CRP), the Rho independent terminators (t1 and t2) and the structural genes (*bglG,B,F,H,I* and *K* respectively) in case of *bgl*, and the three structural genes *proV*, *proW* and *proX*, driven by promoters P1 and P2 in case of *proU*. The H-NS binding sites in both *bgl* and *proU* are indicated with vertical hatched bars. H-NS binding upstream to the promoters is termed *bgl*- and *proU*-URE, respectively, while the binding region located downstream, within the structural genes is termed *bgl*- and *proU*-DRE, respectively.

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 under osmotic stress (Gowrishankar, 1989; May et al., 1989). 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; Rajkumari et al., 1996b;

Lucht et al., 1994a). Like in the case of *bgl,* the repression of *proU* by H-NS is highly specific and requires the presence of flanking sequence upstream and downstream to the promoter (*proU*-URE and *proU*-DRE) (Rajkumari et al., 1997) (Lucht et al., 1994b) (Barr et al., 1992) (Overdier and Csonka, 1992) (Owen-Hughes et al., 1992; Dattananda et al., 1991), and binding of H-NS to the downstream regulatory region represses open complex formation at the promoter (Jordi and Higgins, 2000). However, the highly specific repression of *proU* by H-NS observed *in vivo* could not be reproduced *in vitro* (Jordi et al., 1997).

2.6 Objectives of the current study.

This study addresses the mechanism and modulation of H-NS mediated repression of gene expression, using the *E.coli bgl* and *proU* operons as reporters. Accordingly, the results obtained are presented in three sections; the first section addresses the specific mechanism of repression of *bgl* by H-NS and its parallels with the repression of *proU*, the second section focuses on the modulation of H-NS mediated repression of the *bgl* operon, and the identification of the post-transcriptional osmoregulation of *proU* is presented in the final chapter. The obtained results are discussed and integrated into a model.

3. Experimental procedures

3.1 Media

LB

For 1 l	10g	Bacto Trypton
	5g	Yeast Extract
	5g	NaCl
For plates add	15g	Bacto Agar

SOB

For 1 l	20g	Bacto Tryptone
	5g	Bacto Yeast Extract
	0.5g	NaCl
	1.25ml	2M KCl
	Adjust pH to 7.0 with NaOH,	

After autoclaving just before use add 10ml 1M $MgCl_2$ per liter

SOC

Per liter of SOB add 19.8ml 20% Glucose to SOB.

20 x M9:

140 g Na_2HPO_4 x 2 H_2O
60 g KH_2PO_4
20 g NH_4Cl
H_2O ad 1 l

M9 Medium (prepare from sterile solutions):

20 x M9	50 ml
0.1 M $CaCl_2$	1 ml
1 M $MgSO_4$	1 ml
1 mM $FeCl_3$	0.5 ml
Carbon source 1% final concentration:	
20 % Glucose	50 ml
or 80 % Glycerol	12.5 ml

If required:

1 mg/ml Vitamin B1	1 ml
4 mg/ml amino acids	5 ml
10% casamino acids	66 ml
H_2O	
final volume	1 l

M9-plates

Autoclave 15 g Bacto-Agar
900 ml H_2O

Add, sterile 20 x M9	50 ml
0.1 M $CaCl_2$	1 ml
1 M $MgSO_4$	1 ml
1 mM $FeCl_3$	0.5 ml
Carbon source: 1 % final concentration	

Add, if required:

1 mg/ml Vitamin B1	1 ml
4 mg/ml amino acids	5 ml
10% casamino acids	66 ml

Bromthymol blue plates (BTB-plates)

15g Bacto Agar
1g Yeast-Extract
1g Trypton
5g NaCl
add 900 ml H_2O, autoclave

Add sterile:

1 ml 1 M $MgSO_4$
1 ml 0,1 M $CaCl_2$
1 ml Vitamin B1 (stock solution 1mg/ml, filter sterilize)
0,5 ml $FeCl_3$ 1mM
20 ml 10% (w/v) Casaminoacids
50 ml sugar (e.g. 10 % Salicin, 20% Lactose, etc.)
10 ml BTB stock solution (2% bromthymol blue in 50% EtOH, 0,1N NaOH)

Antibiotics if required.
The medium should be turquoise, if medium is green add NaOH, if it is blue add HCl.

MacConkey Lactose plates

40 g MacConkey Lactose Agar
H_2O
Final volume 1 l

X-gal

Final concentration is 40 µg/ml in LB / NB / minimal plates etc.

X-Gluc

Final concentration is 20 µg/ml in LB / NB / minimal plates etc.

3.2 Antibiotics, sugars, Aminoacids

Antibiotics

	stock solution	final conc.
ampicillin	50mg/ml in 50 % EtOH	50 µg/ml
chloramphenicol	30 mg/ml in Ethanol	15 µg/ml
kanamycin	10 mg/ml in H_2O	25 µg/ml
rifampicin	100mg/ml in Methanol	100µg/ml
spectinomycin	50 mg/ml in 30% EtOH	50µg/ml
tetracyclin	5mg/ml in 70 % Ethanol	12 µg/ml

Sugars

	stock sol.	final conc.
Glucose	20%	1 %
Glycerol	80 %	1 %
Salicin	10%	0.5 %

Casamino acids

Final conc. 10% in H_2O
Dissolve by heating and filter through Schleicher & Schuell folded filters, then autoclave.

3.3 Standard Molecular Biology Techniques

Standard Molecular Biology applications like restriction enzyme digestions, ligations and other enzymatic reactions, PCR, plasmid purification, auto-radiography were performed as described in(Sambrook and Russell, 2001) or according to the manufacturer's instructions.

3.4 Plasmids

Large scale preparations of plasmid DNAs were performed using the plasmid maxiprep/midiprep kit (Promega) according to manufacturer's instructions. A list of plasmids used in the study, with brief descriptions is given in the Table 1. Details of the plasmid constructions are documented in the lab records and sequences are compiled in Vector NTI (Invitrogen).

The plasmids used in the study were derivatives of pACYC, pSC101 or high copy pBR-derivatives (pKK177-3, pUC). The origin of each of the plasmids is also listed in Table 4. The pACYC-derivatives, carry the p15A origin of replication and 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 containing (Prentki et al., 1991) the spectinomycin resistance gene *aadA*, flanked by transcriptional terminators. These plasmids were used for the integration of reporter gene fusions into the chromosome into the *attB* site (see below). The pSC101 derivatives used in the study carry the chloramphenicol resistance gene (*cat*), the *repA* gene and a *lacI*^{q} gene-*lacUV5* or *tac* promoter cassette, followed by a multiple cloning site. The *tac* promoter is flanked by two operators for efficient repression by the *lac* repressor (LacI). Plasmid pKESK18 is a temperature-sensitive derivative of pSC101 (Hashimoto-Gotoh et al., 2000) with a kanamycin resistance gene. The plasmid carries the phage Lambda *cI*-857 allele encoding the temperature sensitive lambda repressor, the Tn*10* transposase gene under control of the phage lambda P_R promoter, and a miniTn*10* transposon (miniTn*10*-Cam^{r}) with a chloramphenicol resistance gene.

The plasmids pKK177-3 has a pBR322 based origin of replication, ampicillin resistance gene (*bla*) and two strong Rho independent transcriptional terminators *rrnB*-T1 and T2 (Brosius and Holy, 1984).

Table 1: Plasmids used in This Study

Name	Relevant structure/description[a] and replicon/resistance[b]	Source
pCP20	FLP recombinase, temperature Sensitive, amp^R.	(Datsenko and Wanner, 2000)
pKD3	Template plasmid for gene deletion. Cam^R	(Datsenko and Wanner, 2000)
pKD4	Template plasmid for gene deletion. kan^R	(Datsenko and Wanner, 2000)
pKD46	λ red recombinase, temperature Sensitive, amp^R.	(Datsenko and Wanner, 2000)
pLDR8	λ repressor, cI-857; int under the control of *λ P_R, pSC101 rep-ts*, Kan^R	(Diederich et al., 1992)
pFDX733	*wt bgl* operon, kan^R	(Schnetz et al., 1987)
pFDX840	*galK rrnBT1 T2* terminators, ampR, *ori-pBR*	Lab collection
pKEKB30	*placUV5 bgl-URE Pbgl +25 lacZ,*p15A, kan^R, spec^R	(Dole et al., 2004a)
pKES15	*attP bgl-URE Pbgl +54 lacZ,*p15A, kan^R, spec^R	Lab collection
pKES99	*attP PlacUV5 lacZ,*p15A, kan^R, spec^R	(Nagarajavel et al., 2007)
pKESD08	*attP bgl-URE Pbgl t1 bgl-DRE lacZ,*p15A, kan^R, spec^R	(Dole et al., 2002)
pKESD20	*attP PlacUV5 t1 bgl-DRE lacZ,*p15A, kan^R, spec^R	(Dole et al., 2002)
pKESD48	*attP PlacUV5 bgl-DRE lacZ,*p15A, kan^R, spec^R	(Dole et al., 2002)
pKESD49	*attP PlacUV5 bgl-DRENT lacZ,*p15A, kan^R, spec^R	(Dole et al., 2002)
pKESK51	*attP PlacUV5 $t1_{RAT}$ bgl-DRE lacZ,*p15A, kan^R, spec^R	(Dole et al., 2002)
pKESK18	cI-857-Tn10 transposase-mTn10-Cm^R, rep-ts pSC101kan^R	(Madhusudan et al., 2005)
pKENV03	*attP PlacUV5 proV'-DRE lacZ,*p15A, kan^R, spec^R	(Nagarajavel et al., 2007)
pKENV34	*attP proU-URE PproU proV'-DRE lacZ,*p15A, kan^R, spec^R	(Nagarajavel et al., 2007)
pKENV64	*placUV5 t1RAT bgl-DRE*, *rrnBT1T2*, PBR, amp^R	(Nagarajavel et al., 2007)
pKENV67	*bgl-URE PlacUV5 $t1_{RAT}$ bgl-DRE, rrnBT1T2*,PBR, amp^R	(Nagarajavel et al., 2007)
pKENV73	*proU-URE PproU proV'-DRE rrnBT1T2*, PBR, amp^R	(Nagarajavel et al., 2007)
pKEM01	*attP ptna-tnaC-tnaAΦlacZ* ,p15A, kan^R, spec^R.	This Study
pKEM02	*attP PlacUV5-Crotr1-lacZ,*p15A, kan^R, spec^R.	This Study
pKEM20	*attP proU-URE PproU proV'-DREΦ lacZ,*p15A, kan^R, spec^R	This Study
pKEM21	*attP proU-URE PproU proV'-DRENT lacZ,*p15A, kan^R, spec^R	This Study
pKEM26	*placI^q-lacI- lacO3 Ptac lacO1-His-rho*,pSC101,Cam^R	This Study

Table 1: Plasmids used in This Study		
Name	Relevant structure/description[a] and replicon/resistance[b]	Source
pKEM31	*rho* (with stopcodon) ,pSC101,Cam^R	This Study
pKEM32	*rho* (without stopcodon) ,pSC101,Cam^R	This Study
pKEM44	*lacI^q- lacO3 Ptac lacO1 HA-rho*, pSC101,Cam^R	This Study
pKEM45	*lacI^q lacO3 Ptac lacO1 HA-MCS*, pSC101,Cam^R	This Study
pKEM46	*lacI^q lacO3 Ptac lacO1 MCS-HA*, pSC101,Cam^R	This Study
pKEM47	*lacI^q lacO3 Ptac lacO1 rho-HA*, pSC101,Cam^R	This Study
pKEM48	*hns* (with stopcodon) , pSC101,Cam^R	This Study
pKEM49	*hns* (without stopcodon) , pSC101,Cam^R	This Study
pKEM50	*lacI^q lacO3 Ptac lacO1 HA-hns*, pSC101,Cam^R	This Study
pKEM51	*lacI^q lacO3 Ptac lacO1 hns-HA*,p SC101,Cam^R	This Study
pKEM52	*placUV5- bgl-DRE-lacZ*, PBR, amp^R	This Study
pKEM53	*placUV5- bgl-DRE$_{NT}$-lacZ*, PBR, amp^R	This Study
pKEM54	*promoterless(NT) bgl-DRE-lacZ*, PBR, amp^R	This Study
pKEM61	*attPlacUV5- proV'-DREΦ lacZ*,p15A, kan^R, $spec^R$	This Study
pKEM63	*lacI^q lacO3 Ptac lacO1-nusA-HA*, pSC101,Cam^R	This Study
pKEM64	*lacI^q lacO3 Ptac lacO1-nusB-HA*, pSC101,Cam^R	This Study
pKEM65	*lacI^q lacO3 Ptac lacO1-nusE-HA*, pSC101,Cam^R	This Study
pKEM66	*lacI^q lacO3 Ptac lacO1-nusG-HA*, pSC101,Cam^R	This Study
pKEM67	*placUV5-proV'-DRE*, PBR, amp^R	This Study
pKEM68	*lacI^q lacO3 Ptac lacO1-greA-HA*, pSC101,Cam^R	This Study
pKEM69	*lacI^q lacO3 Ptac lacO1-greB-HA*, pSC101,Cam^R	This Study
pKEM72	*placUV5-proU-DRE-rrnBT1 in pUC12 amp*^{R}	This Study

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. *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: plasmids which carry a pACYC (p15A) origin of replication and kanamycin, spectinomycin resistance markers also harbor the *attP* site for integration into the chromosome according to

(Diederich et al., 1992). Plasmids carrying pBR origin of replication carry an ampicillin resistance marker. Detailed description of the plasmid construction is documented in lab records and the sequences are compiled in the lab Vector NTI (Invitrogen) database.

3.5 Bacterial strains

The bacterial strains used in this study and their description are listed in Table 2.

Table 2: *E.coli* K-12 strains used in This Study

Strain	Relevant genotype or structure[a]	Source
CAG1843	*F⁻, λ⁻, rph-1, ilvD500::Tn10*	CGSC#7462
CSH50	*bgl° Δ(lac-pro) ara thi (=S49)*	(Miller, 1972)
S524	CSH50 ΔlacZ-Y217 $(gpt\text{-}pro)^+$	(Dole et al., 2002)
S541	S539 Δ*bgl-AC11 ΔlacZ-Y217*	(Dole et al., 2004b)
S544	S524 Bgl+#9912 bgl-CAP (C -66→T)	(Dole et al., 2004a)
S812	MC4100 *hfq1::omega*	(Muffler et al., 1996)
S1193	S541 *attB*::[SpecR *PUV5* bgl_{DRE} *lacZ*]	(Dole et al., 2004b)
S1195	S541 *attB*::[SpecR *PUV5* $bgl_{DRE\text{-}NT}$ *lacZ*]	(Dole et al., 2004b)
S1213	S541 *attB*::[$Spec^R$ bgl_{URE} *Pbgl +25 lacZ*]	(Dole et al., 2004b)
S1553	S541 *sulA3 Δlon* $proC^+$	(Dole et al., 2004a)
S1564	S541 *sulA3 Δlon attB*:: [SpecR *PUV5* $bgl_{DRE\text{-}}$ *lacZ*]	(Dole et al., 2004a)
S1816	S541*attB*:: [$spec^R$ *PUV5* $t1_{RAT}$ bgl_{DRE} *lacZ*]	(Dole et al., 2004a)
S1906	S541 *attB*::[SpecR *PUV5lacZ*]	x pKES99
S1975	S541 *attB*::[$Spec^R$ *Ptna-tnaC-tnaA-lacZ*]	x pKEM02
S1983	S1553 *attB*::[$spec^R$ *Ptna-tnaC-tnaA-lacZ*]	x pKEM02
S1995	S1956 *attB*::[SpecR *PUV5* $bgl_{DRE\text{-}NT}$ *lacZ*]	(Dole et al., 2004a)
S2048	S541 *attB*::[SpecR $ProU_{URE}$*PproU (-315 to +1) lacZ*]	(Nagarajavel et al., 2007)
S2101	S544 *attB*::[SpecR *PUV5* $bgl_{DRE\text{-}NT}$ *lacZ*]	(Dole et al., 2004a)
S2103	S1075 *attB*::[SpecR *PUV5* $bgl_{DRE\text{-}NT}$ *lacZ*]	(Dole et al., 2004a)
S2106	N3431 *lacZ43*(Fs), *rne*-3071(ts), *relA1, spoT1, thi-1*	CGSC#6975
S2137	S541 *attB*::[specR *PUV5* $proV'_{DRE}$ *lacZ*]	(Nagarajavel et al., 2007)
S2142	S2103mut2 *dnaK::cat*	(Madhusudan et al., 2005)
S2226	N3433 *lacZ43*(Fs), *relA1, spoT1, thi-1*	CGSC# 6976
S2501	S541 *attB*::[SpecR $proU_{URE}$ *PproU* $proV'_{DRE}$*(-315 to +303) lacZ*]	(Nagarajavel et al., 2007)
S2608	S541 *attB*::[SpecR $proU_{URE}$ *PproU* $proV'_{DRE}$*(-315 to +303) ϕ lacZ*]	x pKEM20
S2670	S1564 *dnaK::cat*	(Madhusudan et al., 2005)
S2674	S1213 *dnaK::cat*	(Madhusudan et al., 2005)
S2680	S1553 *attB*:: [$Spec^R$ bgl_{URE} *Pbgl +25 lacZ*]	(Madhusudan et al., 2005)
S2710	S2681 *dnaK::cat*	(Madhusudan et al., 2005)
S2712	S1196 *dnaK::cat*	(Madhusudan et al., 2005)
S2888	S1196, *pA1/lacO-1 dnaKJ,* $lacI^q$	(Madhusudan et al., 2005)
S2904	S1213, *pA1/lacO-1 dnaKJ,* $lacI^q$	(Madhusudan et al., 2005)
S2977	S2501 *hfq1::omega1(Kan)*	x T7 (S812)
S2979	S2608 *hfq1::omega1(Kan)*	x T7 (S812)
S3010	S541 Δ$hns::kan_{KD4}$	(Nagarajavel et al., 2007)
S3066	S541 ΔproU::KD3cm	x S665/S672,pKD4
S3077	S541 Δ*proU::KD3frt*	S3066xpCP20
S3122	S1906 Δ$hns::kan_{KD4}$	(Nagarajavel et al., 2007)
S3124	S2048 Δ$hns::kan_{KD4}$	(Nagarajavel et al., 2007)

Table 2: *E.coli* K-12 strains used in This Study		
Strain	Relevant genotype or structure[a]	Source
S3126	S2137 Δ*hns::kan*$_{KD4}$	(Nagarajavel et al., 2007)
S3128	S2501 Δ*hns::kan*$_{KD4}$	(Nagarajavel et al., 2007)
S3130	S2608 Δhns::KD4kan	x S665/S672,pKD4
S3181	S541 *attB*::[SpecR *bgl*$_{URE}$*Pbgl t1*$_{RAT}$ *bgl*$_{DRE}$ *lacZ*]	(Nagarajavel et al., 2007)
S3203	S3181 Δ*hns::kan*$_{KD4}$	x S665/S672,pKD4
S3207	S1195 Δ*hns::kan*$_{KD4}$	x S665/S672,pKD4
S3209	S1816 Δ*hns::kan*$_{KD4}$	x S665/S672,pKD4
S3211	S1193 Δ*hns::kan*$_{KD4}$	x S665/S672,pKD4
S3252	S541 *attB*::[*SpecR PlacUV5-(proU(+1to+303) ϕlacZ*]	x pKEM61
S3296	S1213 Δ*hns::kan*$_{KD4}$	(Nagarajavel et al., 2007)
S3299	S2285 Δ*hns::kan*$_{KD4}$	x S665/S672,pKD4
S3324	S3252 Δ*hns::KD4kan*	x S665/S672,pKD4
S3346	S541 Δ*hns::Kan* $_{KD4FRT}$	(Nagarajavel et al., 2007)
S3352	S2137 *hfq1::omega (Kan*^{R}*)*	x T7 (S812)
S3354	S3252 *hfq1::omega (Kan*^{R}*)*	x T7 (S812)
S3412	S541 *attB*::[SpecR *bgl*$_{URE}$*PUV5+25 lacZ*]	(Nagarajavel et al., 2007)
S3420	S3412 Δ*hns::kan*$_{KD4}$	x S665/S672,pKD4
S3460	S2226 Δ*proU::KD3cm*	x T7 (S3066)
S3462	S2106 Δ*proU::KD3cm*	x T7 (S3066)
S3464	S3346 Δ*proU::KD3cm*	x T7 (S3066)
S3466	S3077 *hfq1::omega (Kan*^{R}*)*	x T7 (S812)
S3701	IBPC633=N3433 rnc105 nadB51::Tn10 (tet)	(Regnier et al., 1991)
S3769	N3431 rnc105 nadB51::Tn10 (tet)	xT7 (S3701)

a: The relevant genotype of the strains (which are all CSH50 derivatives (Miller, 1972)) refers to the *bgl, lac, hns and proU* loci. 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). For other abbreviations, see Table I.

b: Construction of strains by transduction using T4GT7 is explained below. Integration of plasmids into the *attB* site of chromosome was done as described (Diederich et al., 1992) (see below). The deletion of *hns* allele was constructed according to (Datsenko and Wanner, 2000) and is explained in detail below. 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.

3.6 Preparation of competent cells and transformation

$CaCl_2$ 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 pelleted by centrifugation at 3000 rpm for 10 minutes at 4°C. The pellets were resuspended in 12.5ml of ice cold 0.1M $CaCl_2$ and incubated in ice for 20 minutes,followed by centrifugation for 10 minutes at 3000 rpm. The resulting pellet was resuspended in 1ml of 0.1M $CaCl_2$. For transformation 1 to20ng of plasmid DNA or 10µl of ligations in 50µl of TEN buffer was mixed on ice with 100µl of competent cells. The cells were incubated on ice for 20 minutes followed by heat shock at 42°C for 2 minutes and additional 10 minutes incubation 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 2 ml of ice-cold 10% glycerol and pelleted by centrifugation (3000 rpm for 15 minutes). Finally, cells were resuspended in 200 µl 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 40 µl aliquots at -80°C. For transformation 40 µl 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 (BioRad Gene Pulser™) and the electric shock was given for 3 seconds at 1.8 kV. Then 1ml of SOC medium was immediately

added to the cuvettes, and 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.

3.7 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). Briefly, integrations of originless circularized DNA fragments, containing the *attB* sequence and the spectinomycin casette is catalysed by the integrase expressed from a temperature sensitive plasmid with Kanamycin resistance gene (pLDR8). The integrants are selected at 42°C, which inhibits the replication of the plasmid. The integrants are screened for Kanamycin sensitivity to ensure the loss of the plasmid. The strain S541 or its derivatives were first transformed with a temperature sensitive plasmid (pLDR8) expressing the integrase, and the transformants were selected at 28°C on LB kanamycin plates. Plasmids carrying 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 religation was used to transform competent cells of S541/pLDR8. At 37°C the integrase gene is expressed, which promotes recombination between the λ *attB* and *attP* sites resulting in integration of the DNA fragment. The transformants were selected at 42°C on LB spectinomycin plates to select for the integrase catalyzed integration of the DNA fragment into *attB*. In addition, at 42°C 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 mentioned below (primer sequences documented in lab records). 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

3.8 Transduction with phage T4GT7

T4-Topagar
6g Bacto-Agar (Difco)
10g Bacto-Tryptone (Difco)
8g NaCl
2g Tri-Natriumcitrate-Dihydrate
3g Glucose
add 1l H2O

The technique is based on generalized transduction, which makes use of the bacteriophage T4*GT7* to transfer DNA between bacteria (Wilson et al., 1979). Briefly, 100µl of the overnight culture to be transduced was incubated with 10µl, 5µl, and 2µl of T4GT7 lysate prepared from the cells, which carried the allele 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 three to four times, to get rid of the contaminating phages and the transfer of the gene was verified by PCR.

3.9 Deletion of genes according to Datsenko and Wanner

Deletion of genes 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 30 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 a target gene was generated using primers carrying homology to the target chromosomal region and to antibiotic resistance cassettes of plasmids pKD3 and pKD4. This PCR generates a fragment carrying the chloramphenicol or kanamycin resistance genes, flanked by a short homology to upstream and downstream sequences

of the target gene. In addition, the resistance genes are flanked by FRT sites, which allow 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. Competent cells were prepared from cultures grown in LB 10 mM L-Arabinose for induction of λ red-recombinase. The recombinants were selected at 37°C on LB chloramphenicol or kanamycin plates, respectively. The loss of the helper plasmid was confirmed by sensitivity to ampicillin and the deletion of the target gene was confirmed by PCR. Two independent colonies were stored in the laboratory strain collection and used in further experiments.

3.10 β-galactosidase assays

The β-galactosidase assays were performed as described (Miller, 1992). Briefky, cells were grown in M9 medium containing 1% (w/v) glycerol, 0.66 % (w/v) casamino acids (Difco), and 1 µg/ml Vitamin B1, or LB or LB medium with various NaCl concentrations, as stated in the figure legends. Routinely, cultures were inoculated to an OD_{600} of 0.1 to 0.15 from fresh over-night cultures grown in the same medium and grown at 37°C, 42°C or 30°C as indicated. Isopropyl-β,D-thiogalactopyranoside (IPTG) (1mM), tryptophan (50µg/ml), bicyclomicin (gift from Fujisawa Pharmaceutical Co., Ltd. Osaka, Japan) (20µg/ml) was added to this fresh culture, where indicated. Cells were harvested at an OD_{600} of 0.5. The enzyme activities were determined at least 3 times from at least two independent integration derivatives. Standard deviations were less than 10 %.

3.11 Transposon mutagenesis

Transposon mutagenesis screens were performed using pKESK18 (Table 1) carrying a miniTn*10*-cat^r transposon. In this plasmid replication is temperature sensitive and also expression of the transposase is repressed at 28°C and induced at 42°C. Thus at 28°C the plasmid replicates while the transposase is not expressed. Upon a temperature shift expression of the transposase gene and thus transposition is induced, while replication of the plasmid stops, allowing the selection of transposon

mutants on chloramphenicol plates at 42°C. All the mutants that were characterized carry single miniTn*10*-cat^r transposon insertions.

Strains to be mutagenised were transformed with plasmid pKESK18 and grown at 28°C in LB medium containing kanamycin and chloramphenicol. To select for transposon mutants, dilutions were plated on MacConkey Lactose (Difco) chloramphenicol plates and incubated at 42°C. Mutants with a change in the Lactose phenotype were re-streaked and their Bgl-phenotype was tested on bromthymolblue salicin indicator plates. From the mutants that showed a double phenotype change, the insertion of the miniTn*10* transposon was mapped by sequencing of chromosomal DNA by a semi-random, two-step PCR protocol (ST-PCR) as described in (Chun et al., 1997). Briefly, in a first semi-specific PCR reaction a 'random primer' (S360 GGCCACGCGTCGACTAGTACNNNNNNNNNNGATC) and a miniTn*10* specific primer (S357 GGCAGGGTCGTTAAATAGCCGCTTATGT or S358 CGGTATCAACAGGGACACCAGGATTTATTTATTCT). The amplification products of this first PCR reaction were re-amplified in a second PCR using a primer (S361 GCTCTAGAGGCCACGCGTCGACTAGTAC) that matches to the 'random primer' S360 and a nested miniTn*10* specific primer (S359 GCTCTAGAGATCATATGACAAGATGTGTATCCACCTTAACT). The PCR products were gel purified and sequenced with primer S359.

3.12 SDS-PAGE and Western blotting

Cultures were grown in LB at 30°C or 37°C to an OD_{600} of 0.5. IPTG (1mM) was added where indicated. Cultures were stopped on ice, cells were harvested by centrifugation and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10% Glycerol, 62.5mM Tris-HCl pH6.8, 2% SDS, 0.05% Bromophenol blue) (Laemmli, 1970) at a concentration of 0.05 OD_{600} per 10 µl sample buffer. Five µl (0.025 OD) were separated on a 12% sodium dodecyl sulfate SDS-PAGE using a SE600 16 cm gel electrophoresis unit (GE Healthcare). The gel was blotted onto a 0.45 µm pore size poly vinylidene difluoride (PVDF) transfer membrane using a TE70 semidry blotting apparatus (GE Healthcare). The blot was handled using standard Western blotting protocol (Gallagher et al.,

2004). Monoclonal mouse antisera directed against DnaK(1 µg/ml) (Stressgen Bioreagents) or Monoclonal rat antiserum directed against the HA tag (0.2 µg/ml) (Roche Diagnostics) were used as the primary antibodies. Alexa Fluor®680 rabbit anti mouse immunoglobulin G (IgG, H+L; Molecular Probes) (0.5 µg/ml) or Alexa fluor 680-conjugated goat anti-rabbit IgG (H+L; Molecular Probes) (0.5 µg/ml) were used as the secondary antibodies. Visualization and quantification was done using the Odyssey® Imaging System (Li-Cor Biosciences) according to the instructions of the manufacturer.

3.13 RNA analysis by Northern blotting

(Modified from (Ausubel, 2005))

20x SSPE:	3M NaCl, 100mM NaH2PO4·H2O, 10mM EDTA
100x Denhardt solution:	10g Ficoll 400, 10g polyvinylpyrrolidone, 10 g BSA (pentax fraction V), H_2O to 500 ml
20×SSC:	3 M NaCl, 0.3 M Na_3citrate×$2H_2O$, 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 RNeasy Mini Kit (QIAGEN) according to manufacturer's instruction. 7.5 µg of total RNA in 5µl of DEPC H_2O 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) 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 the blot was 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).

For preparation of the RNA probe, 0.2pmol of PCR product containing the T7 RNA polymerase promoter sequence was *in vitro* transcribed in a 20µl reaction containing the following:

0.2pmol	PCR product
1µl	10mM ATP, GTP, CTP stock
1µl	100µM UTP
2.5µl	α-32P UTP (800ci/mmol, 20mCi/ml)
4µl	5xTranscription buffer (Fermentas)
DEPC H_2O	to 20µl
1µl	T7RNA polymerase 20U/µl (Fermentas).

The reaction mix was incubated at 37°C for 45 minutes. The unincorporated nucleotides were removed by passing through a Nick Sephadex^{TM} G50 columns (GE Healthcare) and eluted in 400µl of 10mM Tris-Cl pH8.0.

3.14 Chloroacetaldehyde (CAA) footprinting

E.coli strain S541 and its isogenic *Δhns* mutant (S3346) were transformed with the relevant plasmids (see results). A fresh overnight culture was diluted to OD600 of 0.1 in 8 ml LB with ampicillin or in LB 0.01 M NaCl/0.3 M NaCl with ampicillin. The cultures were grown at 37°C to an OD600 of 0.5 with aeration, after which the cells were spun down and resuspended in 8ml of M9 minimal medium with

B1 and casaaminoacids. Rifampicin was added to a concentration of 200µg/ml and the cells were shaken at 37°C for 5 minutes, where indicated. Chloroacetaldehyde footprinting was performed essentially as described (Guerin et al., 1996). Chloroacetaldehyde (50% in water from FLUKA) was added to a final concentration of 3% and the cells were shaken at 37°C for 10 minutes. Cells were spun down and washed once with 8 ml M9 minimal medium. Then the plasmid was isolated by alkaline lysis, and the CAA modifications within the non-template strand in the relevant DNA portion of the plasmid were analysed by primer extension. To this end 200 fMol of [32P]-end labeled primer was added and the plasmid DNA was denatured with 0.3M NaOH by heating at 90°C for 5 minutes, allowed to cool to room temperature and ethanol precipitated. The pellet was resuspended in Klenow buffer (New England Biolabs, Inc.), incubated at 45°C for 5 minutes for primer annealing and snap cooled. Then 10 µl of dNTPs (1 mM each) and 2 units of Klenow (exo-) (New England Biolabs, Inc.) were added, and the samples were incubated at 45°C for 12 minutes for primer extension. After ethanol precipitation, the pellet was resuspended in 10 µl formamide loading buffer (80% formamide, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.05% xylene cyanol, and 0.05% Bromophenol blue) and 5 µl loaded onto a 6% denaturing polyacrylamide sequencing gel (6% acrylamide:bis-acrylamide 19:1, 7M urea, 0.9 x TBE). The sequencing ladders were generated with the same labeled primer using the T7-sequencing kit (USB), with the following modifications to the manufacturers recommendations: 200 fMol of [32P]-end labeled primer with 2 µg of plasmid DNA were denatured by incubating with 8µl of 2M NaOH for 10 minutes at room temperature, subsequently were ethanol precipitated and resuspended in 12 µl H_2O. Then, 2 µl annealing buffer, 4 µl labeling mix (1.375 µM dNTP, 333.5 mM NaCl), and 2 µl of diluted T7-DNA polymerase were added. After 5 minutes of incubation at room-temperature, 4.5 µl aliquots of this were added to 2.5 µl of the A, C, G, and T termination mixes, respectively, and incubated for 5 minutes at 37°C, the reaction was stopped 5 minutes later by adding 5 µl stop solution (provided in the Kit).

3.15 Primer Extensions

For primer extension 5 pMol of the oligonucleotide was end-labeled with [γ^{32}P]-ATP (50 µCi, 6000 Ci/mMol) using 20 units of T4-Polynucleotide Kinase (Fermentas). Unincorporated nucleotides were removed by passing the sample through a Sephadex-G50 NICK-column (GE-Healthcare). 5µg of the total RNA were incubated with 50 fMol of the [^{32}P]-labeled oligonucleotide in a total volume of 10 µl for 5 minutes at 65°C and cooled on ice. dNTPs (2 µl, 10 mM dNTP mix), 4 µl 5x cDNA buffer, 1 µl 0.1 M DTT, 2 µl H_2O, and 1 µl (15 units) Thermoscript reverse transcriptase (Invitrogen) were then added, and the samples were incubated at 50°C for 45 minutes. The reaction was stopped by heating for 5 minutes to 85°C, the sample was extracted with phenol and chloroform, and then the first strand cDNA was ethanol precipitated and resuspended in 5µl H_2O. 5µl of Stop solution (T7-sequencing kit, USB) was added and the samples were separated next to a sequencing ladder on a denaturing sequencing gel (6% acrylamide:bis-acrylamide 19:1, 7M urea, 0.9 x TBE)

4 Results

4.1 Repression by H-NS binding within the transcription unit.

Data presented in this chapter have in part been published in "Nagarajavel V., Madhusudan S., S. Dole, A. R. Rahmouni, and K. Schnetz (2007) Repression by binding of HNS within the transcription unit. *J. Biol. Chem.* 282:23622-23630."

Transcription elongation is not a continuous process, the elongating RNA polymerase frequently stalls shortly and immediately resumes elongation, or gets paused by sequence specific read-blocks, or protein induced roadblocks. The paused or arrested RNA-polymerase can be released from the template by termination factor Rho (Richardson, 2002). Binding of H-NS within the transcription unit can potentially hinder the process of transcription elongation. The repression by H-NS binding to the *bgl*-DRE has been shown to be affected by termination factor Rho, indicating that the process of transcription elongation is affected by H-NS (Dole et al., 2004b). Further, dual reporter assays with the *bgl*-DRE positioned between two different reporters have shown that H-NS bound to the *bgl*-DRE weakly (2-fold) reduces transcription read-through (Nagarajavel, 2007). However, in case of *proU*, it has been shown that H-NS binding to the DRE affects transcription initiation prior to the open complex formation (Jordi and Higgins, 2000). Therefore, the following questions were addressed (Fig. 5). Does H-NS bound to the *bgl*-DRE acts as a roadblock to the elongating RNA polymerase that induces prolonged pausing? Does H-NS bound to the *bgl*-DRE repress transcription initiation at the promoter?

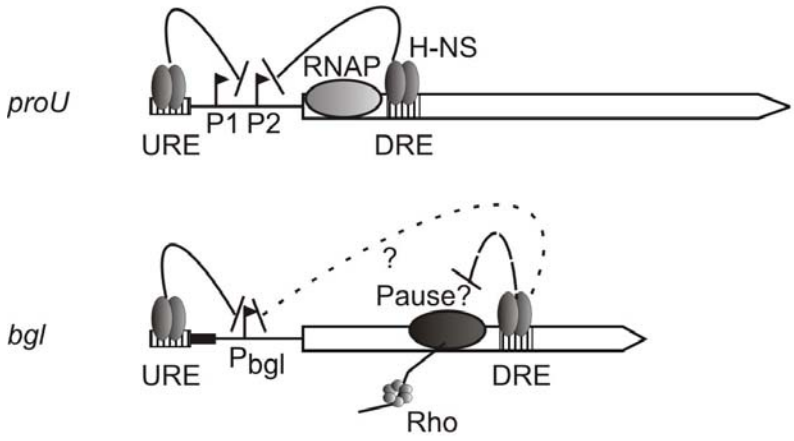


Figure 5. Does H-NS induce pausing of the RNA polymerase within the *bgl* transcription unit or repress transcription initiation? H-NS bound to the *proU-DRE* inhibits transcription initiation (Jordi and Higgins, 2000). H-NS at the *bgl-DRE* has a weak effect on transcription elongation. The repression by H-NS at *bgl*-DRE is affected by termination factor Rho (Nagarajavel, 2007). It is thus possible that H-NS could act as a barrier to the RNA polymerase and cause the pausing of the polymerase within the *bgl* transcription unit. Alternatively or in addition, H-NS bound the the *bgl*-DRE could repress transcription initiation.

4.11 Footprinting for paused RNA polymerase excludes the possibility of H-NS acting as a roadblock to the elongating polymerase.

Binding of H-NS could act as a physical barrier, obstructing the movement of RNA polymerase within the *bgl* transcription unit, and therefore induce a pause or arrest of the transcription elongation complex. Such pausing events can be visualized by *in situ* footprinting using the single strand specific probe Chloracetaldehyde (CAA) (Epshtein et al., 2003) (Toulme et al., 2005). CAA modifies unpaired Cytosine, Adenine and to a lesser extent Guanine residues. The DNA within the transcription bubble is unwound and single stranded. During active transcription the translocation kinetics of the elongating RNA polymerase is high; this prevents CAA from modifying the nucleotides in the transcription bubble. Whereas, when RNA polymerase pauses, the kinetics is altered to give enough time for CAA access to the transcription bubble and modify the nucleotides. These CAA induced modifications are subsequently visualized by primer extensions to precisely map the location of the paused RNA polymerase complex.

To investigate weather H-NS acts as a roadblock to the elongating RNA polymerase, the wild-type and an *hns* mutant harboring high copy plasmids containing either the constitutive *lacUV5* promoter followed by the *bgl*-DRE sequence from +95

to +972 relative to *bgl* transcription start and the *lacZ* gene fusion (pKEM53) or the promoter less *bgl*-DRE fused to *lacZ* (pKEM54) (Fig. 6a) (as a control to determine if the CAA footprints are dependent on transcription or reflect the DNA structure) were treated with CAA. The primer extension was carried out with the oligo S487, mapping at positions just upstream the *bgl*-DRE sequence. A mock reaction without addition of CAA was included to differentiate the reactivity induced by CAA and the non-specific stops during primer extension. The primer extension products were separated on a denaturing sequencing gel next to a sequencing ladder generated with the same primer used for the probing experiments.

The CAA footprints show a clear reactivity at positions +477 to +484 relative to the transcription start site (Fig. 6b, compare *wt* with mock). However, this reactivity was also seen in the *hns* mutant, where it was more pronounced than in the *wt* and is thus independent of H-NS (Fig. 6b). This reactivity is dependent on transcription, because no reactivity was observed when the control plasmid pKEM54 lacking a promoter was probed in the *hns* mutant (Fig 6b, NT). These data demonstrate that RNA polymerase pauses at positions +477 to +484 relative to the transcription start of *bgl*. This pausing is intrinsic and is 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.

A closer look at the sequence revealed a stem loop structure present at positions +459 to +476, and immediately upstream the RNA polymerase pause site detected with CAA footprinting (Fig. 6c). Such a stem loop structure upstream a pause signal can prevent backtracking of the paused RNA polymerase and cause an arrest of the transcription elongation complex (Toulme et al., 2005). However, so far no significant role of this intrinsic pause site could be defined in *bgl* regulation (Nagarajavel, 2007).

In conclusion, footprinting with CAA showed no evidence of H-NS dependent pausing within the transcription unit in *bgl*. Likewise, in *proU* no H-NS dependent pause could be detected by CAA-footprinting (data not shown). These data show that H-NS does not act as a roadblock to the elongating RNA polymerase, although H-NS bound to the *bgl*-DRE weakly (2-fold) reduces transcription elongation (Nagarajavel, 2007). The data suggest that H-NS instead, affects transcription at an earlier step in the transcription cycle.

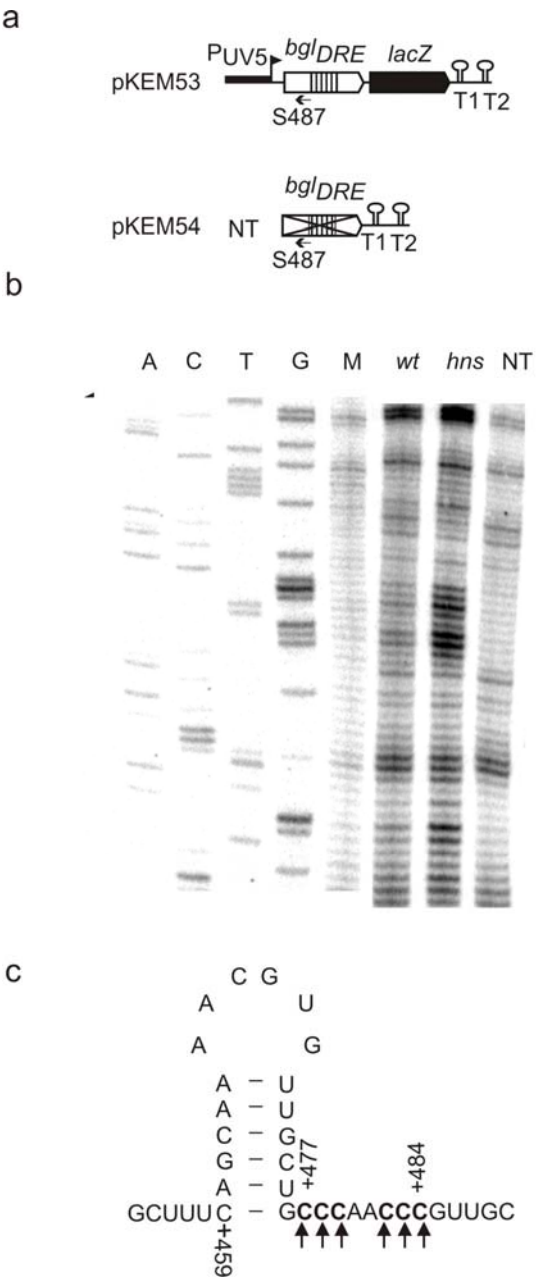


Figure 6. RNA polymerase pauses within the *bgl-DRE*:
(a): Schematic representation of the plasmids used for *in situ* CAA Footprinting. pKEM53 carries the *lacUV5* promoter-*bgl*-DRE-*lacZ* in a pBR backbone, while pKEM54 is the high copy plasmid with the *bgl*-DRE without a promoter. T1 and T2 refer 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. **(b)**: Representative gel showing the RNA polymerase pausing at position +477 to +484 (marked with filled arrows) 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 to the No Transcription control. The plasmids were transformed into S541 **(*wt*)** and S3346 **(*hns*)** and probed with CAA, primer extension done with S487. **(c)**: The reactive site mapping between +477 to+484 is shown, with the bold arrows marking the reactive bases. A stem loop structure (as predicted by MFOLD) formed between the bases from +459 to+476 is also shown. The secondary structure was determined with the web tool available at http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi

4.12 H-NS binding to the bgl-DRE inhibits transcription initiation prior to the open complex formation.

To analyze weather H-NS bound to the *bgl*-DRE affects transcription initiation, as shown before for *proU* (Jordi and Higgins, 2000), CAA footprinting of the promoter was performed. In this experiment wild-type and *hns* mutant cells were

transformed with high copy plasmids carrying either a *lacUV5* promoter fused to the *bgl*-DRE (pKENV64) or a *lacUV5* promoter flanked by the *bgl*-URE and the *bgl*-DRE fusion (pKENV67) (Fig. 7A). Both plasmids carry in addition, the *rrnB-T1T2* terminators distal to the *bgl*-DRE (Fig. 7a). Transformants of the wild-type and the *hns* mutant were grown to exponential phase and treated with CAA. In addition, a second set of cultures were treated with Rifampicin (200µg/ml) for 5 minutes prior to the addition of CAA (experimental procedures). Rifampicin traps RNA polymerase in the open complex by blocking extension of RNA synthesis beyond +2 or +3 and thus allows accumulation of the otherwise transient open intermediates (McClure et al., 1978).

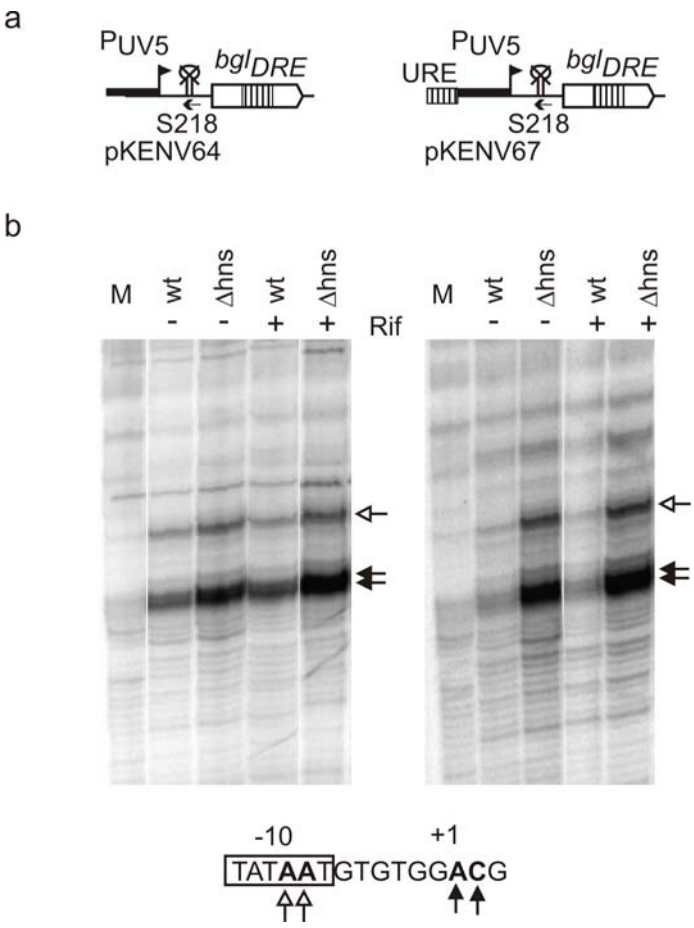


Figure 7. H-NS when binding to the *bgl*-DRE represses open complex formation: (a): Schematic representation of the plasmids used for *in situ* CAA Footprinting, pKENV64 carries the *lacUV5* promoter/*bgl*-DRE/*lacZ* in a pBR backbone, while pKENV67 is the high copy plasmid with the *bgl*-URE/*lacUV5* promoter/*bgl*-DRE/*lacZ*. T1 and T2 refer to rrnB-T1 and T2 terminators. The primer (S218) used for primer extension and the position to which it matches in the bgl_{DRE} is shown. **(b)**: Representative gel showing CAA footprinting of RNA polymerase *lacUV5* promoter complexes in the wild-type (S541) and *hns* mutant (S3346). The left panel shows the result obtained for transformants of plasmid pKENV64, and the right panel for transformants of plasmid pKENV67. The position of the reactivity is indicated in the sequence at the bottom. Rifampicin was added to 200 µg/ml 5 minutes prior to CAA where indicated.

The CAA footprinting of the *lacUV5* promoter-*bgl*-DRE (pKENV64) fusion revealed a clear reactivity at the -10 region and the transcription start site (at positions +1 and +2) (Fig. 7b). This reactivity, corresponding to the open complex was weaker in the wild-type than in the *hns* mutant. Similar result was obtained with the *bgl*-URE-*lacUV5* promoter-*bgl*-DRE fusion (pKENV67) (Fig. 7b). Strikingly, in the wild-type,

open complex formation detected at the *lacUV5* promoter was significantly decreased when the promoter was flanked by both the URE and DRE (pKENV67) as compared to the *lacUV5* promoter followed by *bgl*-DRE alone (pKENV64) (Fig, 7b compare the reactivities observed in *wt* with pKENV64 to those observed with pKENV67). In the *hns* mutant, open complex formation was high irrespective of the presence of the URE (Fig. 7b). In all cases, upon addition of rifampicin the reactivities specific for open complexes were enhanced similarly (Fig. 7b). Taken together the data suggest that H-NS, when binding to the *bgl*-DRE, represses transcription initiation prior to open complex formation. Repression is more effective in the presence of both the URE and the DRE, suggesting synergy in repression by H-NS bound to both the URE and DRE.

4.13 Synergy in repression of bgl and proU by H-NS bound to the URE and DRE.

Complete repression of the *bgl* operon by H-NS requires upstream and downstream regulatory elements to which H-NS binds (Dole et al., 2004b; Schnetz, 1995), while the URE and the DRE alone cause only a moderate repression (Dole et al., 2004b; Nagarajavel, 2007), suggesting synergy in repression by H-NS bound to the *bgl*-URE and the *bgl*-DRE. This is confirmed by CAA footprinting (shown above), the open complex formation at the *lacUV5* promoter is repressed more efficiently when both the *bgl*-URE and DRE are present than in the presence of the *bgl*-DRE alone (Fig. 7b).To compare H-NS mediated repression of *bgl* with that of *proU*, a set of chromosomal *proU-lacZ* fusions carrying the *proU*-URE and *proU*-DRE, or the *proU*-URE or DRE alone were analyzed. Due to the osmoregulation of the *proU* operon, the expression of the *proU-lacZ* fusions was determined from cells grown in LB with low to high osmolarity (containing 0.01, 0.05, 0.1, 0.2, or 0.3 M NaCl) at steady state conditions (Experimental procedures).

At low osmolarity, the *proU* promoter flanked by the URE and the DRE was repressed 16-fold by H-NS (LB 0.01 M NaCl) (Fig. 8a). At high osmolarity (LB 0.3 M NaCl) the expression increased 20-fold in the wild-type and the promoter was not repressed by H-NS (Fig. 8a). In the presence of the URE alone, the *proU* promoter was not significantly repressed by H-NS (1.5-fold at low osmolality, and 0.9-fold at high osmolarity) and the expression of the *proU*-URE-promoter-*lacZ* fusion increased

approximately 3.5-fold from low to high osmolarity (Fig. 8b). The repression mediated *via* the *proU*-DRE alone was tested using *proU* promoter followed by *proU*-DRE fused to *lacZ* gene (Fig. 8c). The *proU* promoter was repressed 5-fold by H-NS at low osmolarity, while at high osmolarity the expression increased ~3-fold, and repression by H-NS dropped to 2-fold (Fig. 8c). In comparison, the *lacUV5* promoter driven *proU*-DRE-*lacZ* fusion was not osmoregulated, and the repression by H-NS was approximately 4-fold (at low and high osmolality) (Fig. 8d). The expression of the *proU* promoter alone, lacking both the URE and the DRE fused to *lacZ* increased 2-fold from low to high osmolarity (Fig. 8e), and this construct was not repressed by H-NS. These data 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., 1994) (Rajkumari et al., 1996a) (Jordi and Higgins, 2000), and the osmoregulation is specific to the *proU* promoter and not to repression by H-NS. Further, the data clearly demonstrate that repression by the *proU*-URE and DRE is synergistic, like in *bgl* (Fig. 7 and 8).

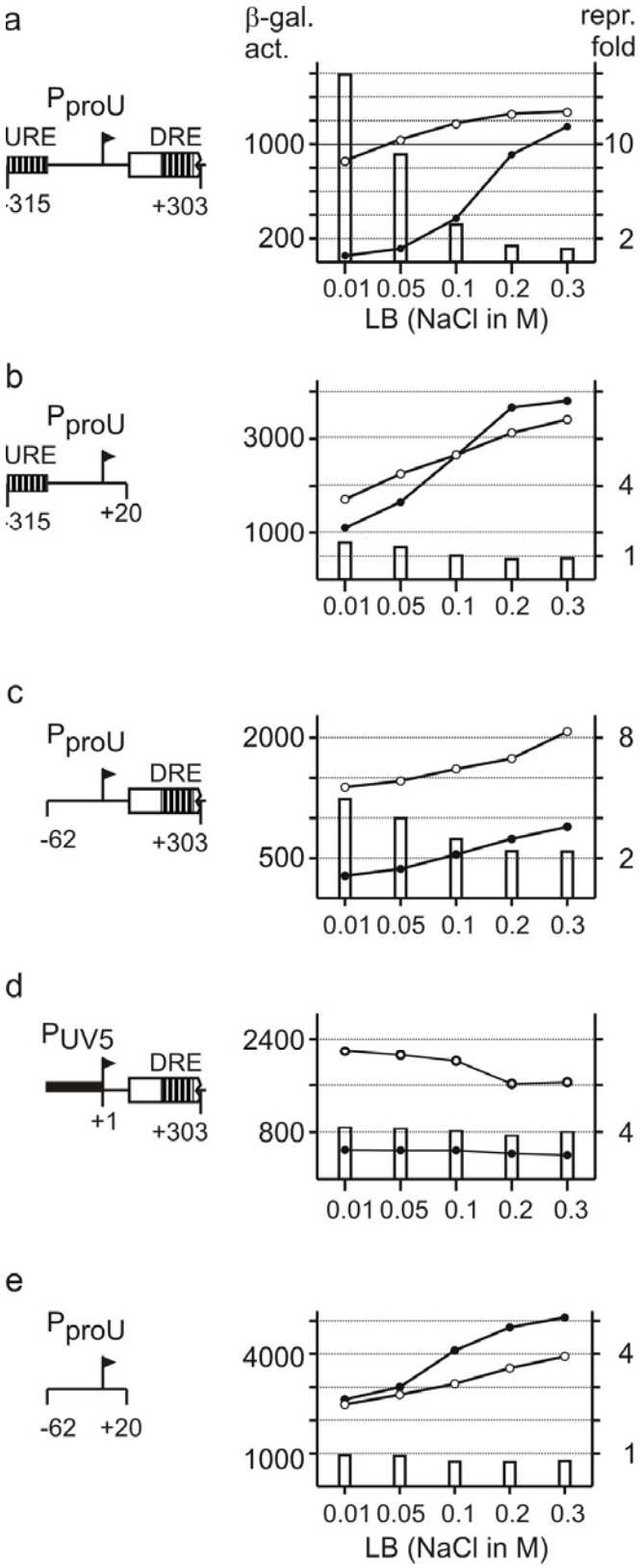


Figure 8. Synergy in repression by H-NS in the *proU* operon: The chromosomal integrants of *proU*-acZ fusions containing $proU_{URE}$ and $proU_{DRE}$ **(a);** the $proU_{URE}$ alone **(b);** $proU_{DRE}$ alone expressed from *proU* promoter **(c);** $proU_{DRE}$ alone expressed from *lacUV5* promoter **(d);** and the *proU* promoter alone **(e);** are 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 filled circles (*wt*) and white 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 c) S3699, S3742 d) S2137, S3126 and e) S369, S3740 respectively.

4.14 Poising of the RNA polymerase at the proU promoter under low osmolariy.

H-NS has been shown to repress the *proU* promoter at a step of transcription initiation prior to open complex formation (Jordi and Higgins, 2000), and transcription initiation at the *proU* promoter is osmoregulated. For Sigma S dependent osmoregulated promoters, it has been shown that RNA polymerase is poised at the promoter at low osmolarity but not at high osmolarity (Gralla and Vargas, 2006). An RNA polymerase that is bound to a promoter, but showing no transcriptional activity is defined to be 'poised' at the promoter.To analyze whether RNA polymerase is likewise poised at the sigma 70 dependent *proU* promoter and how this correlates with repression by H-NS, open complex formation at the *proU* promoter was determined by CAA footprinting. To this end, pKENV73, a high copy number plasmid carrying the *proU* promoter flanked by the *proU*-URE and DRE was used (Fig. 9a). Transformants of the wild-type and *hns* mutant with this plasmid were grown in LB of low and high osmolarity (LB 0.01 M NaCl and 0.3 M NaCl) to mid exponential phase, and CAA was added directly or 5 minutes after rifampicin addition.

At all conditions, a clear reactivity within the -10 region and from positions -5 to +1 was apparent (Fig. 9b). In the wild-type the reactivity specific for open complex formation was rather weak for cells grown at low as well as high osmolarity (Fig. 9b). This confirms that H-NS represses the transcription initiation at the *proU* promoter at a step prior to open complex formation (Jordi and Higgins, 2000). In the *hns* mutant the reactivity was strong for cells grown at low osmolarity (Fig. 9b, 0.01M). Interestingly, in the *hns* mutant grown at high osmolarity the reactivity specific for open complex formation was significantly reduced (Fig. 9b). Taken together these data suggest that at low osmolarity RNA polymerase is poised at the *proU* promoter. At high osmolarity poising of RNA polymerase at the promoter and also repression by H-NS are reduced.

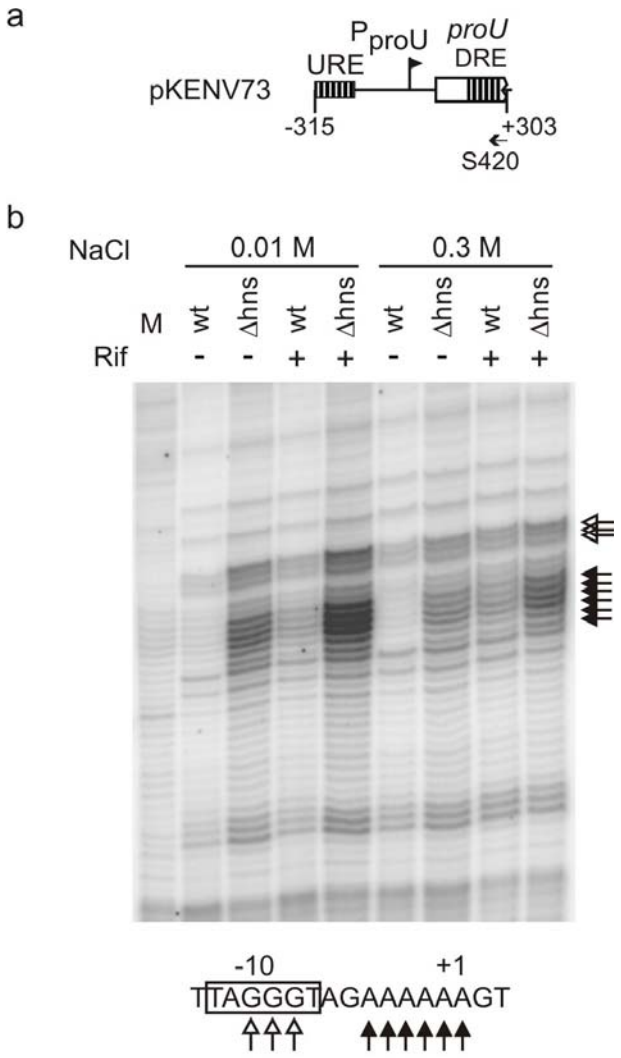


Figure 9. RNA polymerase is 'poised' at the *proU* promoter at low osmolarity: **(a)** Schematic representation of the plasmid used for *in situ* CAA Footprinting, pKENV73 carries the *proU*-URE/promoter *proU*/*proU*-DRE in a pBR backbone. T1 and T2 refer to rrnB-T1 and T2 terminators. The primer (S420) used for primer extension and the position to which it matches in the *proU*-DRE is shown. **(b)**: Representative gel showing CAA footprinting of RNA polymerase *proU* promoter complexes in the wild-type (S541) and *hns* mutant (S3346) cells grown in LB medium with 0.01M NaCl (low osmolarity) and 0.3M NaCl (high osmolarity). The reactivities corresponding to the -10 box are marked with empty arrows, while the bold arrows show the reactivities observed between -5 to +1. The reactive bases are indicated in the sequence at the bottom. Rifampicin was added to 200 µg/ml 5 minutes prior to CAA where indicated.

To summarize, the analysis of repression of *bgl* and *proU* by H-NS revealed several parallels. Firstly, repression of *bgl* and *proU* by binding of H-NS to the URE and DRE is synergistic. Secondly, H-NS represses transcription initiation at a step prior to open complex formation. Thirdly, repression via the DRE correlates inversely to the promoter activity, and in *bgl* H-NS bound downstream reduces transcription read-through (Nagarajavel et al., 2007). These parallels support a model for H-NS mediated repression of the *bgl* and *proU* operons (see discussion).

4.2 Modulation of repression by H-NS bound within the transcription unit

Data presented in this chapter have in part been published in "Madhusudan S., Paukner A., Klingen Y., and K. Schnetz (2005) Independent regulation of H-NS-mediated silencing of the *bgl* operon at two levels: upstream by BglJ and LeuO and downstream by DnaKJ. *Microbiology.* 151:3349-3359."

Repression of a promoter by binding of H-NS within the transcription unit presumably occurs by the same mechanism as repression by binding of H-NS upstream of a promoter; H-NS may either exclude binding of RNA polymerase or trap it at the promoter (Shin et al., 2005). However, in contrast to repression by H-NS that binds upstream of a promoter, H-NS bound downstream is exposed to transcription. In fact, previsous data suggest that transcription and repression by H-NS through the *bgl*-DRE mutually influence each other. Firstly, H-NS bound to the *bgl*-DRE reduces transcription through the *bgl* transcription unit 2-fold (Nagarajavel, 2007). Secondly, repression by H-NS through the *bgl*-DRE is affected by termination factor Rho and translation. Termination factor Rho is essential for the repression by H-NS, while translation of the *bgl* mRNA counteracts the repression (Dole et al., 2004b). Thirdly, repression by H-NS through the DRE inversely correlated to the transcription rate (Nagarajavel, 2007). Taken together this indicates that H-NS bound to the *bgl-DRE* in addition to repressing transcription initiation, may obstruct or slow down transcription elongation and thus expose the elongation complex to proteins that modify it. These proteins include termination factor Rho, GreA and GreB, and the Nus proteins (Liu et al., 1996). Slowed transcription elongation by frequent short pauses would allow termination factor Rho to catch up with the polymerase to bring about termination (Richardson, 2002). Among the Nus factors, NusA is known to prolong the pause of the RNA polymerase (Gusarov and Nudler, 2001). Factors like GreA or GreB, stimulate cleavage of the protruding 3' end of the RNA generated by back tracking, in order to generate a new free 3'OH within the catalytic center (Toulme et al., 1999; Nudler, 1999).

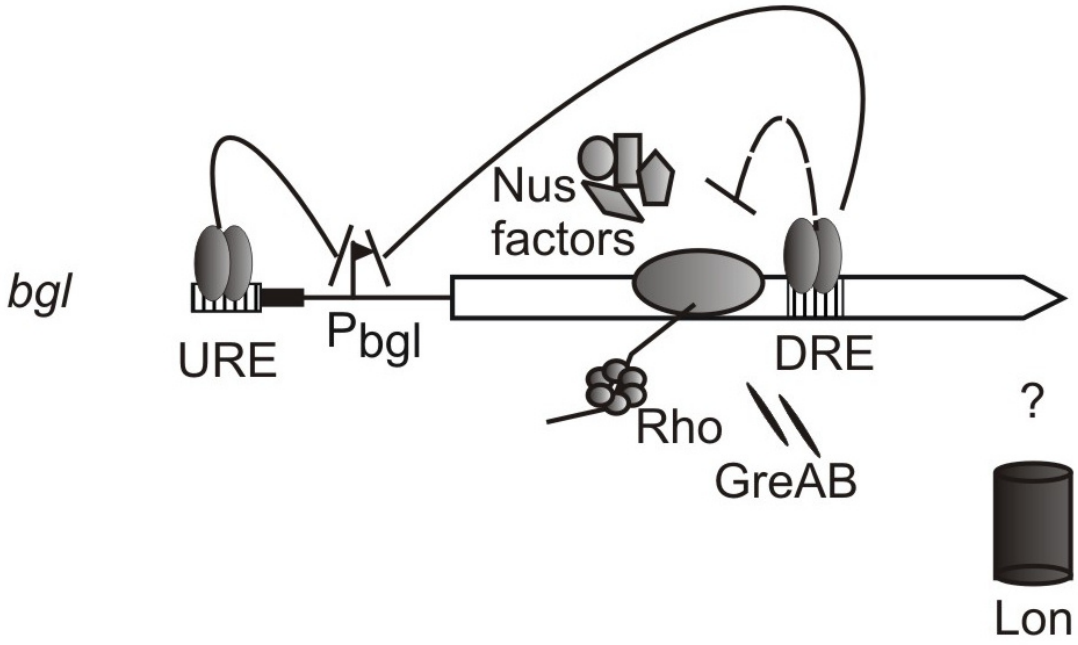


Figure 10. Modulation of repression by H-NS through *bgl*-DRE by the Lon protease. H-NS weakly reduces transcription through the *bgl* transcription unit, termination factor Rho affects the repression by H-NS through *bgl*-DRE (Nagarajavel, 2007). H-NS mediated repression of transcription through the *bgl*-DRE is modulated by protease Lon (Dole et al., 2004a). This modulation by Lon could be achieved by targeting the H-NS Nucleoprotein complex, or Lon could affect the levels of Rho, the Nus factors, or the GreA and GreB proteins; all of which are known to modulate transcription elongation.

In this work, modulation of repression by H-NS through the *bgl*-DRE by the ATP-dependent protease Lon was addressed. Lon was identified in a Transposon mutagenesis screen for factors that modulate repression by H-NS through the *bgl*-DRE. The repression by H-NS thorugh the *bgl*-DRE was more efficient in *lon* mutants, while repression through the *bgl*-URE was not affected by Lon (Dole et al., 2004a). Lon protease is involved in degrading abnormal proteins and regulation of important cellular functions like radiation resistance, cell division, adapting to nutritional downshifts, capsular biosynthesis (Gottesman, 1996). The modulation of the H-NS-mediated repression by Lon could be in several possible ways (summarized in Fig. 10). Lon could target a component of the repressing nucleoprotein complex formed by H-NS. Although it has been excluded that the H-NS homologue StpA, which is a known Lon substrate (Johansson and Uhlin, 1999) is required for the repression of *bgl* (Dole et al., 2004a), another protein that interacts with H-NS or H-NS itself could be the target. Another possibility is that Lon affects the levels of termination factor Rho, or another protein that modulates transcription elongation,

like the Nus factors or GreA and GreB. These possibilities are addressed in the current chapter. In addition, a transposon mutagenesis screen was performed "to identify putative Lon targets" that could modulate the repression by H-NS through the *bgl*-DRE.

4.21 A dnaKJ mutation suppresses the enhanced repression by H-NS in the lon mutant.

As a non biased approach to identify the putative Lon substrate responsible for the enhanced repression by H-NS in the *lon* mutant, a transposon mutagenesis screen was performed (Experimental procedures). To avoid mutations that map *in cis* to the operon, a double phenotype screening strategy was used (schematically shown in Fig. 11). The *lon* mutant strain S2103 (containing the allele *lon-107*::miniTn*10*-Tc^r, Table I), which was screened for suppressor mutations, carries a *bgl* operon allele (*bgl*-CRP$^+$) and a *lacUV5* promoter-*bgl*-DRE$_{NT}$-*lacZ* fusion. In this *bgl* operon allele the promoter is activated by a point mutation improving the CRP-binding site. This *bgl* allele confers a Bgl-positive phenotype in the wild-type, but a Bgl-negative phenotype in the *lon* mutant (Fig. 11). The additional *lacUV5* promoter driving the expression of *bgl*-DRE$_{NT}$-*lacZ* fusion, where the *bgl$_{DRE}$* carries mutation of the start codon and two additional AUG triplets at position 3 and 27 to GCG thereby eliminating translation of *bglG*, likewise confers a Lac positive phenotype in the wild-type, but a Lac negative phenotype in the *lon* mutant (Fig. 11). The mutagenesis screen of this double reporter strain yielded 3 independent insertion mutations with a clear phenotype change to Bgl$^+$ and Lac$^+$, all of which mapped at the *dnaKJ* locus (Fig. 11). All three insertion mutants (designated as alleles *dnaKJ*-M1, M2 and M3) carry a miniTn*10* insertion that disrupts the *dnaK* promoter. The insertions map 63 bp upstream of the *dnaK* ATG start codon in all cases. One of the insertions (*dnaK*-M3) is associated with a 29 bp deletion (indicated by an open arrowhead in Fig. 11). This result suggests that the DnaKJ chaperone system may affect repression by H-NS through *bgl*-DRE in the *lon* mutant.

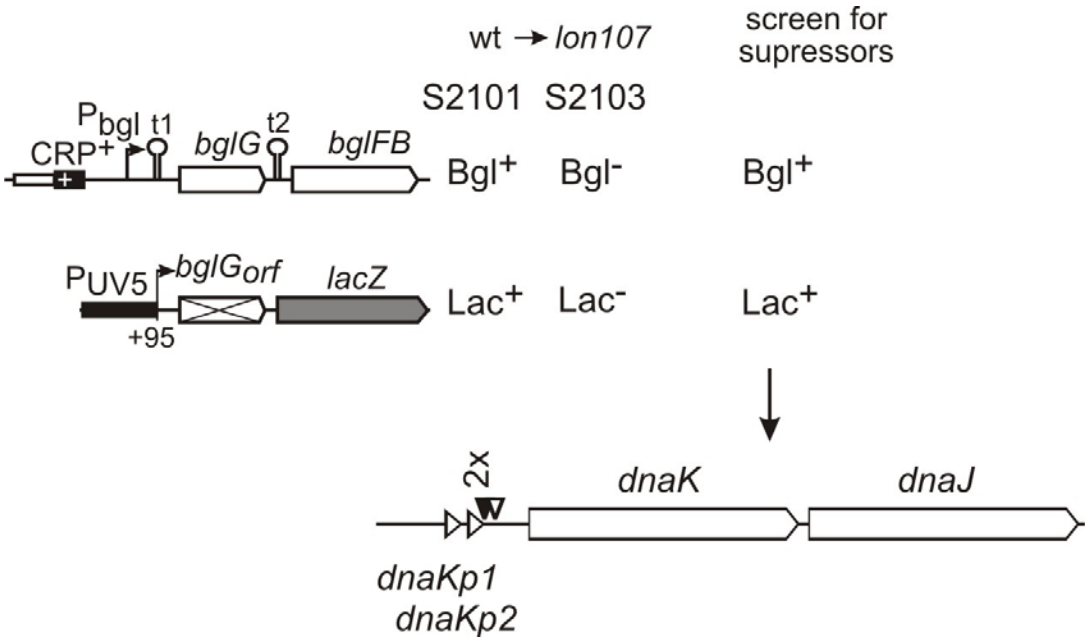


Figure 11. Mutagenesis screen for suppressors of enhanced repression by H-NS in the *lon* mutant: Strain S2103 is a *lon* mutant that carries an activated *bgl* operon and a fusion of the *bgl* regulatory region to the *lac* operon. The *bgl* operon is activated by a point mutation that improves the CRP-binding site (allele *bgl*-CRP$^+$). The expression of the *bglGorf -lacZ* fusion, which carries the downstream regulatory element of *bgl*, is directed by the constitutive *lacUV5* promoter. The *lon* mutant is Bgl and Lac negative due to more efficient *bgl* operon silencing by H-NS, while the phenotype is Bgl and Lac positive in the corresponding wild-type strain S2101. Insertions in the *lon* mutant that showed a Bgl and Lac positive phenotype were selected. Three suppressor mutants were isolated in the screen. All three mutants map 5' to the *dnaK* coding region. The insertion sites of mTn*10*-Cm^r mutations obtained are indicated by arrow heads. One of the insertions associated with a 29 bp deletion is indicated by an open arrowhead.

4.22 The dnaKJ::miniTn10 (dnaKJ-M2) mutation specifically suppresses the H-NS repression through the bgl-DRE.

The possible role of DnaKJ in *bgl*-URE and *bgl*-DRE mediated repression by H-NS, and the modulation of the latter by Lon were addressed using *bgl*-URE and the *bgl*-DRE$_{NT}$/ *lacZ* reporter constructs. For this purpose, the expression of a chromosomal URE-*bgl* promoter fused to the lacZ gene (pKEKB30) and the constitutive *lacUV5* promoter followed by the *bgl*-DRE and the *lacZ* gene (pKESD49) (Dole et al., 2004a) were compared among wild-type, *lon, dnaKJ*-M2, and the *lon dnaKJ*-M2 double mutants (Fig. 12). The expression of the URE-*bgl* promoter construct remained unchanged between the wild-type, *lon, dnaKJ*-M2, and the *lon dnaKJ*-M2 double mutant (Fig. 12a). This suggests that like Lon, the DnaKJ chaperone system does not affect repression by H-NS through the *bgl*-URE. In contrast, the expression level of β-galactosidase directed by the *bgl*-DRE$_{NT}$/ *lacZ*

reporter, which decreases 2-fold in the *lon* mutant (Dole et al., 2004a) and (Fig. 12b) was affected by DnaKJ. In the *dnaKJ*-M2 mutant the expression level increased ~2-fold as compared to the wild-type. Further, in the *lon dnaKJ*-M2 double mutant the expression level increased ~4-fold as compared to the *lon* single mutant (Fig. 12b). These data show that the DnaKJ chaperone system specifically affects the repression of *bgl* by H-NS through the DRE. Furthermore, the DnaKJ effect is independent of Lon.

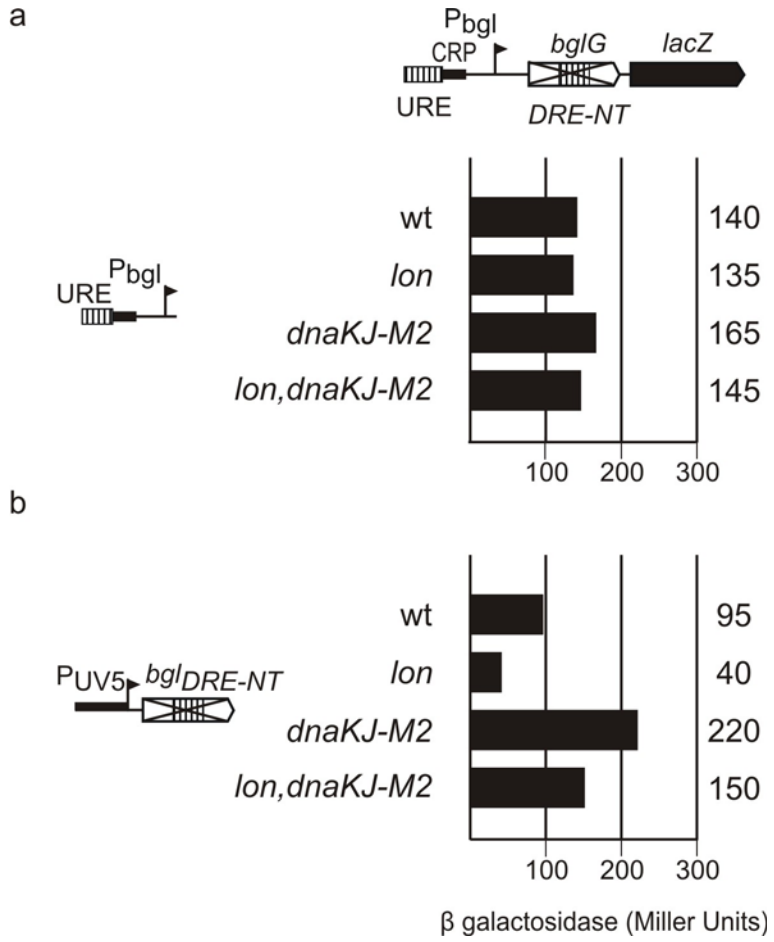


Figure 12. The *dnaKJ-M2* mutation specifically suppresses the H-NS repression of transcription elongation at the *bgl-DRE*. The expression of chromosomal *bgl*-URE/*bgl* promoter/*lacZ* fusion (pKEKB30) and the *lacUV5* promoter/*bgl*-DRE_{NT}/*lacZ* fusion (pKESD49) was analysed in the wt, *lon, dnaK-M2,* and the *lon dnaK-M2* mutants. Cells were grown in LB media at 37°C for β-galactosidase assay. The β-galactosidase values are plotted as bars along the x-axis, and given numerically to the right of the bars. Strains used are shown in the order wt, *lon, dnaK-M2* and the *lon dnaK-M2*: (a) S1213, S2680, S2674, and S2710,and (b) S1196, S1564, S2712 and S2670.

4.23 The DnaKJ chaperone system is imperative for the repression of transcription through bgl by H-NS.

In the *dnaKJ*-M2 mutant, the miniTn*10* insertion maps upstream of the *dnaK* open reading frame. Therefore, constitutive expression of the *dnaKJ* operon maybe directed by the promoter of the chloramphenicol resistance gene located within the miniTn*10* transposon. To analyze whether an increase or a decrease in the cellular levels of DnaKJ affects the repression through the *bgl*-DRE, an additional *dnaKJ* mutant was used, in which the *dnaKJ* promoter is replaced by a *lacI* PA1/*lacO* cassette (Tomoyasu et al., 1998). In this situation, *dnaKJ* expression requires IPTG and DnaKJ levels are very low when cells are grown without IPTG.

The role of DnaKJ levels on H-NS mediated repression through the *bgl*-DRE was tested in the PA1/*lacO dnaKJ* strain using the *lacUV5* promoter/*bgl*-DRE_{NT} /*lacZ* reporter (Fig. 13a). The expression of this reporter increased 5-fold when the cells were depleted of the DnaKJ chaperone system, and repression was even more effective when expression of DnaKJ was induced with IPTG (Fig. 13a, compare + and – IPTG). However, DnaKJ has no effect on repression by H-NS through the *bgl*-URE (Fig. 13b). These data indicate that DnaKJ is required for H-NS-mediated repression through the *bgl*-DRE, but not for repression through the *bgl*-URE. To corroborate this finding, the DnaK levels in the wild-type, the *dnaKJ*-M2 mutant and the PA1/*lacO dnaKJ* strain (+/- IPTG) were analyzed in a Western blot using DnaK specific antibodies (Fig. 13c). The quantitative Western analysis demonstrated that DnaK levels are ~2-fold reduced in the *dnaKJ*-M2 mutant as compared to the wild-type. In the PA1/*lacO dnaKJ* strain, the DnaK level was 5-fold lower than the wild-type level when cells were grown without IPTG, while the DnaK protein level upon induction with IPTG was much higher than in the wild-type strain (17-fold increased) (Fig. 13c). Thus, the repression of transcription through *bgl* by H-NS is more effective when DnaKJ is present.

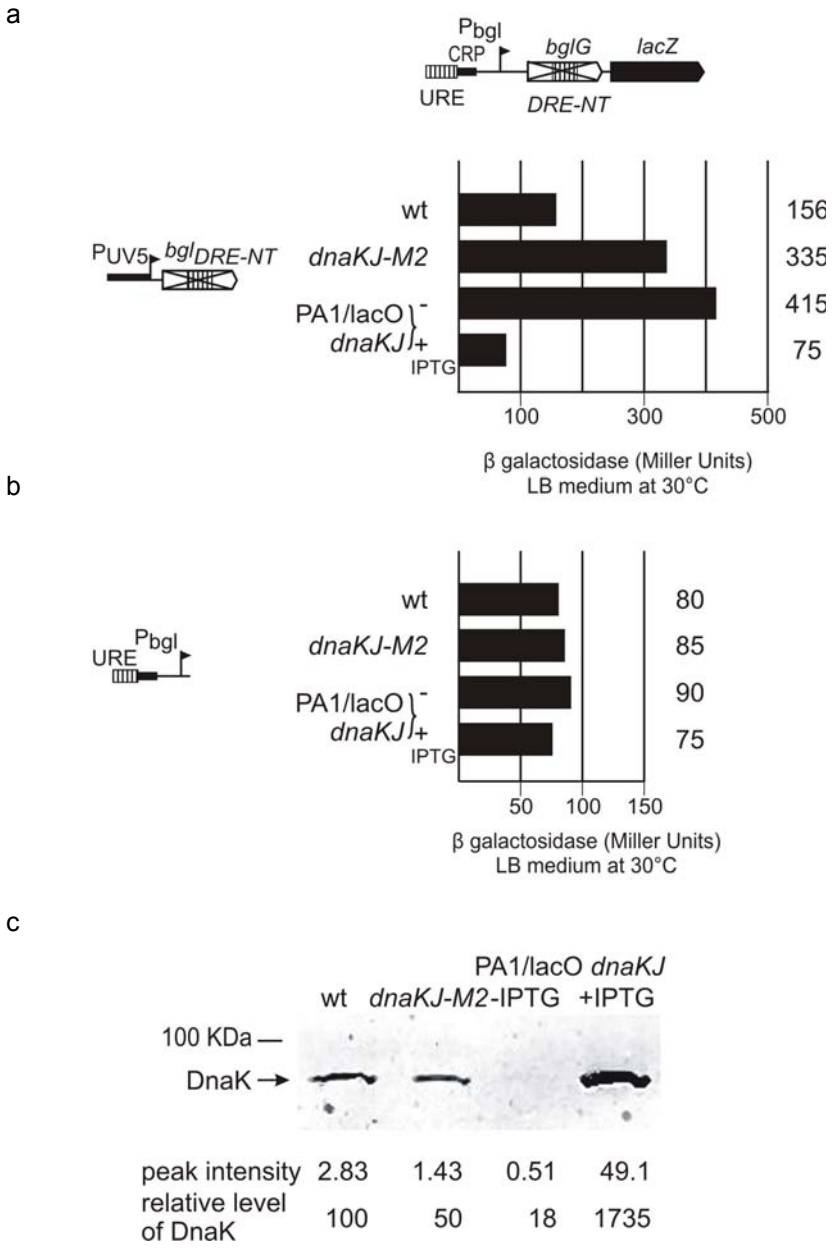


Figure 13. The DnaKJ chaperone system is imperative for the repression by H-NS bound to the *bgl-DRE*.The expression of chromosomal *lacUV5* promoter/*bgl*-DRE$_{NT}$/*lacZ* fusion (pKESD49) **(a)** and the *bgl*-URE/*bgl*promoter/*lacZ* fusion (pKEKB30) **(b)** was analysed in wt, *dnaK-M2* and the PA1/*lacO dnaKJ* strain. In the latter the *dnaKJ* promoter is replaced by a *lacI* PA1/*lacO* cassette and the expression of *dnaKJ* thus requiries IPTG induction). Both the constructs have *lacZ* fused transcriptionally. Cells were grown in LB media at 30°C with 1mM IPTG, where indicated. The β-galactosidase values measured for the respective strains are plotted as bars along the x-axis. The values on the right show the Miller units. Strains used are shown in the order wt, *dnaK-M2* and the PA1/*lacO dnaKJ* ; S1196, S1564, S2712, and S2888 (a) and S1213, S2680, S2674 and S2904 (b). **(C)** A quantitative Western blot analysis of DnaK protein levels expressed in the wild-type (S1196), the *dnaKJ*::miniTn*10* mutant (allele *dnaKJ*-M2; strain S2712), and the PA1/*lacO dnaKJ* strain (S2888) grown without (-IPTG) and with IPTG (+IPTG) at 30°C in LB to OD_{600}=0.5. The Western blot and the result of the quantitative analysis given as peak intensity as well as relative protein levels as compared to the wild-type (wt level set as 100 arbitrary units) are shown.

4.24 Inhibition of termination factor Rho neutralizes the effect of Lon on repression by H-NS through bgl-DRE.

The mutagenesis screen to identify supressors of the Lon effect yielded insertions only in the *dnaKJ* locus. The screen however, cannot pick up insertions in essential genes. The essential termination factor Rho was already shown to contribute to the repression by H-NS bound to the *bgl*-DRE (Dole et al., 2004b). To check if Rho is the Lon target that modulates repression by H-NS bound to the *bgl*-DRE, the expression level of the *bgl*-DRE-*lacZ* reporter (explained earlier Fig. 12) was measured in the *wt* and *lon* mutant grown (in LB at 37°C) without or with sub-lethal concentrations of bicyclomyin (20µg/ml). Bicyclomycin is a specific inhibitor of transcription termination factor Rho (Magyar et al., 1996). The expression of the *bgl*-DRE_{NT}-*lacZ* reporter decreases two-fold in the *lon* mutant (as shown before) (Fig. 14). Upon addition of bicyclomycin the expression level increased, confirming the contribution of Rho to repression. Interestingly, with bicyclomycin there was no difference in the expression levels between the wild-type and the *lon* mutant (Fig. 14). Thus, inhibition of termination factor Rho with bicyclomycin completely neutralizes the 2-fold regulation by Lon. These data suggest that termination factor Rho, or some other factor involved in transcription termination could be a Lon target.

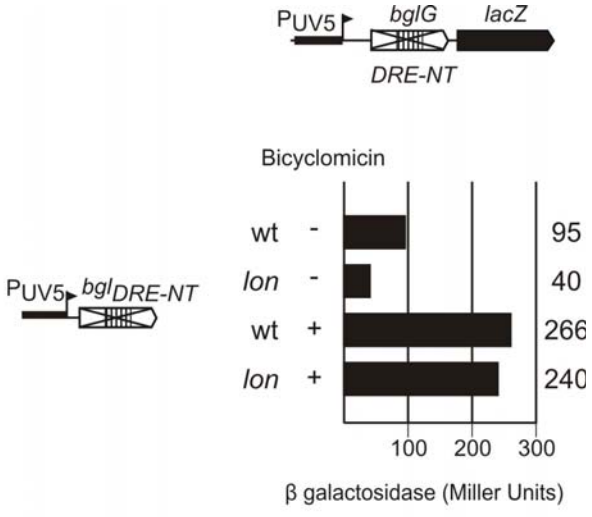


Figure 14. Inhibition of termination factor Rho neutralizes the effect of Lon on the repression by *bgl-DRE*: the expression of chromosomal *lacUV5* promoter/ *bgl*-DRE_{NT}/ *lacZ* fusion (pKESD49) was analysed in wt, and the *lon* mutant. Cells were grown in LB media at 37°C with 20µg/ml bicyclomicin where indicated. The β-galactosidase values in units measured for the respective strains are plotted as bars along the x-axis. The values on the right show the values in Miller units. Strains used are shown in the order wt, *lon*; S1196, S1564.

The possibility that Lon regulates Rho, or another factor involved in transcription termination was addressed by estimating the expression of two other systems involving Rho-dependent transcription termination. For this, the expression of a *tna* operon/*lacZ* fusion and a *lacUV5* promoter-*λtR1* terminator-*lacZ* fusion was compared between the wild-type and a *lon* mutant, with and without addition of bicyclomicin. The *tna* operon codes for tryptophanase, the enzyme that hydrolyses tryptophan. The operon is regulated by tryptophan dependent antitermination. The *tna* leader sequence codes for a short peptide TnaC, which has a critical tryptophan residue at which the ribosomes stall in the absence of tryptophan in the growth medium (Yanofsky, 2000;Yanofsky, 1987). This allows termination factor Rho to load onto the *boxA* sequence that is free of ribosomes, and terminate the transcription at the *rho* dependent terminators located in the leader upstream of *tnaA*, the first structural gene of the operon (Stewart et al., 1986). In presence of tryptophan, the ribosomes do not stall at the *tnaC* leader; hence prevent the access of Rho to a *boxA* sequence. Access to the *boxA* motif is essential for termination, and its blockage by the ribosomes leads to expression of the system. The expression of a *tna* promoter-*tnaC-tnaA'lacZ* translational fusion (in which the *lacZ* gene is translationally fused to the first 20 amino acids of *tnaA* gene) (Konan and Yanofsky, 2000) was determined in cells grown in minimal M9 B1 medium with 0.2% glycerol and 0.05% casa aminoacids. Tryptophan was added for induction of antitermination, and bicyclomycin for inhibition of Rho, where indicated (Fig. 15). Under uninduced condition (without tryptophan), the expression of the *tna-lacZ* fusion was 2-fold reduced in the *lon* mutant (Fig. 15a), as in the case of *bgl* (Fig. 12b). Inhibition of Rho with bicyclomicin led to an approximately 20-fold increase of expression in both wild-type and *lon* mutant. However, the expression in the *lon* mutant was still 2-fold reduced even on inhibition of Rho (Fig. 15a). Induction with tryptophan increased the expression by 10-fold in the wild-type and 20-fold in the *lon* mutant, to reach similar levels (Fig. 15a and b). Under these conditions (with tryptophan) the addition of the bicyclomycin caused a three-fold further increase in expression (Fig. 15b). Taken together, in the *lon* mutant expression of the *tna-lacZ* reporter decreases 2-fold (under uninduced conditions), similar to *bgl*. However, inhibition of Rho with bicyclomycin does not neutralize the *lon* effect, as it does in *bgl*.

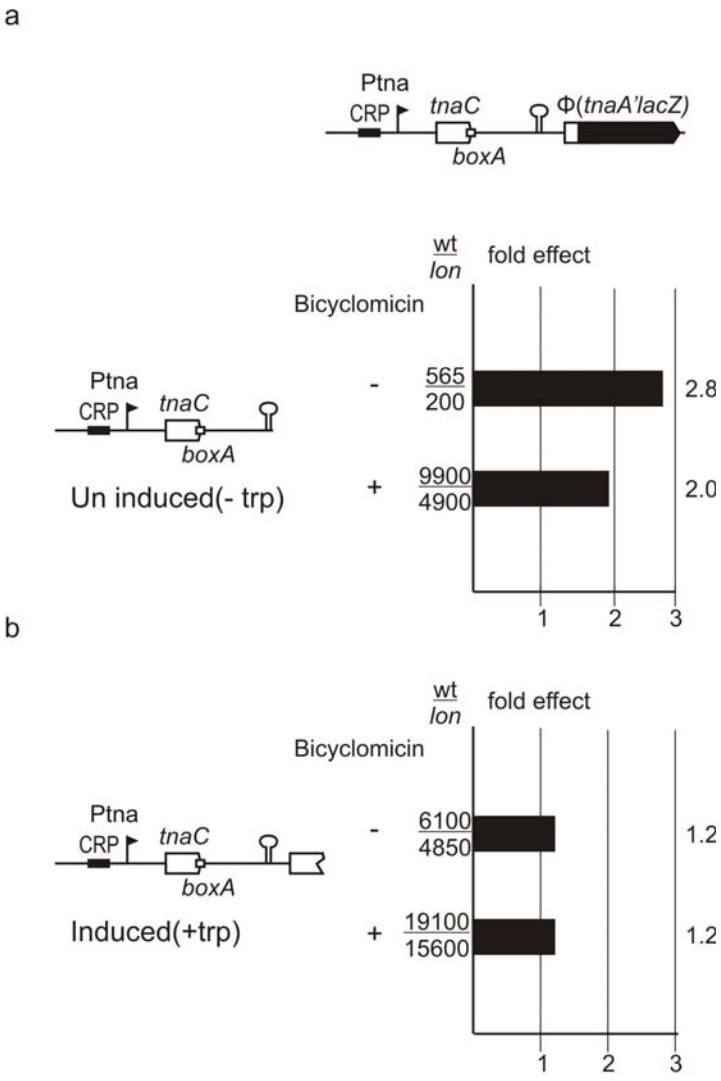


Figure 15. Inhibition of termination factor Rho does not neutralize the effect of Lon on the expression of the *tna* operon. The expression of chromosomal *tna* promoter´-*tnaC-tnaA'lacZ* translational fusion (pKEM02) was analysed in the wt and the *lon* mutant. Cells were grown grown in Minimal M9B1 medium with 0.2% glycerol and 0.05% Casa aminoacids, in uninduced (without tryptophan) and induced (with 50μg/ml tryptophan) states at 37°C. 20μg/ml bicyclomicin was added where indicated. The numbers shown at the left of the bar chart represent the β-galactosidase values. The values on the right and the bars show the ratio of the expression in the wild-type to that in the *lon* mutant. Strains used are shown in the order wt, *lon*; S1975, S1983.

Rho-dependent transcription termination at the phage lambda tR1 terminator is mediated primarily by the rut (Rho utilization) element that encompasses two RNA regions *rutA* and *rutB*, which are separated by a *boxB* RNA motif (Graham et al., 1998) (Richardson, 1996). As a folded hairpin structure, the *boxB* acts as a clamp that holds *rutA* and *rutB* side by side for optimal interactions with Rho leading to efficient termination (Vieu and Rahmouni, 2004). As the second system regulated by Rho, the *λtR1* terminator along with the *rut* sites and the *boxB* (which overlap with the *cro* gene of the phage) was cloned under the control of *lacUV5* promoter and the expression of the *lacUV5* promoter-*λtR1* terminator-*lacZ* fusion was compared between wild-type and *lon* mutant, with and without addition of bicyclomicin (Fig. 16).

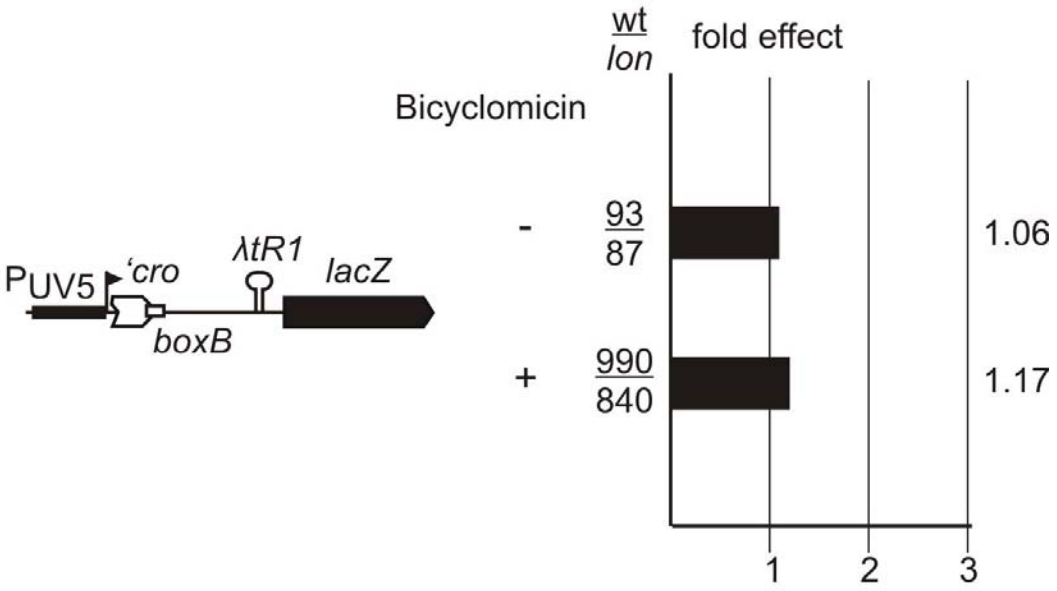


Figure 16. Lon does not affect the termination brought about by Rho at the *λtR1* terminator. The expression of a chromosomal *lacUV5* promoter-*λtR1* terminator-*lacZ* fusion (pKEM02) was analysed in wt, and the *lon* mutant. Cells were grown in LB media at 37°C with 20µg/ml bicyclomicin, where indicated. The fold difference in the expression of the construct between the wild-type and the *lon* mutant is plotted along the x-axis and shown numerically to the right. The β-galactosidase values are represented at the left. Strains used are shown in the order wt, *lon*; S1976, S1980.

The expression of the *λtR1-lacZ* fusion did not vary significantly between the wild-type and the *lon* mutant (Fig. 16). Inhibition of Rho by the addition of bicyclomicin increased the expression of the construct around 10-fold, as expected (Fig. 16). This result like in the case of the *tna* operon (Fig. 15) is in contrast with the one obtained with the *bgl*-DRE (Fig. 14).

4.25 Termination factor Rho and the Nus factors involved in transcription termination are not targets for Lon protease.

Expression of the *bgl* and the *tna-lacZ* fusions is modulated by Lon, while expression of the *λtR1*-terminator-*lacZ* is not. Further, in *bgl* inhibition of Rho abrogates the regulation by Lon, but not in *tna*. These apparently contradicting results suggest that neither Rho nor another factor of transcription termination is a Lon substrate, whose proteolysis might be important for expression of *bgl.* Nonetheless, in parallel to the genetic analysis shown above (Figs. 14 to 16) the protein stability of

factors important in transcription pausing and termination was analyzed in the wild-type and *lon* mutant. This analysis included termination factor Rho (Richardson, 2002), the Nus factors involved in transcription termination; NusA, NusB, NusG, NusE (Nudler and Gottesman, 2002), and two transcription elongation factors; GreA and GreB (Borukhov et al., 2005). For this, a series of low copy plasmids (pSC101 background) carrying C- terminally HA tagged versions of the candidate genes under the control of the IPTG inducible *tac* promoter were constructed. The plasmids also carry the *lacI*^{q} gene (Table 4 and Experimental Procedures). The wild-type (S541) and the *lon* mutant (S1553) strains were transformed with the respective plasmids and the expression of the candidate genes was induced by IPTG. After inhibition of protein synthesis with Spectinomycin (Johansson and Uhlin, 1999), the stability of the respective proteins was compared between the wild-type and the *lon* mutant by Western blotting (Experimental Procedures). Additionally, the chromosomal copy of the *nusA* gene was replaced by a C-terminally HA tagged version, by gene replacement (Datsenko and Wanner, 2000) (Experimental Procedures). The stability of the chromosomal *nusA-HA* was determined similarly (Experimental Procedures), in the wild-type and the *lon* mutant (Fig. 17b).

The results summarized in Figure. 17, show that the stability of all the candidate proteins tested did not vary significantly between the wild-type and the *lon* mutant. This suggests that the regulation of the H-NS mediated repression of *bgl* by the Lon protease is not mediated by the degradation of a factor involved in transcription termination.

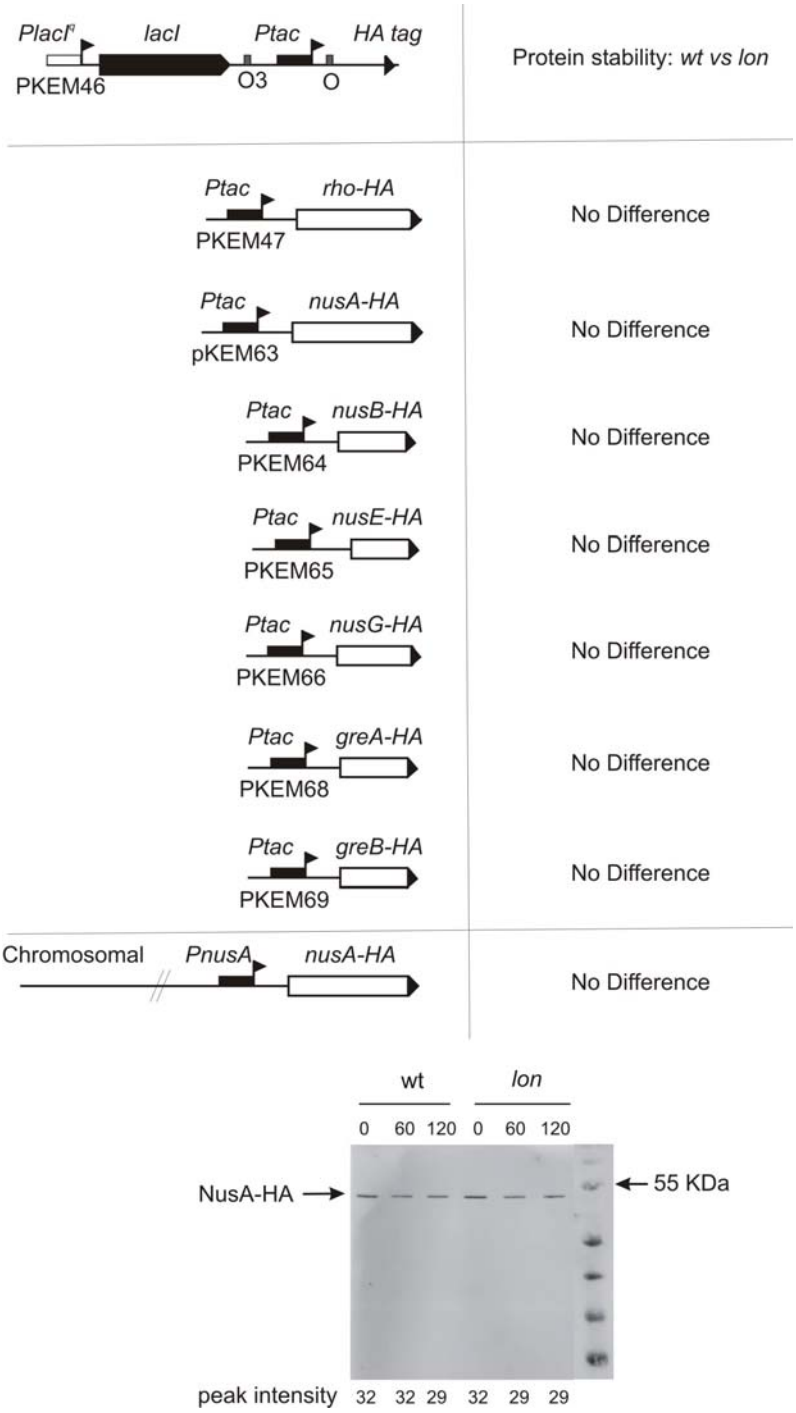


Figure. 17: (a) A series of plasmids derieved from pKEM46 (a pSC101 plasmid with lacI gene and p*tac* with the lac operators O and O3 followed by a HA tag sequence for C-terminal tagging of proteins) were constructed. The plasmids were transformed into wild-type and *lon* mutant strains. The expression of the tagged proteins was induced by 1mM IPTG for 1 hour, after which, the protein synthesis was inhibited with Spectinomycin(100µg/ml). After inhibition of protein synthesis, samples were drawn at 30 min intervals for 120 minutes. Equal OD cells from the two strains were lysed and subjected to SDS-PAGE and Western Blotting (Experimental Procedures). The signal intensities, indicative of protein amounts in the wild-type and the *lon* mutant were compared. **(b)** The stability of the chromosomal *nusA-HA* was estimated in the wild-type and *lon* mutant strains, as explained before. The peak intensities of the protein over the indicated times do not vary significantly between the wild-type and the *lon* mutant. The strains used were wild-type: S541 and *lon* mutant: S1553.

4.26 Protease Lon does not alter the stability of H-NS.

To address the possibility that protease Lon modulates repression directly by proteolysis of H-NS, a C-terminally HA tagged version of the *hns* gene was cloned into a low copy plasmid (pSC101 background) under the control of the IPTG inducible *tac* promoter. The plasmid also carries the *lacI*^{q} gene (pKEM51) (Table. 4 and Experimental Procedures). The wild-type (S541) and the *lon* mutant (S1553) strains were transformed with the plasmid and the expression of the *hns-HA* was induced by IPTG. After inhibition of protein synthesis with Spectinomycin, the stability of the protein was compared between the wild-type and the *lon* mutant by Western blotting (Experimental Procedures). The quantitative Western blot showed that the stability of H-NS does not vary significantly between the wild-type and the *lon* mutant (Fig. 18). This indicates that the Lon protease does not affect repression of *bgl* by H-NS bound to the *bgl-DRE* through degradation of H-NS.

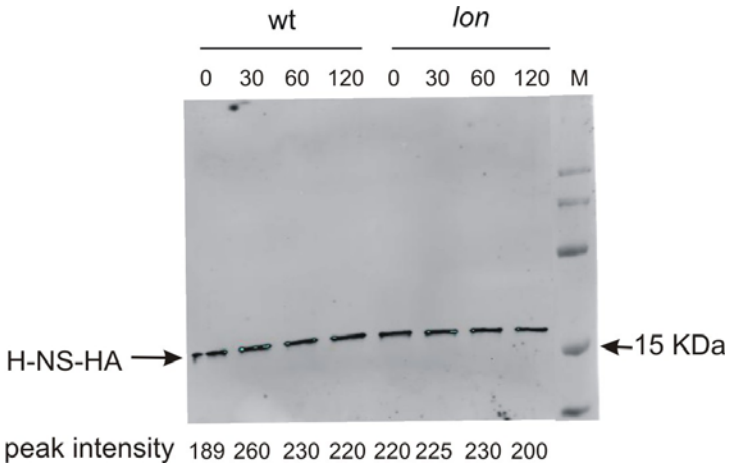


Figure. 18: Stability of H-NS protein in the wildtype and the lon mutant: pKEM51, a pSC101 derivative plasmid with *lacI*^{q} gene and p*tac* promoter with the lac operators O and O3 followed by a C-terminally tagged *hns* gene was transformed into wild-type (S541) and *lon* (S1553) mutant strains. The expression of the *hns-HA* was induced by 1mM IPTG for 1 hour, after which, the protein synthesis was inhibited with Spectinomycin (100µg/ml). After inhibition of protein synthesis, samples were drawn at 30 min intervals for 120 minutes. Equal OD cells from the two strains were lysed and subjected to SDS-PAGE and Western blotting (Experimental Procedures). The signal intensities of the corresponding bands, indicative of protein amounts in the wild-type and the *lon* mutant are shown below the blot. The strains used were wild-type: S541 and *lon* mutant: S1553.

4.27 Could the Lon effect be mediated by a factor involved in mRNA processing?

Repression of *bgl* by binding of H-NS within the transcription unit is regulated 2-fold by Lon and by termination factor Rho (Dole et al., 2004b), and inactivation of Rho abrogates the Lon effect. However, the protease Lon does not degrade Rho or any of the Nus factors involved in transcription termination (Fig. 17). In addition, regulation of the *tna* system by Lon is independent of Rho, and Lon does not regulate Rho-dependent termination at *λtR1*. However, expression of the *tna-lacZ* fusion is affected by the specific ribo-endonuclease, RnaseP (Li and Altman, 2003). This may suggest that Lon targets the mRNA turnover machinery. Also, the RNA chaperone Hfq that affects the stability and translation of several RNAs (Valentin-Hansen et al., 2004), has been implicated in the regulation of *bgl* mRNA (Dole et al., 2004a). It is possible that Lon directly or indirectly modulates the activity of the RNA chaperone Hfq. Although Lon does not alter the stability of H-NS (Fig. 18), there is still a possibility that Lon affects Hha, a protein known to interact with H-NS and regulate a set of genes (Madrid et al., 2002). However at present, it is not known if Hha has a role in the regulation of *bgl*.

4.3 Post-transcriptional regulation of proU

The stability of an mRNA directly controls the rate of gene expression by limiting the number of times the message can be translated into protein. In *E.coli*, mRNA degradation is thought to begin with endonucleolytic cleavage at internal sites (Deana et al., 2005), brought about by Rnase E (Carpousis, 2007) and RnaseIII (Drider et al., 2004). RNase III specifically degrades stem-loop structures and cleave on one or both sides of the stem, usually within an internal unpaired region (Pertzev et al., 2006). Rnase E cuts the RNA in single stranded AU rich regions (Mcdowall et al., 1994), and the enzyme has been shown to preferentially bind to and cleave RNAs with a 5'-mono phosphate to those with tri-phosphate (Mackie, 2000). This 5'-end dependency of Rnase E makes the initial cleavage of an RNA rate limiting, since the 5' end of the mRNA carries a tri-phosohate (Celesnik et al., 2007). Thus, owing to the 5'- end dependency of Rnase E, concerted action of key endoribonucleases bring about the initial cleavage of the mRNA, which is further subject to rapid degradation by RnaseE, and by the 3'-exoribonucleases like RnaseII and PnpA (Kushner, 2002).

The disparate cytoplasmic lifetimes of bacterial mRNAs are affected by characteristics of the RNA itself, usually the 5'-untranslated region (UTR). The key feature of these 5'-UTRs that enable them to protect mRNA from degradation include their ribosome-binding site (RBS). Efficient translation has been shown to increase the stability of the mRNA, by limiting the access of the ribonucleases to the processing sites (Deana and Belasco, 2005) (Arnold et al., 1998). Repression of translation initiation by intra or intermolecular basepairing can accelerate mRNA decay due to the Occlusion of the RBS, which would hinder translation initiation (Nahvi et al., 2002). Several noncoding RNAs (ncRNAs) have been shown to control gene expression by base-pairing at or near the RBS of target mRNAs (Gottesman, 2005). Further, the ncRNAs in many cases are thought to be delivered to their mRNA targets by the RNA chaperone Hfq (Valentin-Hansen et al., 2004). The following section is aimed at understanding the post-transcriptional regulation of *proU*. To see if relevant factors like the translation initiation, H-NS, Hfq, and endoribonucleases like RnaseE and RnaseIII affect the stability of the *proU* message (summarized in Figure. 19)

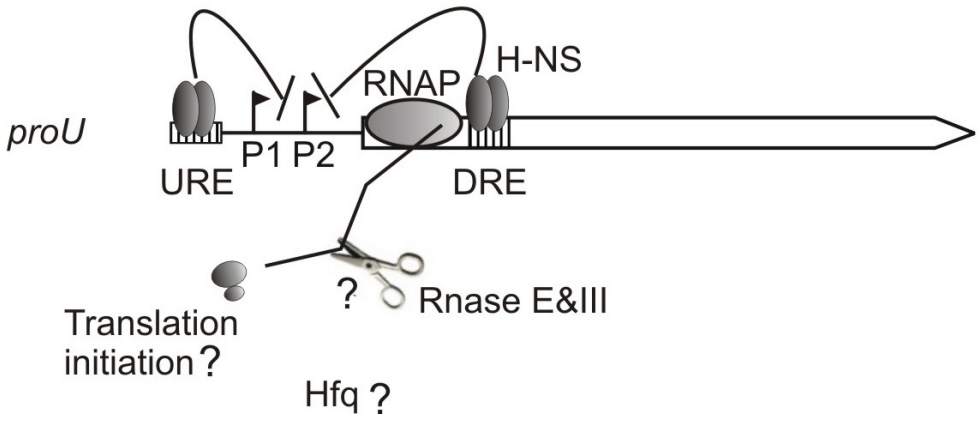


Figure. 19: Post-transcriptional processing of *proU* mRNA: comparison of transcriptional and translational fusions of *proU* to *lacZ* suggests that *proU* is subject to post-transcriptional processing (see below). H-NS bound to the *proU URE* and *DRE* inhibits transcription initiation. It remains to be seen if H-NS bound to the *proU-DRE* affects the stability or the translatibility of the *proU* RNA. It is not known if the translation of *proU* is subject to regulation. If the process requires the action of the RNA chaperone Hfq is not known. Involvement of specific endoribonucleases like Rnase E or RnaseIII in processing of the *proU* message is to be determined.

4.31 proU is subject to post-transcriptional osmoregulation.

The expression of a transcriptional *proU-lacZ* fusion, in which the *proU* promoter is flanked by the URE and DRE, increases 20-fold, from low to high osmolarity (Fig. 8a). Likewise, A translational *lacZ* fusion that carries the *proU* promoter flanked by the URE and the DRE was regulated 80-fold (from low to high osmolarity) (Fig. 20a). However, the expression of transcriptional *lacZ* fusion that carries the *lacUV5* promoter and the *proU*-DRE, was not osmoregulated (Fig. 8c), while the respective translational fusion was osmoregulated. The expression increased approximately 4-fold from low to high osmolarity (Fig. 20b). It is important to note that, unlike the *proU* promoter, the *lacUV5* promoter does not respond to an increase in osmolarity with an increase in activity, in fact it is reported to have a reduced activity at high osmotic conditions (Lee et al., 2004). These data suggest that expression of *proU* is subjected to post-transcriptional osmoregulation.

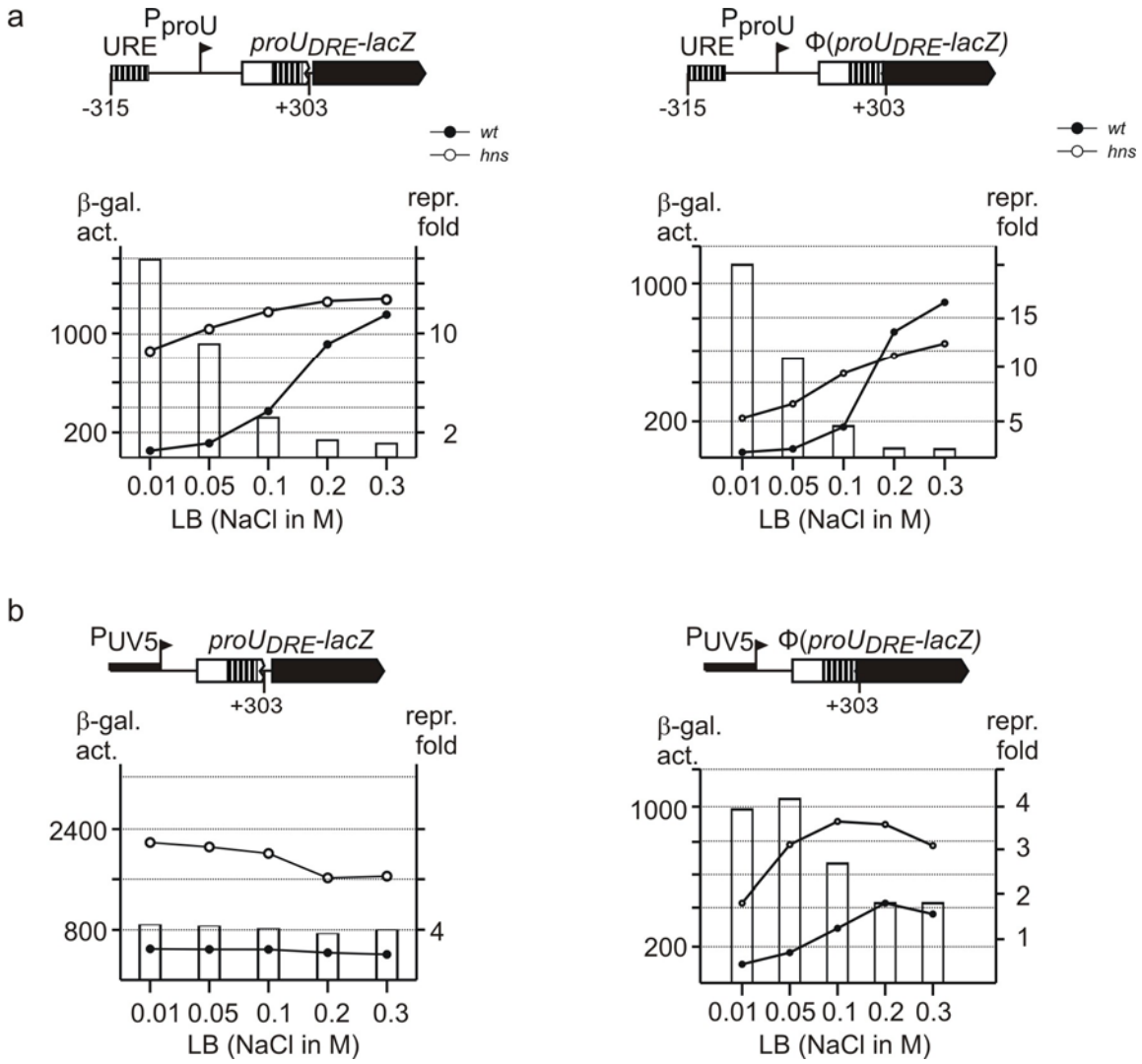


Figure 20. *proU* is subject to post-transcriptional osmoregulation´. The expression of transcriptional and translational *proU*-URE, *proU*-promoter, *proU*-DRE, *lacZ* fusions **(a)** and of transcriptional and translational *lacUV5* promoter, *proU*-DRE, *lacZ* fusion **(b)** was analysed in the wt and *hns* mutant grown in LB media with 0.01M, 0.05M, 0.1M, 0.2M and 0.3M NaCl. The β-galactosidase value obtained is plotted against the respective salt concentration. The values measured in the wild-type are shown with filled circles, and those measured in the *hns* mutant are shown with empty circles. The fold repression by H-NS is represented by the vertical bars. Strains used are shown in the order *proU*-URE/promoter *proU*/*proU*-DRE/*lacZ* transcriptional and translational fusion **(a)** *wt*: S2501, S2608. *hns:* S3128, S3130 and the *lacUV5*promoter/*proV*-DRE/*lacZ* transcriptional and translational fusion **(b)** *wt*: S2137, S3252. *hns*: S2137, S3252.

4.32 The post-transcriptional osmoinduction of proU is reduced in hns mutants.

H-NS bound to the *proU*-URE and DRE inhibits transcription initiation. H-NS is also reported to bind RNA and destabilize it (Brescia et al., 2004). Thus, H-NS could also be involved in the post-transcriptional regulation of the *proU* RNA. The expression of the transcriptional *proU-lacZ* fusion, in which the *proU* promoter is flanked by the URE and the DRE, is repressed 16-fold by H-NS at low osmolarity, while at high osmolarity H-NS has no significant effect (Fig. 20a). The respective translational fusion is likewise repressed 18-fold by H-NS at low osmolarity, and not significantly regulated at high osmolarity (Fig. 20a). However, while there is only a 1.5-fold increase in the expression of the transcriptional fusion in the *hns* mutant (Figures. 9a and 20a), there is a 2-fold increase in the expression of the translational fusion with the increase in osmolarity (Fig. 20a), suggesting a 2-fold post-transcriptional regulation. A similar result was obtained with the transcriptional and translational *lacUV5* promoter driven *proU-lacZ* fusions. In the *hns* mutant the expression of the translational *lacZ* fusion increased 2-fold over the range of osmolarity (Fig. 20b), while expression level of the respective transcriptional fusion did not respond to an increase in osmolarity in the wild-type or the *hns* mutant (Figures. 9c and 20b). Taken together, the post-transcriptional regulation of *proU*, which is 4-fold in the wild-type, is 2-fold in the *hns* mutant. This suggests that H-NS may to some extent be involved in the post-transcriptional osmoregulation of *proU*.

4.33 Hfq is required for efficient expression of proU.

The observation that the osmotic induction of *proU* is regulated post-transcriptionally prompted us to see if the RNA chaperone Hfq has a function in the regulation (Geissmann and Touati, 2004; Valentin-Hansen et al., 2004). For this, the expression of the transcriptional and the translational *proU*-DRE-*lacZ* fusions, which carry the *proU* promoter flanked by the URE, or the *lacUV5* promoter, was compared between the wild-type and the *hfq* mutant (Fig. 21).

The expression of the transcriptional *proU-lacZ* fusion, in which the *proU* promoter is flanked by the URE and the DRE, is about 4-fold lesser in the *hfq* mutant in comparison to the wild-type at low osmolarity, while at high osmolarity the *hfq* effect was significantly reduced to about 2-fold (Fig. 21a). The expression of the

respective translational fusion was similarly affected in the *hfq* mutant, the *hfq* mutant showed 5-fold lesser expression at low osmolarity, and 2-fold lesser at high osmolarity in comparison to the wild-type (Fig. 21a). The induction of *proU* over the range of osmolarity was significantly higher in the *hfq* mutant, the transcriptional fusion showed a 50-fold induction (as opposed to the 20-fold induction in the wild-type), while the translational fusion showed a 250-fold induction in the *hfq* mutant (as opposed to the 80-fold induction in wild-type) (Fig. 21a). A similar result was obtained with transcriptional and translational *lacUV5* promoter driven *proU-lacZ* fusions. The translational fusion showed a 4 and 5-fold increase in expression in response to an increase in osmolarity in the wild-type and *hfq* mutant respectively, and the *hfq* mutant showed a 3-fold reduced expression in comparison to the wild-type (Fig. 21b). The respective transcriptional fusion did not respond to an increase in osmolarity in the wild-type or the *hfq* mutant, but the expression level was about 3-fold lower in the *hfq* mutant (Fig. 21b). Taken together, the expression of both the transcriptional and translational fusions driven by the *proU* as well as the *lacUV5* promoters is about 4 to 5-fold lesser in the *hfq* mutant in comparison to the wild-type at low osmolarity, while the difference in expression is reduced at high osmolarity. This suggests that the RNA chaperone Hfq is required for the efficient expression of *proU* operon at low osmolarity, and to a lesser extent at high osmolarity.

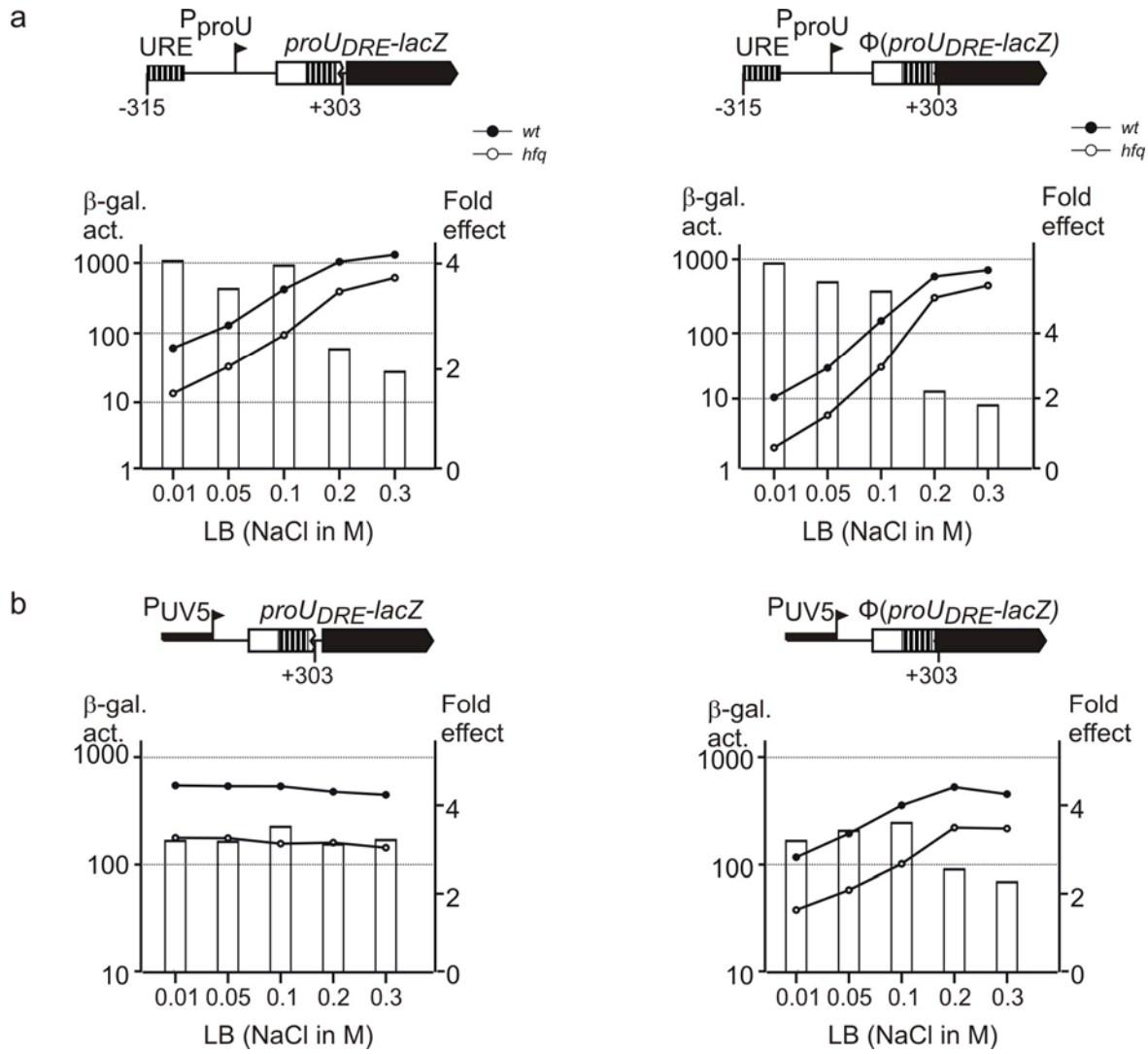


Figure 21. Hfq is required for efficient induction of *proU*: the expression of chromosomal *proU*-URE/promoter *proU*/*proU*-DRE/*lacZ* transcriptional and translational fusions **(a)** and the *lacUV5*promoter/*proU*-DRE/*lacZ* transcriptional and translational fusion **(b)** was analysed in *wt* and *hfq* mutant Cells grown in LB media with 0.01M, 0.05M, 0.1M, 0.2M and 0.3M NaCl. The β-galactosidase value obtained is plotted against the respective salt concentration. The values measured in the wild-type are shown with filled circles, and those measured in the *hfq* mutant are shown with empty circles. The vertical bars represent the fold effect in the *hfq* mutant (the fold reduction in the expression in comparison to the wild-type). Strains used are shown in the order *proU*-URE/promoter *proU*/*proU*-DRE/*lacZ* transcriptional and translational fusion **(a)** *wt*: S2501, S2608. *hfq:* S2977, S2979 and the *lacUV5*promoter/*proV*-DRE/*lacZ* transcriptional and translational fusion **(b)** *wt*: S2137, S3252. *hfq*: S3352, S3254.

4.34 Deletion analysis shows that the complete proU-DRE is necessary for post-transcriptional osmoinduction

The *proU* operon is regulated post-transcriptionally and by pleiotrophic regulators like H-NS and Hfq. To see if there is a specific feature in the sequence that brings about post-transcriptional osmoregulation, a series of in-frame deletions of the *proU-DRE* translational fusion to *lacZ* was constructed (Fig. 21). The plasmids pKEM58, pKEM60 and pKEM62 (Table. 1) carry the *lacUV5* promoter, the *proU*-DRE and the *lacZ* gene translationally fused into the *proV* reading frame. Plamid pKEM56 carries the sequence from +1 to +93 (with *lacZ* fused to the 11th amino acids of *proV*), plasmid pKEM60 carries the sequence from +1 to +144 (28 amino acids) and plasmid pKEM62 carries the sequence from +1 to +222 (54 amino acids). Further, another plasmid pKES139 was used, which carries a replacement of sequences from position +1 to +60 including the *proV* ribosome binding site, by the Shine-Dalgarno sequence of the *lacZ* gene. This plasmid was used to analyze whether the ribosome binding site is important for the post-transcriptional regulation (Fig. 21 and Table 1). The plasmids were integrated into the chromosomal *attB* site (Experimental procedures) and the expression levels directed by these deletion constructs were compared with that of the translational *lacUV5* promoter, *proU* (+1 to +303), *lacZ* fusion carrying the complete *proU-DRE* (Fig. 21). The comparison revealed that the complete *proU-DRE* is necessary for the post-transcriptional osmoinduction (Fig. 22). While the translational *lacUV5* promoter, *proU-DRE*(+1 to +303), *lacZ* fusion was 4-fold osmoregulated, the deletion constructs were not osmoregulated (Fig. 22). Interestingly, upon replacement of the *proU* 5'-UTR with the *lacZ* Shine-Delagarno sequence, a 1.6-fold osmoregulation was retained (Fig. 22). Taken together these data suggest that the post-transcriptional osmoinduction is not based on regulation of translation initiation. Thus, it was next analyzed whether the *proU* mRNA is processed by RNases and whether its stability is osmoregulated. Processing of the mRNA would affect expression of the translational *proU-lacZ* fusion, but not necessarily that of the transcriptional *proU-lacZ* fusion, which in a processed but stable form would retain the *lacZ* Shine-Dalgarno sequence.

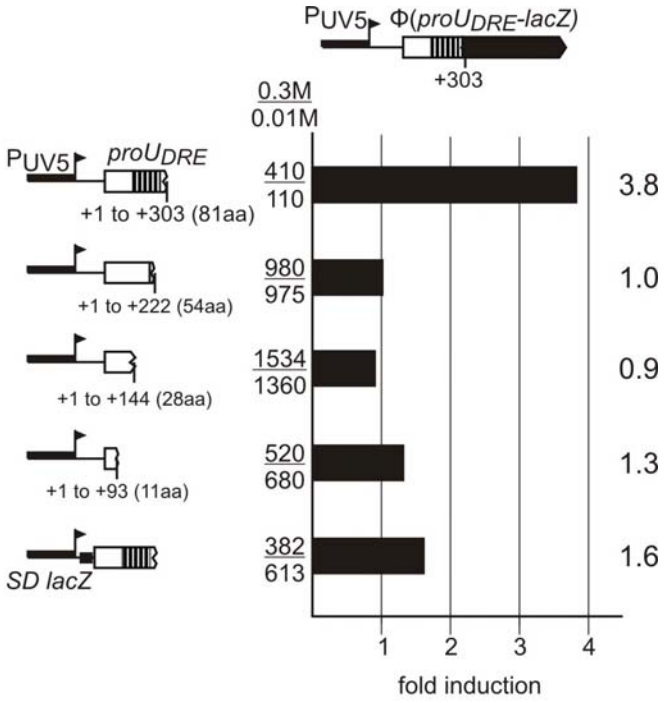


Figure 22. Deletion analysis of *proU*: expression of chromosomal the *lacUV5*promoter/*proV*-DREΦ*lacZ* translational fusion in *wt* was compared to that of the *proU* +1 to +222, +1 to +144, +1 to +93 and *lacUV5* promoter/ (Δ+1 to+60) *SDlacZ proU-DRE* Φ*lacZ* construct. Cells were grown in LB media with 0.01M, and 0.3M NaCl. The numbers in the left are the β-galactosidase values measured at 0.3M NaCl/ the values at 0.01M NaCl, expressed in Miller units The bars indicate the fold induction; the ratio of the expression of the respective constructs under high osmolarity (0.3M NaCl) to that under low osmolarity (0.01M NaCl). The fold induction is shown in the right. Stranis used were: S3252 (+1 to+303), S3254 (+1 to +222), S3142 (+1 to +144), S3140 (+1 to +93), and S3083 (Δ+1 to+60) *SDlacZ proU-DRE* Φ*lacZ*.

4.35 The half-life of the proU mRNA is not significantly altered

The post-transcriptional osmoregulation is not based on osmoregulation of translation initiation. To check if the stability of the *proU* RNA is altered by osmolarity, the decay kinetics of the *proU*-mRNA at low and high osmolarity was analyzed in wild-type and in *hns* and *hfq* mutants. For this, the *lacUV5* promoter-*proU-DRE* (+1 to +303) fragment, followed by the *rrnBT1* terminator was cloned into a pBR derived high copy plasmid, pKEM72 (Fig. 23 and Table. 1). The size of the *proU* transcript encoded by this construct is 350 bases and thus easily detectable in Northern blots. A *proU* specific RNA probe that matches the *proU* sequence from +184 to +303 (schematically shown is Fig. 23)

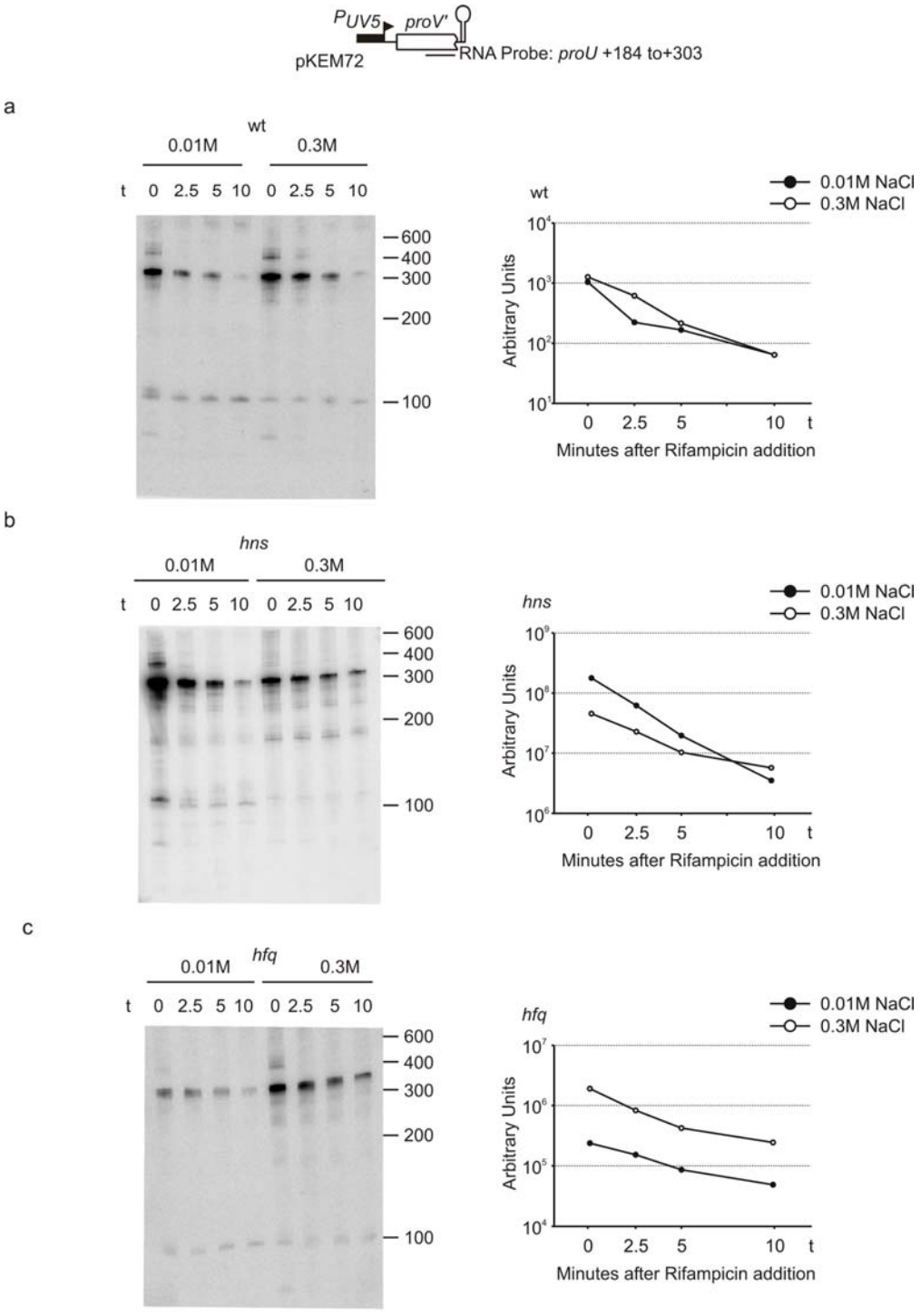


Figure 23. The decay kinetics of the proU mRNA under low and high osmolarity conditions in wild type, *hns* and *hfq* mutants: the *lacUV5*promoter/*proU-DRE* followed by *rrnB*T1 terminator expressed from a high copy plasmid (pKEM72) used for the northern analysis is shown along with the probe used for hybridization, the position at which the probe matches with the *proU* mRNA is also indicated. Transformants of **(a)** *wt*, **(b)** *hns*, and **(c)** *hfq* mutant strains were grown at 37°C in low (0.01M NaCl) and high (0.3M NaCl) osmolarities to an OD of 0.5. Transcription was inhibited with 100µg/ml of Rifampicin. Aliquots were drawn at 0, 2.5, 5 and 10 minutes after Rifampicin addition. Total RNA isolated and 10µg of the RNA run on 6% gel with Urea (Experimental procedures), blotted and hybridized. The signal intensities of the full length obtained from the wild type and the mutants, at low and high osmolarities are plotted against the time after Rifampicin addition. The strains used are listed in the order *wt*, *hns*, and *hfq* mutant; S3077, S3464, and S3466.

Transformants of the wild-type, the *hns* and *hfq* mutants with this plasmid were grown in medium of low (0.01M NaCl) and high (0.3M NaCl) osmolarity to an OD_{600} of 0.5. Then transcription was inihibted by the addition of Rifampicin (100µg/ml), and total RNA was isolated from cells prior to Rifampicin addition (0 minutes) and 2.5, 5, and 10 minutes after inhibition of transcription. Ten micrograms of the total RNA was separated on a 6% denaturing acrylamide gel, followed by Northern blotting and hybridization (Experimental procedures). The blot was hybridized with a *proU* specific RNA probe, and the signal intensities were quantified with ImageQuant™ (GE Healthcare). The following results and interpretations are based on a single experiment, and must be confirmed with repetition; hence they are to be treated as indications rather than facts.

The Northern analysis shows that the half life of the *proU* mRNA is not altered by an increase in the osmolarity in the wild type and *hfq* mutant (Fig. 23a and c). Plotting of the quantified signal intensities of the RNA against time (after inhibition of transcription with Rifampicin) yielded fairly linear decay kinetics for the *proU* mRNA in the wild-type at low and high osmotic conditions (Fig. 23a). The RNA stability was likewise not significantly changed in the *hfq* mutant (Fig. 23c). However, in the *hns* mutant the decay kinetics of the *proU* message seems to be osmoregulated (Fig 23b). The slope of the plotted decay kinetic observed under low osmolarity was slightly steeper in comparison to that observed under high osmolarity (Fig. 23b). This observation corelates with the expression of the respective *proU-lacZ* fusion (Fig. 20), suggesting that H-NS is involved in the post-transcriptional regulation of *proU*.

Interestingly, the northern analysis also revealed a truncation product of around 100 bases along with the full length *proU* mRNA of 350 bases (Fig. 23). This truncation product was apparent in the wild-type as well as the *hns* mutant at low osmolarity under steady state condition (Fig. 23a and b; 0 minutes at 0.01M). To analyze this further, the RNA isolated prior to Rifampicin addition (0 minutes, corresponding to the steady state level of the RNA) from the wild-type, *hns* and *hfq* mutant cells grown at low and high osmolarities, from the stability experiment was subjected to northern blotting and hybridization as explained before. The blot was hybridized with the *proU* RNA probe.

The analysis of the steady state level of the *proU* mRNA reveals the full length (350 bases) and a truncated product (approximately 100 bases) in the wild-type, and *hns* mutant under low osmolarity (Fig. 24). Also, at low osmolarity, the amount of the full length RNA is 15-fold higher in the *hns* mutant in comparison to the wild-type, while the *hfq* mutant shows a 3-fold reduction in the full length RNA in comparison to the wild-type (Fig. 24). Under high osmolarity conditions the difference between the amount of the full length RNA in the wild-type and the *hns* mutant is reduced to 1.5-fold (as opposed to the 15-fold difference at low osmolarity) (Fig. 24). Also at high osmolarity, the amount of the full length product was only 1.5-fold lesser in the *hfq* mutant in comparison to the wild type (Fig. 24). This is in agreement with the regulation of the *proU-lacZ* fusion, where in the *hfq* mutant the values were 5-fold lower than that of the wild-type at low osmotic condition, and merely 1.5-fold different at high osmolarity (Fig. 21). It is also evident from the Northern blot that in the wild-type, albeit a 1.5-fold increase in the full length product, there was no quantifiable truncation detected at high osmolarity (Fig. 24). Likewise, in the *hns* mutant there was no quantifiable truncation detected at high osmolarity and in the *hfq* mutant, the expression level was too low to detect the truncations at low or high osmolarity (Fig. 24). These data indicate that the processing of *proU* mRNA possibly happens more efficiently at low osmotic conditions, this notion however contradicts the fact that the decay kinetics of the *proU* mRNA does not significantly change between low and high osmolarity (Fig. 23a). However, the truncations strongly suggest that the *proU* mRNA is subjected to processing by the RNAses. This is analyzed further in the following section.

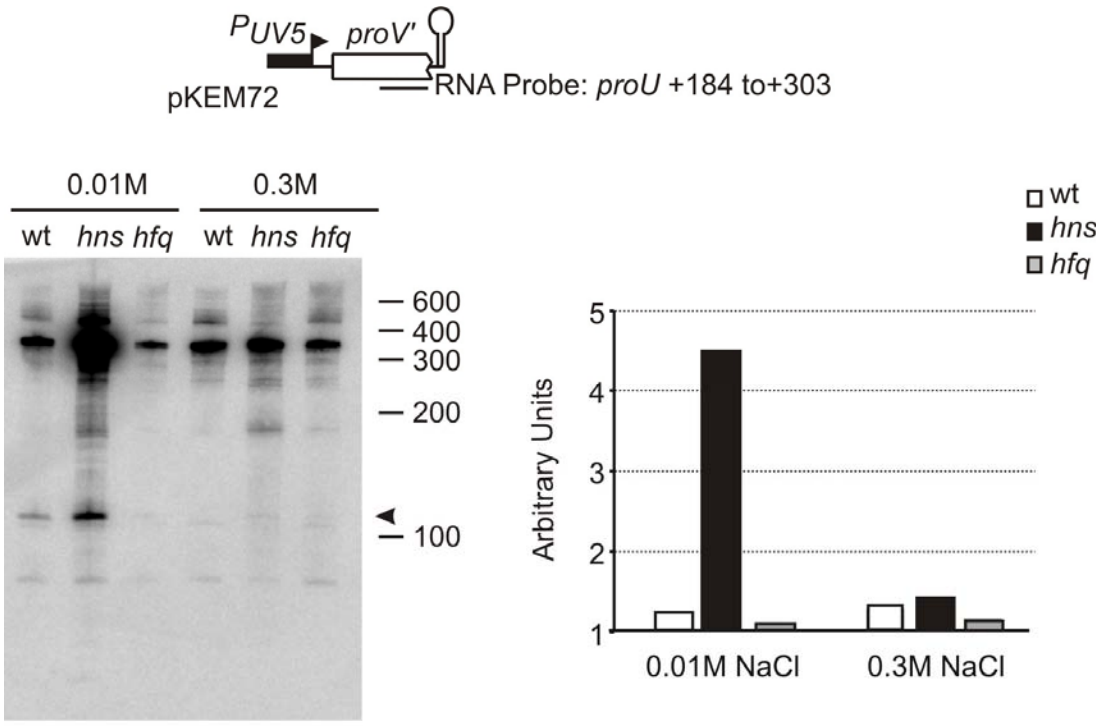


Figure 24. A truncated product is apparent in wild type, *hns* and *hfq* mutants : the *lacUV5*promoter/*proU-DRE* followed by *rrnB*T1 terminator expressed from a high copy plasmid (pKEM72) used for the northern analysis is shown along with the probe used for hybridization, the position at which the probe matches with the *proU* mRNA is also indicated. Transformants of *wt*, *hns*, and *hfq* mutant strains were grown at 37°C in low (0.01M NaCl) and high (0.3M NaCl) osmolarities to an OD of 0.5. Total RNA isolated and 10µg of the RNA run on 6% gel with Urea (Experimental procedures), blotted and hybridized with the *proU* specific probe. The expected full length RNA is around 350 bases. A specific truncation observed at around 100 bases is marked with the arrow head. The signal intensities of the full length observed in the wild type and the mutants, at low and high osmolarities are plotted as Arbitrary Units. The band intensities were quantified with ImageQuant™ (GE Life Sciences). The strains used are listed in the order *wt*, *hns*, and *hfq;* S3077, S3464, and S3466.

4.36 RNAse III and RNAse E are involved in processing the proU mRNA.

Northern analysis suggests that *proU* mRNA is subject to processing by RNAses (Fig. 24). To identify the enzyme involved in processing the *proU* mRNA, the steady state level of the *proU* mRNA was analyzed in the wild-type, the RNAse E (*rne*), and in Rnase III (*rnc*) mutants, grown in low and high osmolarity conditions. Since RNAse E is an essential protein, the temperature sensitive *rne-3701* allele encoding a misfunctional RNAse E protein at 44°C (Table. 3, Experimental procedures) was used, and all the cultures were grown to exponential phase at 28°C, then shifted to 44°C for 20 minutes before RNA isolation. Since the *rnc rne* double mutant failed to grow at low or high osmolarity, it was grown in LB (0.08 M). Total

mRNA isolated from the respective transformants with pKEM72 (*lacUV5* promoter, *proU-DRE*, *rrnBT1*) was subjected to Northern analysis and hybridization with the *proU* (+184 to +303) RNA probe.

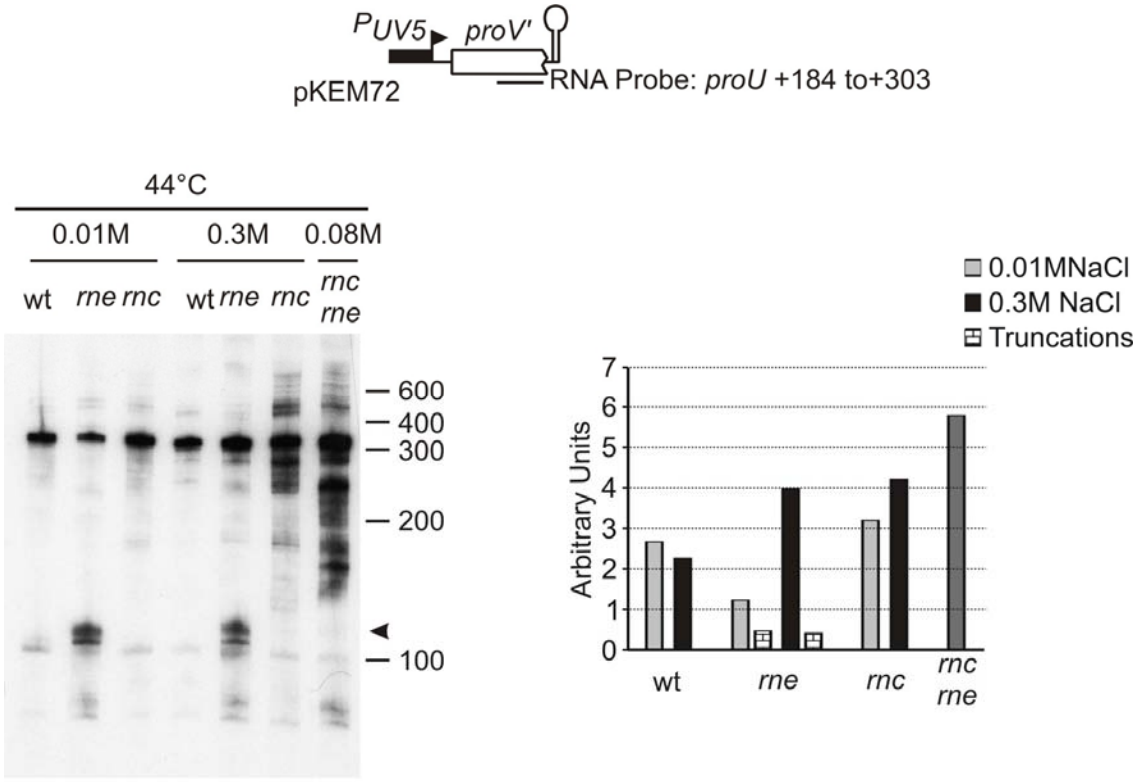


Figure 25. The *proU* mRNA is subjected to processing by RNAseIII and RnaseE: the *lacUV5*promoter/*proU-DRE* followed by *rrnB*T1 terminator expressed from a high copy plasmid (pKEM72) used for the northern analysis is shown along with the probe used for hybridization, the position at which the probe matches with the *proU* mRNA is also indicated. Transformants of *wt*, *rne*, *rnc* and *rnc rne* double mutant strains were grown at 28°C in low (0.01M NaCl) and high (0.3M NaCl) osmolarities to an OD of 0.5, and shifted to 44°C for 20 mins. Total RNA isolated and 10µg of the RNA run on 6% gel with Urea (Experimental procedures), blotted and hybridized with the *proU* specific probe. The expected full length RNA is around 350 bases. A specific truncation observed at around 100 bases is marked with the arrow head. The signal intensities of the full length product (and the truncation observed in the *rne* mutant) obtained from the wild type and the RNAse mutants, at low and high osmolarities are plotted as Arbitrary Units. The band intensities were quantified with ImageQuant™ (GE Life Sciences). The strains used are listed in the order *wt*, *rnc*, *rne*, and *rnc rne;* S2226, S3701, S2106 and S3769.

The northern analysis confirmed that the *proU* mRNA is subject to processing and that the RNAseIII and RNAse E are involved in the processing (Fig. 25). Under low osmotic conditions (0.01M) the full length product and a faint truncated product was detected in the wild-type, the *rne* mutant showed a decrease in the amount of the full length product in comparison to the wild-type; but a dramatic accumulation of the truncations was very evident. The *rnc* mutant showed elevated amount of the full length product in comparison to both the wild-type and *rnc* mutant, intriguingly there

was no detectable truncation in the *rnc* mutant (Fig. 25). These data suggests that the full length product is stabilized in the *rnc* mutant and that RNAse III possibly catalyzes the initial processing of the *proU* mRNA. At high osmolarity, only the full length RNA was detected in the wild type, the *rne* mutant showed an increase in the full length amounts in comparison to that at low osmolarity. Concomitantly, the ratio of the full length to truncation in the *rne* mutant was elevated at high osmolarity (Fig. 25). No truncations were detectable in the *rnc* mutant (Fig. 25). The *rne rnc* double mutant behaves similar to the *rnc* single mutant, with no *rnc* specific truncation (100 bases) visible (Fig. 25). This confirms the notion that RNAse III brings about the initial processing of the *proU* mRNA, which is then subsequently degraded in RNAse E dependent manner. The fact that the amount of accumulated truncations in the *rne* mutant is higher at low osmolarity suggests an interesting possibility that the processing of the *proU* mRNA by RNAse III is more effective at low osmolarity (see Discussion for further details).

4.37 RNAse III processes the proU mRNA at position +217.

To map the position of the RNAseIII cleavage site in the *proU* RNA, primer extensions were performed from total RNA isolated of transformants of the wild-type, and the *rne*, *rnc*, and *rne rnc* mutants with plasmid pKEM72 (*lacUV5* promoter/*proU-DRE*/*rrnBT1*). The wild type and the *rnc* and *rne* single mutante were grown in LB of low and high osmolarity medium, while the double *rnc rne* mutant was grown in LB with 0.08M NaCl. The RNA was subjected to primer extension using an end labeled primer (S420) that maps at *proU* positions (+281 to+303), and the samples were separated on a sequencing gel next to a sequence ladder generated with the same primer, using pKEM72 as template (Fig. 26).

The primer extension analysis corroborate the results from the Northern hybridization and map the processing site of the *proU* mRNA to a G residue at position +217 relative to the transcription start (Fig. 26). As observed in the Northern analysis, at low osmolarity, a weak processing signal was detectable in the wild-type, in the *rne* mutant the amount of the full length extension product was lesser in comparison to the wild-type, but there was a dramatic accumulation of the processed product (Fig. 26). This processing maps to a G residue at position +217, as deduced from the sequence ladder (Fig. 27). In the *rnc* mutant the processing at +217 was not

detectable, and only the full length signal was visible (Fig. 26). At high osmolarity, the processing signal was hardly detectable in the wild-type, and it was considerably reduced in the *rne* mutant, as compared to the signal at low osmolarity (Fig. 26). The *rnc* mutant showed an accumulation of the full length product alone (Fig. 26). The rne rnc mutant also lacked the processing signal (Fig. 26). The analysis also showed another truncation mapping to a T residue at position +245 (Fig. 26). However, this product was visible in all the strains, grown under both high and low osmolarity conditions. Importantly, this truncation was also visible in the *rnc* and *rnc rne* double mutants. Hence, this is not the processed product of the specific initial endonucleolytic processing of the *proU* mRNA by RNAse III at position +217, and is possibly the result of secondary mechanism of processing of the *proU* mRNA (See Discussion for details).

Taken together, the abundance of the truncated product in the *rne* mutant at low osmolarity conditions in comparison to high osmolarity, the stabilization of the full length product in the *rnc* mutant at high osmotic conditions, and the fact that the *rnc rne* double mutant lacks the RNAse III specific cleavage product; evident from both the Northern and primer extension analyses (Fig. 25 and 26) suggests that the initial cleavage of the *proU* mRNA at position +217 is mediated by RNAse III, and that the RNAse III mediated cleavage possibly happens at a higher rate at low osmolarity. Presumably the processed RNA is then further degraded by RNase E.

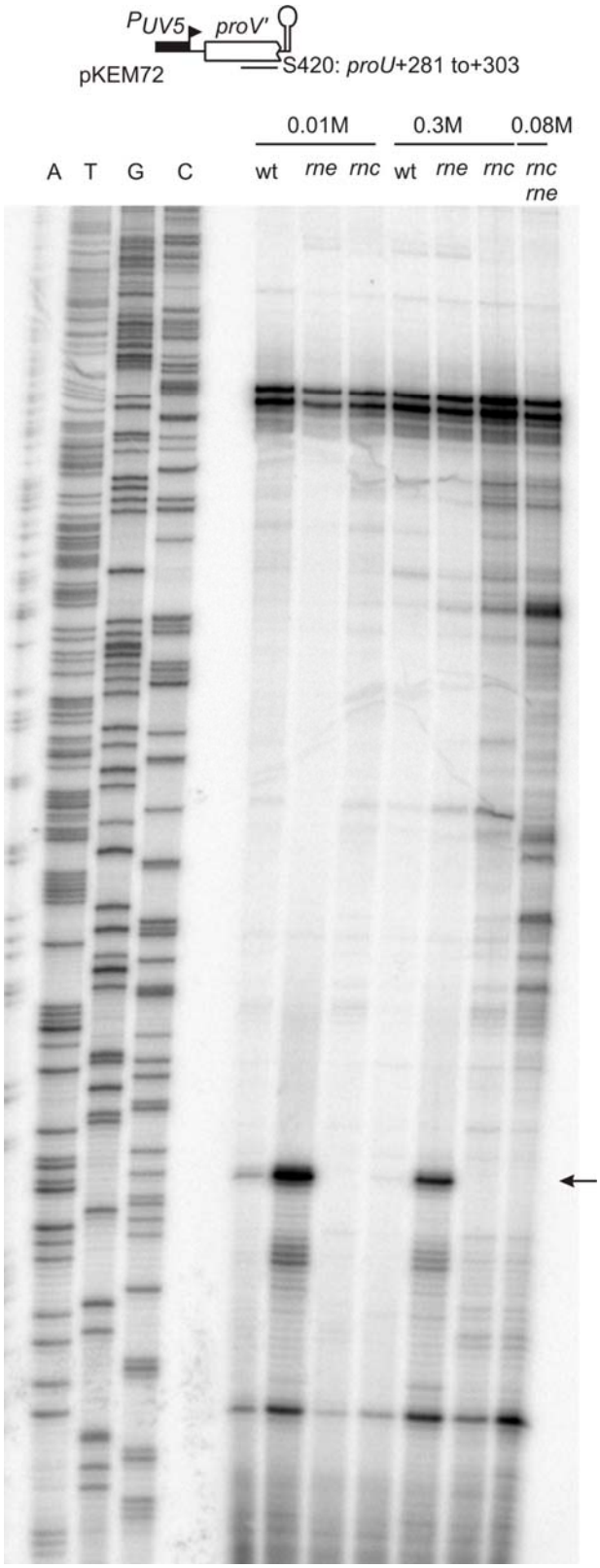


Figure 26. primer extension to map the specific processing of the *proU* mRNA. the *lacUV5*promoter/*proU-DRE* followed by *rrnB*T1 terminator expressed from a high copy plasmid (pKEM72) is shown along with the primer S420 used primer extension, the position at which the primer matches with the *proU* mRNA is also indicated. Transformants of *wt*, *rne*, *rnc* and *rnc rne* double mutant strains were grown at 28°C in low (0.01M NaCl) and high (0.3M NaCl) osmolarities to an OD of 0.5, and shifted to 44°C for 20 mins. Total mRNA isolated and 5µg of the RNA was subject to primer extension with end labeled primer using Thermoscript reverse transcriptase (Invitrogen). The extension products were run on 6% sequencing gel with Urea along with the sequencing ladder generated using the same end labeled primer and pKEM72 as the template. (Experimental procedures). The RNAse III specific truncation observed in the northern analysis at around 100 bases is mapped to a G residue at position +217 (marked with the arrow), and shown below. Another truncation product is also apparent from the gel. It maps to a T residue at +245. However, note that this truncation is visible in the wild type and all the mutants under both high and low osmotic conditions. The strains used are listed in the order *wt*, *rnc*, *rne*, and *rnc rne;* S2226, S3701, S2106 and S3769.

CATTGAAGAAGGCGAG
GTAACTTCTTCCGCTC
+225

4.38 RNAse III processes the proU mRNA in a stretch of highly conserved sequence.

The post-transcriptional regulation of the *E.coli proU* operon is thus far unreported. However, the transcriptional regulation of the *proU* operon by H-NS in *E.coli* and *Salmonella* is analogous. This prompted us to look for conservation of *proU* sequence elements within the *Enterobacteriaceae*. To estimate the conservation of *proU* sequence elements within the *Enterobacteriaceae*, the sequence of the *E.coli* K12 *proU* operon from +1 (transcription start) to +1263 (the end of the *proV* coding region) was used as a blast query against the genomes of the sequenced members of *Enterobacteriaceae*, using the web tool available from NCBI at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. The sequence of different strains of the same species was not significantly different. Therefore, the *proU* sequence alignment is shown only for representative of the species *Escherchia coli* and *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, *Klebsiella pneumoneae*, *Erwinia carotovora* and *Photorabdus luminescens* in Figure 27 (which is presented at the end of the chapter at pages 75 to77, to maintain continuity of the text). The sequence alignment revealed numerous nucleotide polymorphisms (SNPs). Strikingly, a small window between +194 to +308, relative to the transcription start (+1) of the *E.coli proU* sequence is highly conserved in all species (boxed in Fig. 27). This stretch of sequence is identical in *E .coli* (and *S.flexneri*) and *S.typhimurium*, although there is a higher incidence of SNPs in other regions of the sequence of the two species (Fig. 27). Further, this window also shows a high homology with the sequences form the more distantly related *E.carotovora* and *P.luminescens* (Fig. 27). Furthermore, the sequences between +211 to +223 are identical in all 5 species. (The specific processing of the *proU* message by RNAseIII happens at +217). This suggests that the sequence important for post-transcriptional regulation of *proU* is highly conserved among *E.coli* and related bacteria.

Since the sequence around the RNAse III processing site at +217 is identical in *E.coli, S.flexneri, S. enterica* serovar Typhimurium*, E.carotovora* and *P.luminescens,* the predicted secondary structure of the RNA around the processing site of the *E.coli proU* operon was compared with the structures predicted for the sequence from other representative members of the group. The secondary structure predictions were obtained using *mfold*, an online RNA structure prediction tool available at

http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi. Two alternate structures predicted for each of the unique sequences are shown in Figure 28. The conserved residues are represented in bold face. The processing site at the G residue at +217 is shown in red. Since the sequence between +194 to +307 is identical among *E.coli, S.flexneri,* and *S.enterica*, one set of predicted structures is shown for the three species.

Interestingly, the secondary structures look strikingly similar for the analyzed enterobacterial species (Fig. 28). The processing site at +217 lies within a stem loop structure in all of the predicted structures; it is either located at the base of the stem or in the loop (Fig. 28 compare the alternate structures). In case of the *P.luminescens* sequence the predicted structures look very similar, and the G residue at +217 forms the base of a stem loop structure (Fig. 28). The fact that the processing site at +217 is enclosed in a stem loop structure is significant in the light of the fact that RNAse III cleaves RNA at stem loop structures (Pertzev and Nicholson, 2006). The conservation of the sequence and possibly the structure around the processing site among Enterobacteria suggests a common mode of regulation of post-transcriptional osmoregulation operating in the related bacteria, with an interesting possibility of a conserved ncRNA involved in the post-transcriptional regulation of the *proU* operon in Enterobacteria (see Discussion for details).

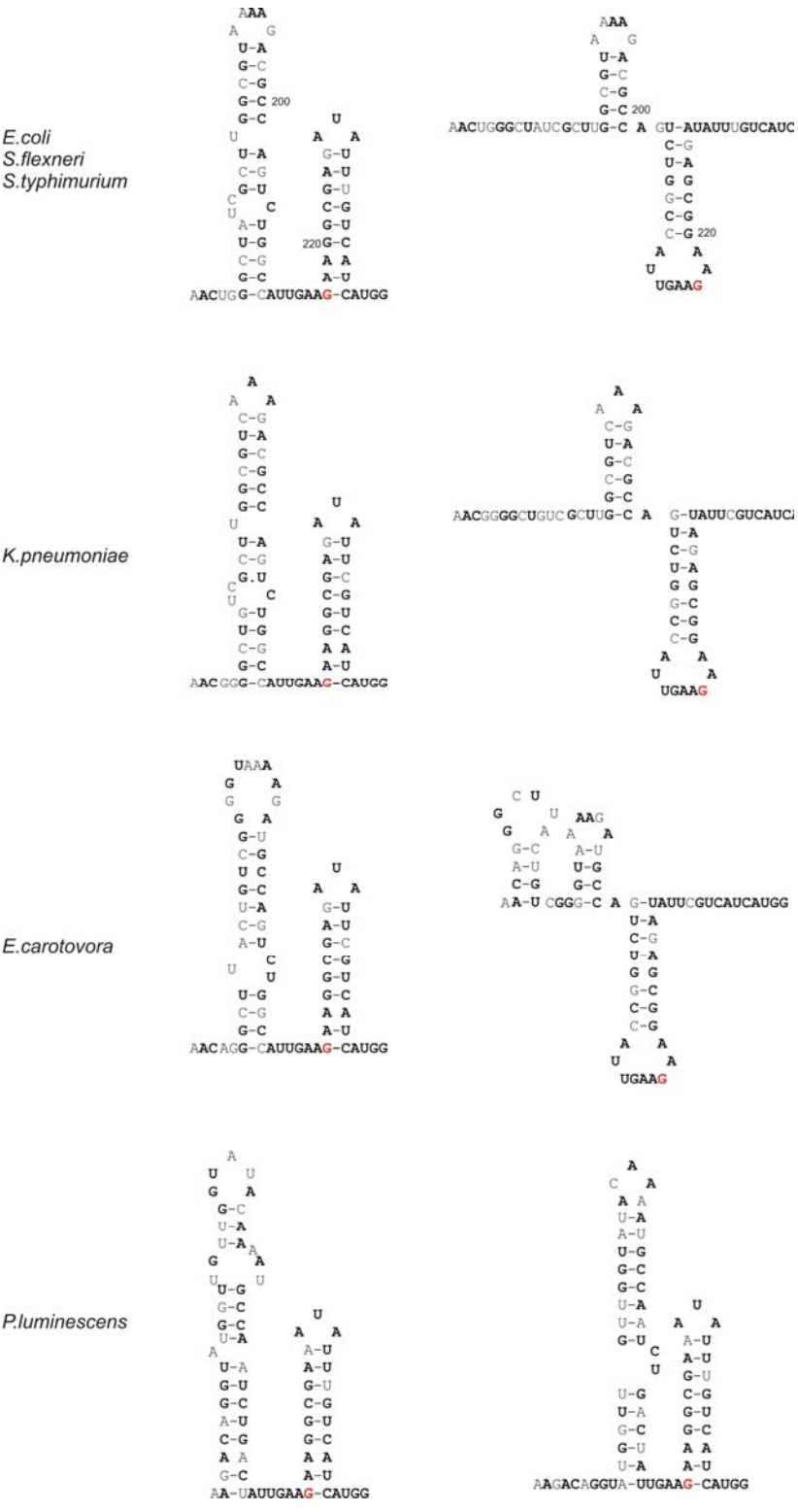
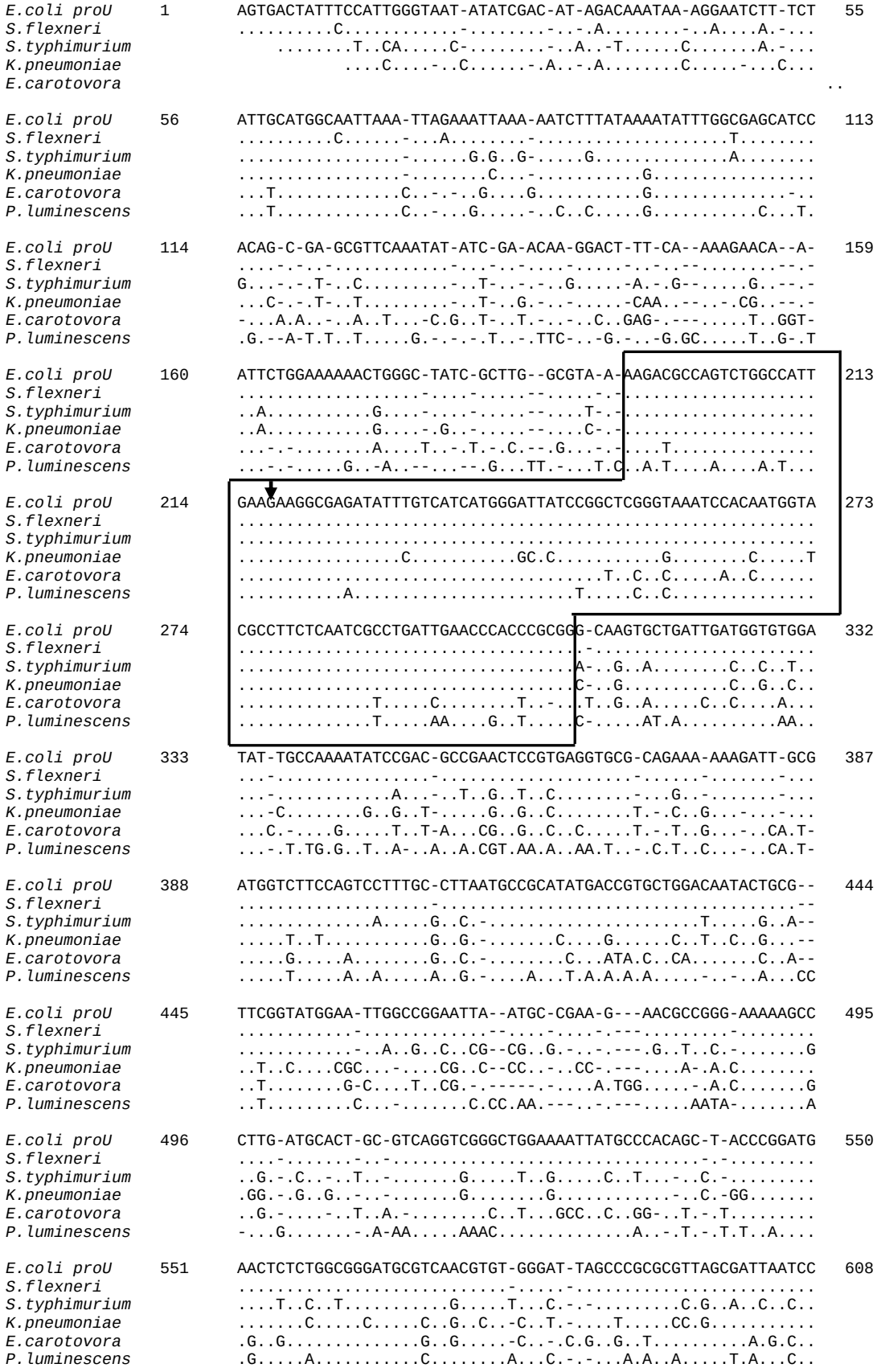


Figure. 28: Comparison of the predicted secondary structure of the highly conserved sequence around the RNAseIII processing site in the *proU* operon of selected Enterobacteria. The secondary structures of the sequence around the RNAse III specific processing site at +217 of the *E.coli proU* operon is compared with the corresponding conserved sequence (determined from the sequence alignment, Fig. 27) present in other enterobacterial species. The structure predictions were obtained using the *mfold* tool available at http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi. Two alternate structure predictions for each of the unique sequence are shown. The conserved residues are shown in bold face; the processing site at +217 (with respect to the *E.coli proU* transcription start) is marked in red.

In summary, the proU operon of *E.coli* is subject to post-transcriptional regulation. H-NS is involved in the post-transcriptional regulation. The RNA chaperone Hfq is required for expression of the *proU* operon at low osmolarity. The *proU* mRNA is processed by RNAse III and RNAse E; with RNAse III making the initial cleavage. The processing seems to happen at a higher efficiency or frequency in low osmotic conditions. The RNAse III mediated initial processing maps to a G residue at proU +217. The sequence and possibly the structural motif around the processing site are highly conserved among Enterobacteria.


E.coli proU 1 AGTGACTATTTCCATTGGGTAAT-ATATCGAC-AT-AGACAAATAA-AGGAATCTT-TCT 55
S.flexneri .....C.....-.....-..-.A.....-..A....A.-...
S.typhimurium .....T..CA.....C-.....-..A..-T.....C.....A.-...
K.pneumoniae ....C....-..C.....-.A..-.A.....C.....-...C...
E.carotovora ..
E.coli proU 56 ATTGCATGGCAATTAAA-TTAGAAATTAAA-AATCTTTATAAAATATTTGGCGAGCATCC 113
S.flexneri .....C.....-...A.....-.....T.....
S.typhimurium .....-.....G.G..G-.....G.....A.....
K.pneumoniae .....-.....C...-.....G.....
E.carotovora ...T.....C..-.-..G....G.....G.....-..
P.luminescens ...T.....C..-...G.....-..C..C.....G.....C...T.
E.coli proU 114 ACAG-C-GA-GCGTTCAAATAT-ATC-GA-ACAA-GGACT-TT-CA--AAAGAACA--A- 159
S.flexneri ....-.-..-.....-...-..-....-.....-..-..-.....--.-
S.typhimurium G...-.-.T-..C.....-..T-..-.-..G.....-A.-.G--.....G..--.-
K.pneumoniae ...C-.-.T-..T.....-..T-..G.-.-.....-CAA..-.-..-.CG..--.-
E.carotovora -...A.A..-..A..T...-C.G..T-..T.-.-..C..GAG-.---.....T..GGT-
P.luminescens .G.--A-T.T..T.....G.-.-.-.T..-.TTC-..-G.-..-G.GC.....T..G-.T
E.coli proU 160 ATTCTGGAAAAAACTGGGC-TATC-GCTTG--GCGTA-A-AAGACGCCAGTCTGGCCATT 213
S.flexneri .....-.....-.....--.....-.-.....
S.typhimurium ..A.....G....-....-.....--....T-.-.....
K.pneumoniae ..A.....G....-.G..-.....--....C-.-.....
E.carotovora ...-.-.....A....T..-.T.-.C.--.G...-.-....T.....
P.luminescens ...-.-.....G..-A..-...--.G...TT.-...T.C..A.T....A....A.T...
E.coli proU 214 GAAGAAGGCGAGATATTTGTCATCATGGGATTATCCGGCTCGGGTAAATCCACAATGGTA 273
S.flexneri .....
S.typhimurium .....
K.pneumoniae .....C.....GC.C.....G.....C.....T
E.carotovora .....T..C..C.....A..C.....
P.luminescens .....A.....T.....C..C.....
E.coli proU 274 CGCCTTCTCAATCGCCTGATTGAACCCACCCGCGGG-CAAGTGCTGATTGATGGTGTGGA 332
S.flexneri .....-.....
S.typhimurium .....A-..G..A.....C..C..T..
K.pneumoniae .....C-..G.....C..G..C..
E.carotovora .....T.....C.....T..-...T..G..A.....C..C....A...
P.luminescens .....T.....AA....G..T.....C-.....AT.A.....AA..
E.coli proU 333 TAT-TGCCAAAATATCCGAC-GCCGAACTCCGTGAGGTGCG-CAGAAA-AAAGATT-GCG 387
S.flexneri ...-.....-.....-.....-.....-...
S.typhimurium ...-.....A...-..T..G..T..C.....-...G..-.....-...
K.pneumoniae ...-C.....G..G..T-.....G..G..C.....T.-.C..G...-...-...
E.carotovora ...C.-....G.....T..T-A...CG..G..C..C.....T.-.T..G...-..CA.T-
P.luminescens ...-.T.TG.G..T..A-..A..A.CGT.AA.A..AA.T..-.C.T..C...-..CA.T-
E.coli proU 388 ATGGTCTTCCAGTCCTTTGC-CTTAATGCCGCATATGACCGTGCTGGACAATACTGCG-- 444
S.flexneri .....-.....--
S.typhimurium .....A.....G..C.-.....T.....G..A--
K.pneumoniae .....T..T.....G..G.-.....C....G.....C..T..C..G...--
E.carotovora .....G.....A.....G..C.-.....C...ATA.C..CA.....C..A--
P.luminescens .....T.....A..A.....A..G.-....A...T.A.A.A.A.....-..-..A...CC
E.coli proU 445 TTCGGTATGGAA-TTGGCCGGAATTA--ATGC-CGAA-G---AACGCCGGG-AAAAAGCC 495
S.flexneri .....-.....-...-....-.---.....-.....
S.typhimurium .....-..A..G..C..CG--CG..G.-..-.---.G..T..C.-.....G
K.pneumoniae ..T..C....CGC...-....CG..C--CC..-..CC-.-.....A-.A.C.....
E.carotovora ..T.....G-C....T..CG.-.-----.-....A.TGG.....-.A.C.....G
P.luminescens ..T.....C...-.....C.CC.AA.---..-.---.....AATA-.....A
E.coli proU 496 CTTG-ATGCACT-GC-GTCAGGTCGGGCTGGAAAATTATGCCCACAGC-T-ACCCGGATG 550
S.flexneri ....-.....-..-.....-.....-.....-.-.....
S.typhimurium ..G.-.C..-..T..-.....G.....T..G.....C..T...-..C.-.....
K.pneumoniae .GG.-.G..G..-..-.....G.....G.....-..C.-GG.....
E.carotovora ..G.-....-..T..A.-.....C..T...GCC..C..GG-..T.-.T.....
P.luminescens -...G.....-.A-AA.....AAAC.....A..-.T.-.T.T..A....
E.coli proU 551 AACTCTCTGGCGGGATGCGTCAACGTGT-GGGAT-TAGCCCGCGCGTTAGCGATTAATCC 608
S.flexneri .....-.....-.....
S.typhimurium ....T..C..T.....G.....T...C.-.-.....C.G..A..C..C..
K.pneumoniae .....C.....C.....C..G..C..-C..T.-....T.....CC.G.....
E.carotovora .G..G.....G..G.....-C..-.C.G..G..T.....A.G.C..
P.luminescens .G.....A.....C.....A...C.-.-...A.A..A.....T.A...C..

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E.coli proU      609    GGATATAT-TATTAATGGACGAAGCCTTCTCGGCGCTCGATCCATTAATTCGCACCGAGA   667
S.flexneri              ........-.................................................
S.typhimurium           T.....C.-.........T.....G..T..C..C................T.....A.
K.pneumoniae            C.....-.C.G........T...........T...........GC.C.....T.....A.
E.carotovora            C.....CC-....G.....T..G..A.....C..A........GC....C..T..T..A.
P.luminescens           A.....T.-.GC.............A..T..T..C..T.............T..A..A.

E.coli proU      668    TGCAGGATGAGCTGGTAAAATTACA-GGCGAAACATC-AGCGCACCATTGTCTTTATTTC   725
S.flexneri              ........................-...........-....................
S.typhimurium           ................G........-...........-....................
K.pneumoniae            ............CA.C........-......G....-.A.....T..C..........
E.carotovora            ..........T.A..C...C.C..-.T.CCGT....-.A.....T..C........C..
P.luminescens           ....A.....A...C...GCC.G..A...-..T..G.C.-..G..T..C........C..

E.coli proU      726    CCACGATCTTG-ATGAAGCCATGCGTATTGGCGACCGAATTGCCATTATGCAAAATGGTG   784
S.flexneri              ...........-.................................................
S.typhimurium           ...........-....G..T................A.G.....................C.
K.pneumoniae            ...T..C....-.............A........T..T..C.................C.
E.carotovora            G........G.-........G.....C..C..T.....G........C.....CGGC....
P.luminescens           T..T...-...G....G..A........C..A.....G...............G.C....

E.coli proU      785    AAGTGGTACAGGTCGGCACACCGGATGAAAT-TCTC-AATAATCCGGCGAATGATTATGT   842
S.flexneri              ................................-....-....................
S.typhimurium           .G..C........T..T..G........G..-C..G-...........A...........
K.pneumoniae            .......C...........T...........A.-.A-...........C...........
E.carotovora            .....A.T........T..G..C.....G..-C..G-.....C..A..C.....C.....
P.luminescens           TT..T..G........G.....T.........--..TG........A......A.C.....

E.coli proU      843    CCGTACCTTCTTCCGTGGCGTTGATATTAGTCAGGTATTCAGTGCGAAAGATATTGCCCG   902
S.flexneri              ....................................G.......................
S.typhimurium           ...C..G..............G..............C..T..C..C..............
K.pneumoniae            G..C...........C.....G........C...........C.................
E.carotovora            G..C.....T.....C..T..C.....C..C..C..G..T..C..C........C..G..
P.luminescens           GAAA..T.....T........G.....C..C..T...........T.....C........

E.coli proU      903    CCGGA-CACCGAAT-GGC-TTAATTCGTAAAACCCCTGGCTTCGG-CCCACGTTCGGCAC   958
S.flexneri              .....-........-...-..............T...........-..............
S.typhimurium           T..C.G.-...G-.C..A-..............G..A..T.....T...-..........
K.pneumoniae            ...C.G.-...GT.-...-C.G...........G.....T..T..-...C........G.
E.carotovora            T..T--.G..C.G.-TA.C.................C...G.A..-A..G.....C..G.

E.coli proU      959    TGAAATTA-TTGCAGGATGAAGATCGCGAATATGGCTACGTTATCGAACGCGGTAATAAG   1017
S.flexneri              ........-.....................................................
S.typhimurium           ........-..A.....C.....C..T........T.....C..T..G..T..C.....A
K.pneumoniae            ........-..A...........C..G...........T..C..A..G.........C-.
E.carotovora            .....A.CC..-.....C.....C..T..T..C........

E.coli proU      1018   -TTTGTCGGCG-CA--GTCTCCATCGATTCGCTTAAAACCG-CGTTAA-CGC-AG-CAGC   1069
S.flexneri              -..........-..--.....................-......-...-.-....
S.typhimurium           -..C..G....T.---..G........C..A-......G..G.A....--..C..G.-..
K.pneumoniae            C........-.--.TT.....G..T.....T..G.....A.-.CC.C.G...-C.-G-..

E.coli proU      1070   AAGGTCTTGATGCGGCGCTGATTGATG-CGCCGTTAG-CAGTCGATGCACAAACGCCTCT   1127
S.flexneri              ..........................-.........-....................
S.typhimurium           ....GA....A........T..C..C.A.-..T....T.-..T.....G.....C..A..
K.pneumoniae            .G..GA.C..G.............C.-.C...C.G.-.G..G..A..G.....C..G..

E.coli proU      1128   TAGCGAGT-TGCTCTCTCATGTCGGACAGGCACCCTGTGCGGTGCCCGTGGTCGACGAGG   1186
S.flexneri              ........-.....................................................
S.typhimurium           C.......-..........C.....C.....G.....C........G..T.....T..A.
K.pneumoniae            C......-.C.......C..C.....C..C..T..G..C..C.....G..A..G..T..A.

E.coli proU      1187   ACCAACAGTATGTCGGCATCATTTCGAAA-GGAATGCTGCTGC-GCGCTTTAGATCGTGA   1244
S.flexneri              ..........................-.............-...............
S.typhimurium           .A..C........T.....T.....A...-C.T...T....A.A.-...........C..
K.pneumoniae            .A.....A...A.T...........C...C...-..T....C.A.-...........C..

E.coli proU      1245   GGGGG-TAAATAATGGCTGA  1263
S.flexneri              .....-............
S.typhimurium           .....G...C-.......
K.pneumoniae            .....-.G...C.......
```

Figure. 27: Sequence alignment of the 5' end of the *proU* operon of Enterobacteriaceae. With the sequence of the *E.coli* K12 *proU* operon from +1 (transcription start) to +1263 (the end of the *proV* coding region) a BLAST search against the genomes of the sequenced members of *Enterobacteriaceae* was performed using the web tool available from NCBI at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. Since the sequence of individual strains of the same species was not significantly different, the sequence

alignment of representative sequences from the species *Escherechia coli* and *Shigella flexneri*, *Salmonella enteritica* serovar Typhimurium, *Klebsiella pneumoneae*, *Erwinia carotovora* and *Photorabdus luminescens* is shown. The conserved residues are represented with dots. Gaps in the sequences represented by dashes (-). Polymorphism at a particular position is indicated with the substituted residue. The highly conserved stretch of the sequences between +200 to +300 relative to the *E.coli proU* transcription start is boxed. The RNAse III specific processing site, mapped by primer extension to a G residue at position +217 is indicated with a filled arrowhead.

5 Discussion

The highly specific repression of the *E.coli bgl* and *proU* operons requires binding of H-NS to upstream and downstream regulatory elements (URE and DRE respectively) flanking the promoters (Schnetz, 1995) (Owen-Hughes et al., 1992; Overdier and Csonka, 1992). H-NS binding to the *proU-DRE* represses transcription initiation at the *proU* promoter (Jordi and Higgins, 2000). H-NS bound to the *bgl-DRE* weakly affects transcription through the *bgl* transcription unit, while no such effect was observed in *proU* (Nagarajavel, 2007). This study, in attempting to understand the mechanism and the modulation of repression by H-NS, establishes the following: There is synergy in repression by H-NS bound to the two regulatory elements in *proU*, as observed in *bgl* earlier (Nagarajavel, 2007). H-NS bound to the *bgl-DRE* represses an early step of transcription initiation at the promoter, as observed in *proU* (Jordi and Higgins, 2000). Termination factor Rho affects the repression by H-NS through the *bgl*-DRE, and Lon protease counteracts the repression (Dole et al, 2004b), while here it is shown that the DnaKJ chaperone system is essential for the repression by H-NS through the *bgl*-DRE. Further, the *proU* operon is subject to post-transcriptional osmoregulation. RNAse III brings about initial processing of the *proU* transcript within highly conserved stretch of sequence, a process, which maybe crucial for post-transcriptional regulation. H-NS is possibly involved in the post-transcriptional osmoregulation of *proU*. Although the mechanism of repression of the *bgl* and the *proU* operons by H-NS is very similar, the modulation of the repression seems to be locus specific, with each system requiring specific signals and a specific set of additional factors that in concert bring about the modulation of a similar mode of repression.

5.1 The mechanism of repression by H-NS binding within the bgl and proU transcription units

CAA footprinting performed in order to observe if H-NS bound to the *bgl-DRE* could act as a roadblock to the elongating RNA polymerase could not identify any H-NS dependent pausing events within the *bgl* transcription unit (Fig. 6). An intrinsic, H-NS independent pause was identified within the *bgl* transcription unit between positions +477 to +484 with respect to the *bgl* transcription start (Fig. 6). An RNA secondary structure is predicted to form immediately upstream of the pause signal between positions +454 to +476. However, deletion of the pause and the preceding secondary structure does not affect H-NS repression of *bgl* (Nagarajavel, 2007). The significance of RNA polymerase pausing at this position within the *bgl* transcription unit is currently unknown. However, it is clear that H-NS does not act as a roadblock to the elongating RNA polymerase, although it hinders readthrough.

Footprinting of RNA polymerase at the *bgl* promoter further revealed that, H-NS bound to the *bgl-DRE* inhibits open complex formation, i.e. an early step of transcription initiation like in the case of *proU* (Jordi and Higgins, 2000) (Fig. 7). It is not clear at the moment weather the RNA polymerase at the *bgl* and *proU* promoters is trapped in the closed complex or whether it is excluded from binding by H-NS. Both of these roles have been attributed to H-NS before. A genome wide analysis of H-NS and RNA polymerase distribution along the genome suggests that H-NS excludes RNA polymerase binding (Lucchini et al., 2006), while a different study concludes that H-NS and the RNA polymerase co-localize at promoters (Oshima et al., 2006).

The footprints also corroborate the observation, that repression of the *bgl* and *proU* operons by H-NS bound to the two regulatory elements is synergistic (this work and (Nagarajavel, 2007). It is clear that the open complex detected at the *bgl* promoter is significantly decreased in presence of both URE and the DRE, in comparison to the promoter followed by the DRE alone (Fig. 7). Expression analyses of *proU-lacZ* fusions demonstrated that the combination of the URE and DRE, as it is in the natural context, is required for efficient 16-fold repression. The URE alone has no effect, while the DRE causes a merely 5-fold repression at low osmolarity, which decreases to 2-fold at high osmolarity (Fig. 8). This shows that the repression by H-NS bound to both regulatory elements is synergistic in *bgl* and in *proU*.

H-NS bound to the URE and the DRE, respectively, represses transcription initiation (Fig. 29). It is possible that this process involves DNA loop formation. H-NS is known to zip two DNA strands together (Dorman, 2004; Dame et al., 2002). Recent studies have shown that this DNA bridging by H-NS does not occur when both DNA strands are bound by H-NS, i.e. it does not occur by protein-protein interaction, instead involves binding of H-NS dimers to two DNA double helices and DNA-H-NS-DNA bridge formation (Dame et al., 2006). Therefore, in case of *bgl* and *proU,* it is possible to hypothesize that H-NS bound to the URE traps a downstream segment of DNA into the complex. Likewise, H-NS bound to the DRE may bridge to an upstream DNA segment. In the presence of both the upstream and downstream regulatory elements the DNA bridging may be mutually enhanced resulting in the formation of a stable repressing complex. This possibility has also been discussed in the context of the *proU* operon, with the discovery of two high affinity H-NS nucleation sites (Bouffartigues et al., 2007).

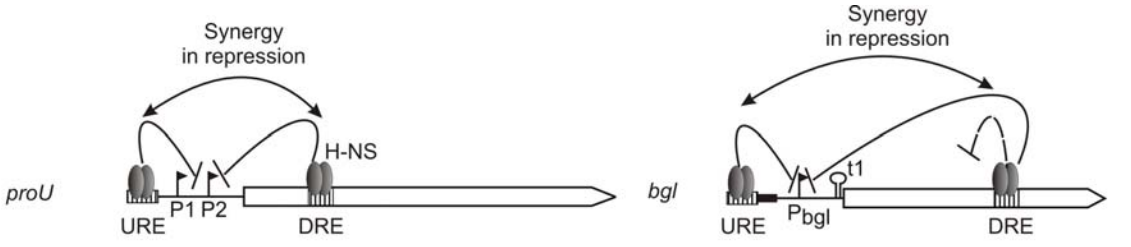

Figure. 29: Mechanism of repression of the *bgl* and *proU* operons by H-NS. H-NS bound to the URE and the DRE in the *bgl* and *proU* operons represses transcription initiation at the respective promoters (Fig. 7) and (Jordi and Higgins, 2000). Additionally, H-NS bound to the *bgl-DRE* weakly affects transcription through the *bgl* transcription unit (Nagarajavel, 2007). The repression by H-NS bound to the two regulatory elements in both *bgl* and *proU* is synergistic (Fig. 8, and(Nagarajavel, 2007; Tendeng and Bertin, 2003).

5.2 Transcription modulates the H-NS mediated repression of the proU and bgl operons.

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). Unlike H-NS bound to the upstream regulatory element, H-NS bound to the downstream regulatory element will encounter transcription by RNA polymerase. The repression by H-NS bound to the DRE in both *bgl* and *proU* is reported to be affected by the transcription rate, wherein low transcription rate favors effective repression (Nagarajavel et al., 2007). This is also apparent from the induction of the *proU* operon (Fig. 8), at low osmolarity when the *proU* promoter activity is low, the *proU* promoter is repressed 16-fold by H-NS bound at the two regulatory sites, while the H-NS bound to the DRE alone represses the promoter 5-fold (Fig. 8a and c). An increase in osmolarity leads to a 2-fold increased activity of the *proU* promoter (Fig. 8e), this increased promoter activity drastically reduces the repression by H-NS (Fig. 8a and e). Considering the fact that an RNA polymerase engaged in active transcription exerts a force of about 20 pN (pico Newton) (Davenport et al., 2000), and a force of about 9 pN is sufficient to disrupt a DNA bridge formed by one H-NS dimer (Dame et al., 2006), it is possible to imagine that a high rate of transcribing RNA polymerase remodels the H-NS nucleoprotein complex. Indeed, H-NS is reported to be excluded from actively transcribed regions of the genome (Lucchini et al., 2006b).

The interesting observation of poising of RNA polymerase at the *proU* promoter at low osmolarity adds a further aspect to the mutual influence of transcription and repression by H-NS (Fig. 9). The clearing of the promoter, i.e. the transition of the RNA polymerase from transcription initiation to elongation, is considered to be a rate limiting step, and in genome scale analyses the RNA polymerase density is high around promoters, suggesting that the RNA polymerase is poised at a majority of promoters (Reppas et al., 2006). At the *proU* promoter RNA polymerase is poised at low osmolarity, but clears the promoter with a better efficiency at high osmolarity (Fig. 9). While the signal that induced the *proU* promoter is not known, σS dependent osmoregulated promoters are activated by an increase in the potassium glutamate concentrations (Gralla and Vargas, 2006).

In case of the *bgl* operon, the LysR-type transcription factor LeuO, and the LuxR-type transcription factor BglJ, possibly in conjunction with another LuxR type transcription factor, RcsB specifically counteract promoter repression by H-NS (Madhusudan et al., 2005) (Paukner. A, Schnetz. K, unpublished observation). Further, BglJ, LeuO and H-NS are involved in a complex inter regulatory network, with H-NS repressing the expression of *bglJ* and *leuO* (Klauck et al., 1997) (Stratmann.T, Schnetz.K, unpublished observation). LeuO in turn controls the expression of H-NS, through the small RNA DsrA (Klauck et al., 1997). Although the activity of the other members of the LysR family is known to be affected by binding of specific cofactors (Ezezika et al., 2007) (Laishram and Gowrishankar, 2007), no such effector molecule that modulates the activity of LeuO under specific conditions has been identified so far

Taken together, the data suggest that a small increase in the activity of the *proU* and *bgl* promoter, respectively, causes complete derepression of the two operons. Increased transcription reduces repression by H-NS bound to the respective DRE possibly by the remodeling of the H-NS nucleoprotein complex by the elongating RNA polymerase (Fig. 30). This would result in a reduction of synergistic repression by H-NS bound at the URE and DRE. However, the mechanisms, by which the promoter is activated in *bgl* and *proU* are different; with the transcription factors LeuO and BglJ involved in the *bgl* promoter activation, and increased promoter clearance at high osmolarity at the *proU* promoter (Fig. 30).

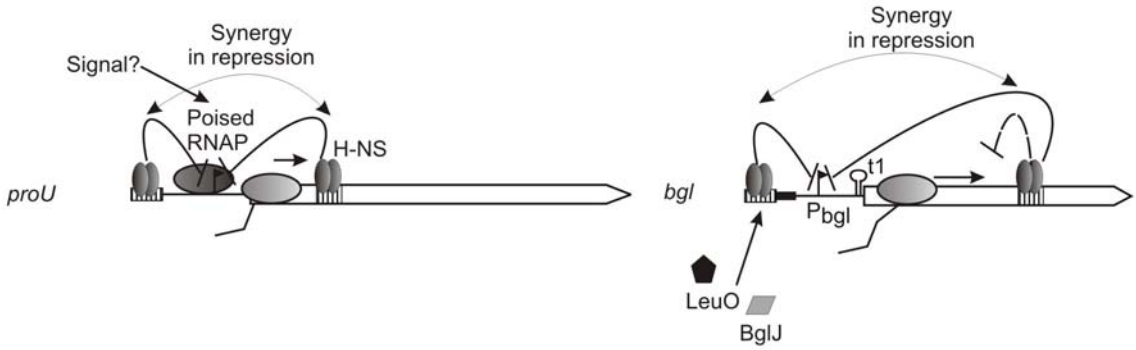

Figure. 30: Modulation of the H-NS mediated repression of the *proU* and *bgl* operons by transcription. At the *proU* promoter the RNA polymerase is poised at low osmolarity, while promoter clearance is increased at high osmolarity (Fig. 9). The signal responsible for enhanced promoter clearance at high osmolarity is thus far unidentified. The transcription factors LeuO and BglJ counteract repression of the *bgl* promoter by H-NS. An increase in the promoter activity with the increase in osmolarity in case of *proU*, and by the action of specific transcription factors in *bgl* leading to an increase in transcription through the transcription units drastically reduces repression by H-NS, possibly by remodeling the H-NS nucleoprotein complex, and reducing synergy of repression.

5.3 Additional factors in the modulation of repression by H-NS bound within the bgl transcription unit.

H-NS bound to the *bgl-DRE* represses transcription initiation at the promoter and, in addition has been shown to weakly reduce transcription through the *bgl* transcription unit (Nagarajavel, 2007). Possibly the nucleoprotein complex formed by H-NS at the *bgl-DRE* affects the translocation of the RNA polymerase through the *bgl* transcription unit. Further, the efficiency of repression by H-NS thorugh the DRE is modulated by the termination factor Rho and the ATP dependent Lon protease (Dole et al., 2004a). Since the stability of a tagged H-NS protein was not altered in the *lon* mutant and since Lon affects only repression through the DRE but not repression by H NS bound to the URE it is unlikely that Lon targets the H-NS nucleoprotein complex. It was therefore assumed, that Lon as Rho targets the processivity of the transcription elongation complex, which causes modulation of H-NS repression. Here, it is shown that the repression by H-NS through the *bgl*-DRE is also modulated by the heat shock-induced DnaKJ chaperone system (Fig. 13), as it was identified in a transposon mutagenesis screen (Fig.12). It was found that DnaKJ is required for repression by H-NS bound to the *bgl* transcription unit, which indicates a modulation of H-NS activity, or the processivity of the elongating RNA polymerase by the chaperone system.

Since termination factor Rho modulates the H-NS mediated repression within the *bgl* transcription unit, it was analyzed if Rho or another factor involved in termination is a Lon substrate. Inhibition of Rho with bicyclomycin leads to a complete neutralization of the Lon effect in *bgl* (Fig. 15). However, bicyclomycin did not neutralize the regulation of *tna* by Lon (Fig. 16), which is a system with characterized Rho-dependent terminator (Stewart et al., 1986). Also the expression of a Rho-dependent λ-terminator *R1/lacZ* fusion was not altered in a *lon* mutant (Fig. 17). These latter results contradict the idea that Lon targets a factor involved in termination. In agreement with this, the stability of tagged constituents of the termination machinery was not altered in the *lon* mutant (Fig. 18). Thus, the mechanism by which protease Lon counteracts the H-NS mediated repression of transcription through the *bgl* transcription unit is presently unknown. Since the expression of the *tna-lacZ* fusion is reduced 2-fold in the *lon* mutant similar to the *bgl-DRE* fusion (Fig. 11 and 16a), and the fact that the *tna* operon has characterized

RNA processing regulating its expression (Li and Altman, 2003), it is possible that Lon could target a constituent of the RNA turnover machinery. It is also not known presently if the *bgl* RNA is subjected to processing by RNAses.

It is evident that the repression by H-NS bound within the transcription unit is modulated by a complex network of pleiotrophic regulators. The DnaKJ chaperone system and termination factor Rho enhance this repression, and Lon reduces it. The seemingly small alterations are significant in the light of the amplification of repression by feedback mechanisms (Radde et al., 2007) (Dole et al., 2002). All of these factors are integrated into a model to depict the modulation of H-NS mediated repression of *bgl* (Fig. 31)

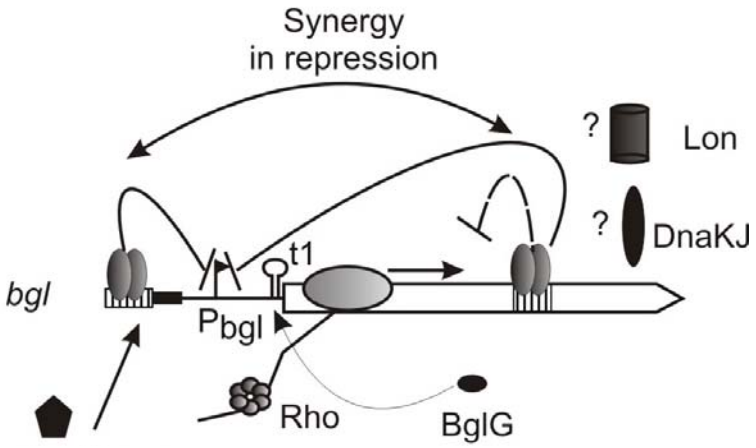

Figure. 31: Modulation of the H-NS mediated repression of *bgl* by Lon. H-NS bound to the *bgl-DRE* weakely affects transcription through the *bgl* transcription unit; termination factor Rho affects the repression by H-NS through *bgl*-DRE (Nagarajavel, 2007). The chaperone DnaKJ abets the repression by H-NS requires by an unkown mechanism. Protease Lon counteracts the H-NS mediated repression through the *bgl*-DRE by an unknown mechanism. An increase in the concentration of BglG antiterminator as a result of the complex interplay between the modulators completely de represses the operon due to antitermination at the t1 terminator (Dole et al., 2002).

5.4 Post-transcriptional osmoregulation of proU.

The *proU* operon is subject to post-transcriptional regulation. This conclusion is based on the finding the osmoregulation of translational *proV-lacZ*-fusion is 4-fold more specific than that of transcriptional *lacZ* fusions. This enhanced specificity of osmoregulation is independent of the promoter, i.e. independent whether the *proU* promoter, which is itself osmoregulated, drives expression or whether the *lacUV5* promoter directs expression (Fig. 8 and Fig. 20). It is also evident that the RNA chaperone Hfq is essential for basal expression of the *proU* operon (Fig. 21) at low

osmolarity and to a lesser extend for high levels of expression at high osmolarity. Again this is independent of the promoter. Further the difference in the expression level in the *hfq* mutant is reflected in lower amounts of the *proU* message present at low osmolarity. However, the stability of the *proU* mRNA in not altered in the *hfq* mutant in dependence of the osmolarity (Fig. 23c). Interestingly, the post-transcriptional osmoregulation was reduced in an *hns* mutant (Fig. 20). H-NS possibly affects the half life of the *proU* RNA with the *proU* mRNA being more stable in the *hns* mutant at high osmolarity (Fig. 23b). Whether H-NS alters the stability or the translatability of the *proU* mRNA directly by binding to it (Brescia et al., 2004), or through an indirect mechanism (by controlling a factor that regulates the stability of the *proU* mRNA) is not known at the moment. This indicates that H-NS, in addition to repressing the *proU* promoter, may alter the stability of the *proU* mRNA.

A specific processing product of the *proU* mRNA was evident from the Northern analyses (Fig. 24). Interestingly, the ratio of the full length RNA to the processed RNA was two-fold increased at high osmolarity, although the decay kinetics of the *proU* message was not altered (Fig. 24). Further, mutant analyses established that the *proU* mRNA is processed by RNAse III and RNAse E. RNase III brings about the initial rate limiting processing of the *proU* mRNA at position +217 (Fig. 25 and 26). Then the processed mRNA is rapidly degraded by RNAse E, which is evident from the accumulation of the processed RNA in the *rne* mutant (Fig. 25). The analyses in *rne* mutants further demonstrated that more processed RNA accumulates at low osmolarity than at high osmolarity (Fig. 25), and that the ratio of the full length to the processed RNA is higher at high osmolarity (Fig. 24). This suggests that the initial cleavage brought about by RNAse III happens at a higher efficiency or frequency at low osmolarity.

Alignment of the *E.coli proU* sequence from +1 to +1263 encompassing the *proV* gene with that of other enterobacterial species showed that a stretch of sequence between about +194 to +307 relative to *E.coli proU* transcription start is highly conserved among enterobacteria. This stretch encompasses the RNAse III processing site at +217. Further, this stretch of sequence is identical between *E .coli* (and *S.flexneri*), and *S.typhimurium*, although there are numerous polymorphisms between the species outside this window of conservation (Fig. 27). Furthermore, secondary structure predictions of the region around the processing site shows that the

processing site is enclosed in a very similar stem loop structure in all the enterobacterial sequences compared (Fig. 28). This suggests that the mechanism of the post-transcriptional regulation of the *proU* operon is conserved among enterobacteria.

What could be the mechanism of post-transcriptional osmoregulation of *proU*? At present a causal relationship between processing and post-transcriptional osmoregulation has not been established, although the RNase III dependent rate of processing seems to be osmoregulated. Likewise, the role of Hfq and H-NS remains enigmatic. However, it is possible to imagine that a noncoding regulatory (ncRNA) thus far unidentified is involved in the post-transcriptional osmoregulation (Fig. 32). Such an ncRNA would have to be osmoresponsive, similar to the *micF* RNA that regulates the expression of the OmpF porin and that is induced by an increase in osmolarity (Ramani et al., 1994). Further, it is known that the translation rate of an mRNA affects its stability (Arnold et al., 1998). Therefore, the putative ncRNA could act either by regulating the processing of the *proU* mRNA or by regulating its translation, which then would affect its processing. For instance, the *sodB* mRNA in *E.coli* is regulated by the ncRNA RhyB by basepairing that inhibits ribosome binding and creates RNAse III and RNAse E processing sites (Afonyushkin et al., 2005). The ncRNA DsrA forms a complex with the *rpoS* mRNA, which leads to the exposition of the translation initiation region of the *rpoS* mRNA, thereby facilitating its translation and stabilization (Lease and Belfort, 2000; Lease et al., 1998). The fact that H-NS affects the level of post-transcriptional regulation and alters the stability of the *proU* mRNA could also be based in the regulation of expression of the putative regulatory RNA.

Recent studies reveal that RNA 'thermosensors' are employed by bacteria during adaptation to heat or cold stress (Narberhaus et al., 2006). Further, an RNa that changes the conformation of its 5'-UTR according to the cytoplasmic Mg^{2+} concentration, thereby determining if the transcription reads through into the structural gene or stops at the UTR, was recently reported in *Salmonella* (Cromie et al., 2006). Thus, one could also speculate that the post-transcriptional osmoregulation of *proU* is an intrinsic property of the *proU* mRNA itself, with the RNA behaving as an 'Osmosensor' (Fig. 32). At low osmolarity the RNA might adopt

a conformation that favors processing by RNAse III, and at high osmolarity a different conformation could reduce processing of the message by RNAse III.

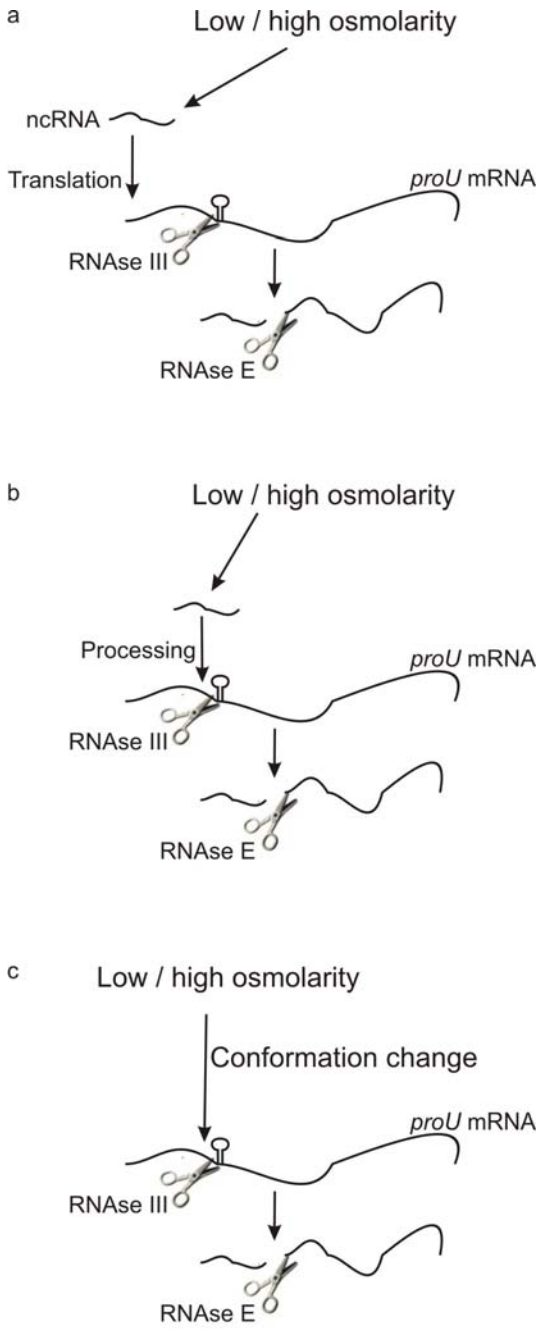

Figure. 32: Possible mechanisms of the post-transcriptional osmoregulation of the *proU* mRNA (a) a thus far unidentified non coding (ncRNA) induced by osmolarity could trigger efficient initial processing of the *proU* message by RNAse III by inhibiting translation, after which the processed RNA is rapidly degraded by RNAse E **(b)** or osmoinduced the ncRNA could stimulate the processing of the proU mRNA by RNAse III by direct interaction. **(c)** Alternatively, the *proU* mRNA could behave as an 'osmosensor' whereby the conformation of the RNA allows processing by RNAse III at low osmolarity and the increase in osmolarity leading to a change in the conformation of the *proU* mRNA making it resistant to the endonucleolytic attack by RNAse III.

5.5 The modulation of H-NS mediated repression is gene specific.

Taken together the data suggest that the mechanism of H-NS mediated repression of the *bgl* and *proU* operons is similar, but that it is modulated in the two systems in a context specific manner. H-NS inhibits transcription initiation in both cases either by occluding RNA polymerase from the promoter or by preventing open complex formation. In both systems a small increase of the promoter activity, which is context specific, is sufficient to relief repression presumably by remodeling of the repressing nucleoprotein complex formed by H-NS. Further, both systems are subject to a second context specific level of regulation that enhances the specificity of regulation (summarized in Figure. 33).

Repression of the *bgl* by H-NS is modulated by binding of transcription factors LeuO and BglJ that counteract repression of the promoter by H-NS. Owing to the amplification of transcription through *bgl* by the antiterminator BglG, small modulations of the repression through *bgl* by H-NS, might lead to small increase in the promoter activity and subsequent transcription through the *bgl* transcription unit. This would result in higher concentrations of BglG antiterminator, which by the positive amplification could cause complete derepression of the operon.

In case of the *proU* operon the promoter activity is osmoregulated by a hitherto unidentified signal that may cause a better promoter clearance of the RNA polymerase at high osmolariry. The second context specific level is the post-transcriptional osmoinduction, which governs the stability of the *proU* mRNA and consequently the levels of the ProVWX transporter proteins.

Taken together, the mechanism of H-NS mediate repression at the *bgl* and *proU* operons is very similar. However, its modulation is a complex mechanism involving numerous additional factors specific to the two systems, and thus is achieved in a context specific manner.

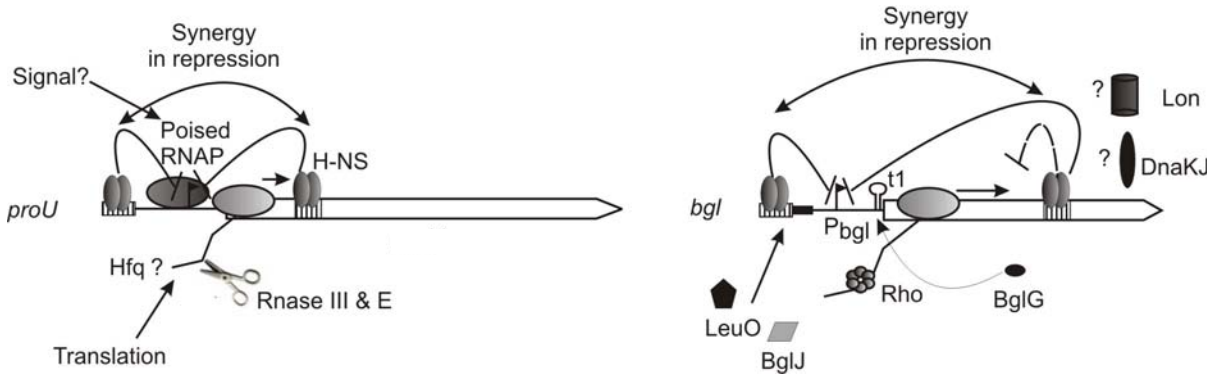

Figure. 33:The similar mode of H-NS mediated repression of the *bgl* and *proU* operons is modulated gene specifically: H-NS bound to the URE and the DRE in *bgl* and *proU* operons repress transcription at the respective promoters. An increase in transcription through the transcription units, achieved by different means (with better promoter clearance of the poised RNA polymerase at the proU promoter, and specific counteraction by transcription factors LeuO and BglJ at the *bgl* promoter) possibly leads to a decrease in cooperativity in repression by H-NS. Incase of *bgl* a small increase in promoter activity, could result in the synthesis of sufficient BglG for anititermination that could lead to efficient de repression of the *bgl* operon. In case of the proU operon, an increased promoter clearance at high osmolarity, and the post-transcriptional osmoregulation, which involves processing by RNAses III and E, leads to manifold enhanced expression of *proU* at high osmolarity. Thus, a similar mode of repression by H-NS in *bgl* and *proU* is modulated in very different ways, suggesting that the modulation of H-NS mediated repression is achieved context specifically.

6. Bibliography

Amit,R., Oppenheim,A.B., and Stavans,J. (2003). Increased bending rigidity of single DNA molecules by H-NS, a temperature and osmolarity sensor. Biophys. J. *84*, 2467-2473.

Arnold,T.E., Yu,J., and Belasco,J.G. (1998). mRNA stabilization by the ompA 5 ' untranslated region: Two protective elements hinder distinct pathways for mRNA degradation. Rna-A Publication of the Rna Society *4*, 319-330.

Artsimovitch,I. and Landick,R. (2000). Pausing by bacterial RNA polymerase is mediated by mechanistically distinct classes of signals. Proc. Natl. Acad. Sci. U. S. A *97*, 7090-7095.

Artsimovitch,I. and Landick,R. (2002). The transcriptional regulator RfaH stimulates RNA chain synthesis after recruitment to elongation complexes by the exposed nontemplate DNA strand. Cell *109*, 193-203.

Atlung,T. and Ingmer,H. (1997). H-NS: a modulator of environmentally regulated gene expression. Mol. Microbiol. *24*, 7-17.

Ausubel,F.M Brent, R., Kensington, E., .Moore, D.D., .Seidman J.G.,and Struhl.K. (2005). Current Protocols in Molecular Biology. *In* . John Wiley & Sons, Inc.).

Badaut,C., Williams,R., Arluison,V., Bouffartigues,E., Robert,B., Buc,H., and Rimsky,S. (2002). The degree of oligomerization of the H-NS nucleoid structuring protein is related to specific binding to DNA. J. Biol. Chem. *277*, 41657-41666.

Barr,G.C., Bhriain,N.N., and Dorman,C.J. (1992). Identification of two new genetically active regions associated with the osmZ locus of Escherichia coli: role in regulation of proU expression and mutagenic effect of cya, the structural gene for adenylate cyclase. J. Bacteriol. *174*, 998-1006.

Bertin,P., Benhabiles,N., Krin,E., Laurent-Winter,C., Tendeng,C., Turlin,E., Thomas,A., Danchin,A., and Brasseur,R. (1999). The structural and functional organization of H-NS-like proteins is evolutionarily conserved in gram-negative bacteria. Mol. Microbiol. *31*, 319-329.

Bloch,V., Yang,Y., Margeat,E., Chavanieu,A., Auge,M.T., Robert,B., Arold,S., Rimsky,S., and Kochoyan,M. (2003). The H-NS dimerization domain defines a new fold contributing to DNA recognition. Nat. Struct. Biol. *10*, 212-218.

Borukhov,S., Lee,J., and Laptenko,O. (2005). Bacterial transcription elongation factors: new insights into molecular mechanism of action. Molecular Microbiology *55*, 1315-1324.

Bouffartigues,E., Buckle,M., Badaut,C., Travers,A., and Rimsky,S. (2007). H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing. Nature Structural & Molecular Biology *14*, 441-448.

Brescia,C.C., Kaw,M.K., and Sledjeski,D.D. (2004). The DNA binding protein H-NS binds to and alters the stability of RNA in vitro and in vivo. J. Mol. Biol. *339*, 505-514.

Brosius,J. and Holy,A. (1984). Regulation of Ribosomal-Rna Promoters with A Synthetic Lac Operator. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences *81*, 6929-6933.

Browning,D.F., Cole,J.A., and Busby,S.J. (2000). Suppression of FNR-dependent transcription activation at the Escherichia coli nir promoter by Fis, IHF and H-NS: modulation of transcription initiation by a complex nucleo-protein assembly. Mol. Microbiol. *37*, 1258-1269.

Caramel,A. and Schnetz,K. (1998). Lac and lambda repressors relieve silencing of the Escherichia coli bgl promoter. Activation by alteration of a repressing nucleoprotein complex. J. Mol. Biol. *284*, 875-883.

Carpousis,A.J. (2007). The RNA Degradosome of Escherichia coli: A Multiprotein mRNA-Degrading Machine Assembled on RNase E. Annu. Rev. Microbiol.

Chun,K.T., Edenberg,H.J., Kelley,M.R., and Goebl,M.G. (1997). Rapid amplification of uncharacterized transposon-tagged DNA sequences from genomic DNA. Yeast *13*, 233-240.

Dalal,R.V., Larson,M.H., Neuman,K.C., Gelles,J., Landick,R., and Block,S.M. (2006). Pulling on the nascent RNA during transcription does not alter kinetics of elongation or ubiquitous pausing. Mol. Cell *23*, 231-239.

Dame,R.T., Luijsterburg,M.S., Krin,E., Bertin,P.N., Wagner,R., and Wuite,G.J. (2005). DNA bridging: a property shared among H-NS-like proteins. J. Bacteriol. *187*, 1845-1848.

Dame,R.T., Noom,M.C., and Wuite,G.J. (2006). Bacterial chromatin organization by H-NS protein unravelled using dual DNA manipulation. Nature *444*, 387-390.

Dame,R.T., Wyman,C., and Goosen,N. (2001). Structural basis for preferential binding of H-NS to curved DNA. Biochimie *83*, 231-234.

Dame,R.T., Wyman,C., and Goosen,N. (2000). H-NS mediated compaction of DNA visualised by atomic force microscopy. Nucleic Acids Res. *28*, 3504-3510.

Dame,R.T., Wyman,C., Wurm,R., Wagner,R., and Goosen,N. (2002). Structural basis for H-NS-mediated trapping of RNA polymerase in the open initiation complex at the rrnB P1. J. Biol. Chem. *277*, 2146-2150.

Datsenko,K.A. and Wanner,B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Sciences of the United States of America *97*, 6640-6645.

Dattananda,C.S., Rajkumari,K., and Gowrishankar,J. (1991). Multiple mechanisms contribute to osmotic inducibility of proU operon expression in Escherichia coli:

demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. J. Bacteriol. *173*, 7481-7490.

Davenport,R.J., Wuite,G.J., Landick,R., and Bustamante,C. (2000). Single-molecule study of transcriptional pausing and arrest by E. coli RNA polymerase. Science *287*, 2497-2500.

Deana,A. and Belasco,J.G. (2005). Lost in translation: the influence of ribosomes on bacterial mRNA decay. Genes Dev. *19*, 2526-2533.

Dehaseth,P.L. and Helmann,J.D. (1995). Open Complex-Formation by Escherichia-Coli Rna-Polymerase - the Mechanism of Polymerase-Induced Strand Separation of Double-Helical Dna. Molecular Microbiology *16*, 817-824.

Diederich,L., Rasmussen,L.J., and Messer,W. (1992). New cloning vectors for integration into the lambda attachment site *attB* of the *Escherichia coli* chromosome. Plasmid *28*, 14-24.

Dole,S., Klingen,Y., Nagarajavel,V., and Schnetz,K. (2004a). 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., Kuhn,S., and Schnetz,K. (2002). Post-transcriptional enhancement of Escherichia coli bgl operon silencing by limitation of BglG-mediated antitermination at low transcription rates. Mol. Microbiol. *43*, 217-226.

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

Dorman,C.J. (2004). H-NS: a universal regulator for a dynamic genome. Nat. Rev. Microbiol. *2*, 391-400.

Dorman,C.J., Hinton,J.C., and Free,A. (1999). Domain organization and oligomerization among H-NS-like nucleoid-associated proteins in bacteria. Trends Microbiol. *7*, 124-128.

Druger-Liotta,J., Prange,V.J., Overdier,D.G., and Csonka,L.N. (1987). Selection of mutations that alter the osmotic control of transcription of the Salmonella typhimurium proU operon. J. Bacteriol. *169*, 2449-2459.

Epshtein,V., Toulme,F., Rahmouni,A.R., Borukhov,S., and Nudler,E. (2003). Transcription through the roadblocks: the role of RNA polymerase cooperation. EMBO J. *22*, 4719-4727.

Esposito,D., Petrovic,A., Harris,R., Ono,S., Eccleston,J.F., Mbabaali,A., Haq,I., Higgins,C.F., Hinton,J.C., Driscoll,P.C., and Ladbury,J.E. (2002). H-NS oligomerization domain structure reveals the mechanism for high order self-association of the intact protein. J. Mol. Biol. *324*, 841-850.

Falconi,M., Gualtieri,M.T., La,T.A., Losso,M.A., and Pon,C.L. (1988). Proteins from the prokaryotic nucleoid: primary and quaternary structure of the 15-kD Escherichia coli DNA binding protein H-NS. Mol. Microbiol. *2*, 323-329.

Gallagher,S., Winston,S.E., Fuller,S.A., and Hurrell,J.G. (2004). Current Protocols in Molecular Biology. *In* . John Wiley & Sons, Inc.).

Geissmann,T.A. and Touati,D. (2004). Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. Embo Journal *23*, 396-405.

Gottesman,S. (2005). Micros for microbes: non-coding regulatory RNAs in bacteria. Trends in Genetics *21*, 399-404.

Gottesman,S. (1996). Proteases and their targets in Escherichia coli. Annual Review of Genetics *30*, 465-506.

Gottesman,S. (2004). Small RNAs shed some light. Cell *118*, 1-2.

Gowrishankar,J. (1989). Nucleotide sequence of the osmoregulatory proU operon of Escherichia coli. J. Bacteriol. *171*, 1923-1931.

Gralla,J.D. and Vargas,D.R. (2006). Potassium glutamate as a transcriptional inhibitor during bacterial osmoregulation. Embo Journal *25*, 1515-1521.

Guerin,M., Leng,M., and Rahmouni,A.R. (1996). High resolution mapping of E.coli transcription elongation complex in situ reveals protein interactions with the non-transcribed strand. EMBO J. *15*, 5397-5407.

Gusarov,I. and Nudler,E. (2001). Control of intrinsic transcription termination by N and NusA: the basic mechanisms. Cell *107*, 437-449.

Hashimoto-Gotoh,T., Yamaguchi,M., Yasojima,K., Tsujimura,A., Wakabayashi,Y., and Watanabe,Y. (2000). A set of temperature sensitive-replication/-segregation and temperature resistant plasmid vectors with different copy numbers and in an isogenic background (chloramphenicol, kanamycin, lacZ, repA, par, polA). Gene *241*, 185-191.

Herbert,K.M., La,P.A., Wong,B.J., Mooney,R.A., Neuman,K.C., Landick,R., and Block,S.M. (2006). Sequence-resolved detection of pausing by single RNA polymerase molecules. Cell *125*, 1083-1094.

Hommais,F., Krin,E., Laurent-Winter,C., Soutourina,O., Malpertuy,A., Le Caer,J.P., Danchin,A., and Bertin,P. (2001). Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. Mol. Microbiol. *40*, 20-36.

Johansson,J. and Uhlin,B.E. (1999). Differential protease-mediated turnover of H-NS and StpA revealed by a mutation altering protein stability and stationary-phase survival of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A *96*, 10776-10781.

Jordi,B.J., Fielder,A.E., Burns,C.M., Hinton,J.C., Dover,N., Ussery,D.W., and Higgins,C.F. (1997). DNA binding is not sufficient for H-NS-mediated repression of proU expression. J. Biol. Chem. *272*, 12083-12090.

Jordi,B.J. and Higgins,C.F. (2000). The downstream regulatory element of the proU operon of Salmonella typhimurium inhibits open complex formation by RNA polymerase at a distance. J. Biol. Chem. *275*, 12123-12128.

Kaidow,A., Wachi,M., Nakamura,J., Magae,J., and Nagai,K. (1995). Anucleate cell production by Escherichia coli delta hns mutant lacking a histone-like protein, H-NS. J. Bacteriol. *177*, 3589-3592.

Klauck,E., Bohringer,J., and Hengge-Aronis,R. (1997). The LysR-like regulator LeuO in Escherichia coli is involved in the translational regulation of rpoS by affecting the expression of the small regulatory DsrA-RNA. Mol. Microbiol. *25*, 559-569.

Konan,K.V. and Yanofsky,C. (2000). Rho-dependent transcription termination in the tna operon of Escherichia coli: Roles of the boxA sequence and the rut site. J. Bacteriol. *182*, 3981-3988.

Kushner,S.R. (2002). mRNA decay in Escherichia coli comes of age. J. Bacteriol. *184*, 4658-4665.

Laemmli,U.K. (1970). Cleavage of Structural Proteins During Assembly of Head of Bacteriophage-T4. Nature *227*, 680-&.

Landick,R. (2006). The regulatory roles and mechanism of transcriptional pausing. Biochem. Soc. Trans. *34*, 1062-1066.

Landick,R., Wang,D., and Chan,C.L. (1996). Quantitative analysis of transcriptional pausing by Escherichia coli RNA polymerase: his leader pause site as paradigm. Methods Enzymol. *274*, 334-353.

Lease,R.A. and Belfort,M. (2000). A trans-acting RNA as a control switch in Escherichia coli: DsrA modulates function by forming alternative structures. Proc. Natl. Acad. Sci. U. S. A *97*, 9919-9924.

Lease,R.A., Cusick,M.E., and Belfort,M. (1998). Riboregulation in Escherichia coli: DsrA RNA acts by RNA:RNA interactions at multiple loci. Proc. Natl. Acad. Sci. U. S. A *95*, 12456-12461.

Li,Y. and Altman,S. (2003). A specific endoribonuclease, RNase P, affects gene expression of polycistronic operon mRNAs. Proceedings of the National Academy of Sciences of the United States of America *100*, 13213-13218.

Lucchini,S., Rowley,G., Goldberg,M.D., Hurd,D., Harrison,M., and Hinton,J.C. (2006a). H-NS Mediates the Silencing of Laterally Acquired Genes in Bacteria. PLoS. Pathog. *2*.

Lucchini,S., Rowley,G., Goldberg,M.D., Hurd,D., Harrison,M., and Hinton,J.C.D. (2006b). H-NS mediates the silencing of laterally acquired genes in bacteria. Plos Pathogens *2*, 746-752.

Lucht,J.M., Dersch,P., Kempf,B., and Bremer,E. (1994a). Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled proU operon of Escherichia coli. J. Biol. Chem. *269*, 6578.

Lucht,J.M., Dersch,P., Kempf,B., and Bremer,E. (1994b). Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled proU operon of Escherichia coli. J. Biol. Chem. *269*, 6578.

Luijsterburg,M.S., Noom,M.C., Wuite,G.J., and Dame,R.T. (2006). The architectural role of nucleoid-associated proteins in the organization of bacterial chromatin: A molecular perspective. J. Struct. Biol. *156*, 262-272.

Mackie,G.A. (2000). Stabilization of circular rpsT mRNA demonstrates the 5 '-end dependence of RNase E action in vivo. Journal of Biological Chemistry *275*, 25069-25072.

Madhusudan,S., Paukner,A., Klingen,Y., and Schnetz,K. (2005). Independent regulation of H-NS-mediated silencing of the bgl operon at two levels: upstream by BglJ and LeuO and downstream by DnaKJ. Microbiology *151*, 3349-3359.

Magyar,A., Zhang,Y.D., Kohn,H., and Widger,W.R. (1996). The antibiotic bicyclomycin affects the secondary RNA binding site of Escherichia coli transcription termination factor Rho. Journal of Biological Chemistry *271*, 25369-25374.

Majdalani,N., Vanderpool,C.K., and Gottesman,S. (2005). Bacterial small RNA regulators. Critical Reviews in Biochemistry and Molecular Biology *40*, 93-113.

May,G., Faatz,E., Lucht,J.M., Haardt,M., Bolliger,M., and Bremer,E. (1989). Characterization of the osmoregulated Escherichia coli proU promoter and identification of ProV as a membrane-associated protein. Mol. Microbiol. *3*, 1521-1531.

McClure,W.R., Cech,C.L., and Johnston,D.E. (1978). Steady-State Assay for the Rna-Polymerase Initiation Reaction. Journal of Biological Chemistry *253*, 8941-8948.

Mellies,J., Brems,R., and Villarejo,M. (1994). The Escherichia coli proU promoter element and its contribution to osmotically signaled transcription activation. J. Bacteriol. *176*, 3638-3645.

Miller,J.H. (1972). Experiments in Molecular Genetics. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Miller,J.H. (1992). A Short Course in Bacterial Genetics: A Laboratory Manual for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 456 pp.).

Mooney,R.A. and Landick,R. (1999). RNA polymerase unveiled. Cell *98*, 687-690.

Mosrin-Huaman,C., Turnbough,C.L., Jr., and Rahmouni,A.R. (2004). Translocation of Escherichia coli RNA polymerase against a protein roadblock in vivo highlights a passive sliding mechanism for transcript elongation. Mol. Microbiol. *51*, 1471-1481.

Muffler,A., Fischer,D., and Hengge-Aronis,R. (1996). The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for rpoS translation in Escherichia coli. Genes Dev. *10*, 1143-1151.

Mukerji,M. and Mahadevan,S. (1997). Characterization of the negative elements involved in silencing the bgl operon of Escherichia coli: possible roles for DNA gyrase, H-NS, and CRP-cAMP in regulation. Mol. Microbiol. *24*, 617-627.

Nagarajavel, V. H-NS mediated repression of *E.coli bgl* and *proU* operons. Ph.D.Thesis, Institute for Genetics, University of Cologne . 2007.
Ref Type: Thesis/Dissertation

Nagarajavel,V., Madhusudan,S., Dole,S., Rahmouni,A.R., and Schnetz,K. (2007). Repression by Binding of H-NS within the Transcription Unit. J. Biol. Chem. *282*, 23622-23630.

Nahvi,A., Sudarsan,N., Ebert,M.S., Zou,X., Brown,K.L., and Breaker,R.R. (2002). Genetic control by a metabolite binding mRNA. Chemistry & Biology *9*, 1043-1049.

Narberhaus,F., Waldminighaus,T., and Chowdhury,S. (2006). RNA thermometers. Fems Microbiology Reviews *30*, 3-16.

Navarre,W.W., Porwollik,S., Wang,Y., McClelland,M., Rosen,H., Libby,S.J., and Fang,F.C. (2006). Selective silencing of foreign DNA with low GC content by the H-NS protein in Salmonella. Science *313*, 236-238.

Neelakanta, G. Genome variations in comensal and pathogenic *E.coli*. Ph.D.Thesis, Institute for Genetics, University of Cologne . 2005.
Ref Type: Generic

Nudler,E. (1999). Transcription elongation: structural basis and mechanisms. J. Mol. Biol. *288*, 1-12.

Nudler,E. and Gottesman,M.E. (2002). Transcription termination and anti-termination in E-coli. Genes to Cells *7*, 755-768.

Ono,S., Goldberg,M.D., Olsson,T., Esposito,D., Hinton,J.C., and Ladbury,J.E. (2005). H-NS is a part of a thermally controlled mechanism for bacterial gene regulation. Biochem. J. *391*, 203-213.

Oshima,T., Ishikawa,S., Kurokawa,K., Aiba,H., and Ogasawara,N. (2006). Escherichia coli Histone-Like Protein H-NS Preferentially Binds to Horizontally Acquired DNA in Association with RNA Polymerase. DNA Res.

Overdier,D.G. and Csonka,L.N. (1992). A transcriptional silencer downstream of the promoter in the osmotically controlled proU operon of Salmonella typhimurium. Proc. Natl. Acad. Sci. U. S. A *89*, 3140-3144.

Owen-Hughes,T.A., Pavitt,G.D., Santos,D.S., Sidebotham,J.M., Hulton,C.S., Hinton,J.C., and Higgins,C.F. (1992). The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. Cell *71*, 255-265.

Pertzev,A.V. and Nicholson,A.W. (2006). Characterization of RNA sequence determinants and antideterminants of processing reactivity for a minimal substrate of Escherichia coli ribonuclease III. Nucleic Acids Research *34*, 3708-3721.

Radde, N., Gebert, J., Faigle, U., Schrader, R., and and Schnetz, K. Modeling feedback loops in the H-NS mediated regulation of the Escherichia coli *bgl* operon. 2007.
Ref Type: Unpublished Work

Rajkumari,K., Ishihama,A., and Gowrishankar,J. (1997). Evidence for transcription attenuation rendering cryptic a sigmaS-dependent promoter of the osmotically regulated proU operon of Salmonella typhimurium. J. Bacteriol. *179*, 7169-7173.

Rajkumari,K., Kusano,S., Ishihama,A., Mizuno,T., and Gowrishankar,J. (1996a). Effects of H-NS and potassium glutamate on sigmaS- and sigma70-directed transcription in vitro from osmotically regulated P1 and P2 promoters of proU in Escherichia coli. J. Bacteriol. *178*, 4176-4181.

Rajkumari,K., Kusano,S., Ishihama,A., Mizuno,T., and Gowrishankar,J. (1996b). Effects of H-NS and potassium glutamate on sigmaS- and sigma70-directed transcription in vitro from osmotically regulated P1 and P2 promoters of proU in Escherichia coli. J. Bacteriol. *178*, 4176-4181.

Richardson,J.P. (2002). Rho-dependent termination and ATPases in transcript termination. Biochimica et Biophysica Acta-Gene Structure and Expression *1577*, 251-260.

Rimsky,S. (2004). Structure of the histone-like protein H-NS and its role in regulation and genome superstructure. Curr. Opin. Microbiol. *7*, 109-114.

Rimsky,S., Zuber,F., Buckle,M., and Buc,H. (2001). A molecular mechanism for the repression of transcription by the H-NS protein. Mol. Microbiol. *42*, 1311-1323.

Sambrook,J. and Russell,D. (2001). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schnetz,K. (1995). Silencing of Escherichia coli bgl promoter by flanking sequence elements. EMBO J. *14*, 2545-2550.

Schnetz,K. and Rak,B. (1988a). Cleavage by EcoO109 and DraII is inhibited by overlapping dcm methylation. Nucleic Acids Res. *16*, 1623.

Schnetz,K. and Rak,B. (1988b). Regulation of the bgl operon of Escherichia coli by transcriptional antitermination. EMBO J. *7*, 3271-3277.

Schnetz,K. and Rak,B. (1992). IS5: a mobile enhancer of transcription in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A *89*, 1244-1248.

Schnetz,K., Toloczyki,C., and Rak,B. (1987). Beta-glucoside (bgl) operon of Escherichia coli K-12: nucleotide sequence, genetic organization, and possible evolutionary relationship to regulatory components of two Bacillus subtilis genes. J. Bacteriol. *169*, 2579-2590.

Schnetz,K. and Wang,J.C. (1996). Silencing of the Escherichia coli bgl promoter: effects of template supercoiling and cell extracts on promoter activity in vitro. Nucleic Acids Res. *24*, 2422-2428.

Schroder,O. and Wagner,R. (2002). The bacterial regulatory protein H-NS--a versatile modulator of nucleic acid structures. Biol. Chem. *383*, 945-960.

Shin,M., Song,M., Rhee,J.H., Hong,Y., Kim,Y.J., Seok,Y.J., Ha,K.S., Jung,S.H., and Choy,H.E. (2005). DNA looping-mediated repression by histone-like protein H-NS: specific requirement of Esigma70 as a cofactor for looping. Genes Dev. *19*, 2388-2398.

Smyth,C.P., Lundback,T., Renzoni,D., Siligardi,G., Beavil,R., Layton,M., Sidebotham,J.M., Hinton,J.C., Driscoll,P.C., Higgins,C.F., and Ladbury,J.E. (2000). Oligomerization of the chromatin-structuring protein H-NS. Mol. Microbiol. *36*, 962-972.

Stewart,V., Landick,R., and Yanofsky,C. (1986). Rho-dependent transcription termination in the tryptophanase operon leader region of Escherichia coli K-12. J. Bacteriol. *166*, 217-223.

Tadigotla,V.R., Maoileidigh,O., Sengupta,A.M., Epshtein,V., Ebright,R.H., Nudler,E., and Ruckenstein,A.E. (2006). Thermodynamic and kinetic modeling of transcriptional pausing. Proc. Natl. Acad. Sci. U. S. A *103*, 4439-4444.

Tendeng,C. and Bertin,P.N. (2003). H-NS in Gram-negative bacteria: a family of multifaceted proteins. Trends Microbiol. *11*, 511-518.

Tomoyasu,T., Ogura,T., Tatsuta,T., and Bukau,B. (1998). Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in Escherichia coli. Molecular Microbiology *30*, 567-581.

Toulme,F., Guerin,M., Robichon,N., Leng,M., and Rahmouni,A.R. (1999). In vivo evidence for back and forth oscillations of the transcription elongation complex. EMBO J. *18*, 5052-5060.

Toulme,F., Mosrin-Huaman,C., Artsimovitch,I., and Rahmouni,A.R. (2005). Transcriptional pausing in vivo: a nascent RNA hairpin restricts lateral movements of RNA polymerase in both forward and reverse directions. J. Mol. Biol. *351*, 39-51.

Umanski,T., Rosenshine,I., and Friedberg,D. (2002). Thermoregulated expression of virulence genes in enteropathogenic Escherichia coli. Microbiology *148*, 2735-2744.

Valentin-Hansen,P., Eriksen,M., and Udesen,C. (2004). The bacterial Sm-like protein Hfq: a key player in RNA transactions. Molecular Microbiology *51*, 1525-1533.

Vieu,E. and Rahmouni,A.R. (2004). Dual role of boxB RNA motif in the mechanisms of termination/antitermination at the lambda tR1 terminator revealed in vivo. J. Mol. Biol. *339*, 1077-1087.

Wilson,G.G., Young,K.Y.K., Edlin,G.J., and Konigsberg,W. (1979). High-frequency generalised transduction by bacteriophage T4. Nature *280*, 80-82.

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