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New Insights into Transcriptional Regulation by H-NS

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Abstract

H-NS, a nucleoid-associated DNA-binding protein of enteric bacteria, was discovered thirty-five years ago and subsequently found to exert widespread and highly pleiotropic effects on gene regulation. H-NS binds to high-affinity sites and spreads along adjacent AT-rich DNA to silence transcription. Preferential binding to sequences with higher AT-content than the resident genome allows H-NS to repress the expression of foreign DNA in a process known as “xenogeneic silencing.” Counter-silencing by a variety of mechanisms facilitates the evolutionary acquisition of horizontally transferred genes and their integration into pre-existing regulatory networks. This review will highlight recent insights into the mechanism and biological importance of H-NS-DNA interactions.

H-NS-- A Nucleoid Protein

H-NS is an abundant DNA-binding protein implicated in the organization of the bacterial chromosome [1]. H-NS and other nucleoid-associated proteins can affect DNA topology at specific loci, thereby modulating gene transcription. Binding by these proteins is proposed to selectively direct supercoiling effects to promoters [2•]. Mutation of *hns* alters the responsiveness of transcription to changes in DNA superhelicity. Regulatory effects of supercoiling are linked to metabolic and environmental conditions. Thus, H-NS has been viewed as a nucleoid structuring protein with global effects on gene expression [3].

H-NS exists primarily as a dimer at low concentrations but can multimerize into higher order complexes [4,5] that form bridges between adjacent DNA helices [6]. Optical tweezers have been used to demonstrate that H-NS dimers or multimers can simultaneously interact with separate DNA binding sites [7••]. H-NS-coated DNA is not self-interactive, suggesting that dimerization or oligomerization must precede DNA binding for bridging to occur. Force measurements suggest that transcription barriers created by H-NS binding are weak (~7 pN) and readily overcome, so that H-NS oligomers pose only a relative barrier to translocating RNA polymerase. DNA bridging is a conserved feature of H-NS homologs found in most gram-negative bacteria [8] that appears to constrain large DNA loops [9••] and helps to account for the effects of H-NS on transcription. However, it must be noted that the relationship between bridging as a general effect and the silencing of specific promoters is not yet clear.

H-NS is more appropriately viewed as a determinant of chromosomal architecture rather than as a general structural component. The analysis of nucleoids treated with urea suggests that

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neither H-NS nor the nucleoid-associated proteins Fis, Dps or StpA is required for a cooperative transition between compacted and partially expanded forms of the chromosome [10]. The bacterial chromosome is organized into topological domains averaging 10kbp, resulting in approximately 400 domains per chromosome. Although H-NS bridging constrains short loop formation, a possible role of H-NS in the formation or maintenance of larger topological domain barriers has been recently highlighted [11,12]. DNA looping has been proposed as a fundamental mechanism for action at a distance in the control of gene expression by proteins. DNA resists bending and twisting due to its inherent rigidity, and a major function of architectural proteins is to reshape DNA and/or modify its stiffness. It has been suggested that looping might require a binding protein to act not only at the end of a loop but also on intervening DNA to enhance flexibility [9]. An assay to study the effects of the nucleoid proteins H-NS, HU and IHF on the *in vivo* flexibility of DNA demonstrated that H-NS destabilizes rather than stabilizes small loops [9]. In contrast, HU promotes DNA looping *in vivo*. Changes in superhelicity do not appear to explain the effects of architectural proteins on DNA looping. Since looping can affect DNA affinity for regulatory molecules and promote cooperative interactions [13], H-NS constraints on looping may account for some of its repressive actions.

Effects of H-NS and other nucleoid-associated proteins on superhelicity are proposed to have important regulatory consequences during changes in environmental conditions [2]. H-NS has long been regarded as a global modulator of gene expression in response to pH, temperature, osmolarity and growth phase [14-16]. A large proportion of *Salmonella* genes induced upon temperature shift from 25°C to 37°C are dependent on H-NS [17], an effect attributed to putative conformational changes in H-NS and reduced DNA binding. However, a large number of H-NS-repressed genes remain repressed at 37°C, suggesting that a simple effect of temperature on H-NS multimerization or DNA binding cannot account for the temperature-dependent derepression of a subset of genes. Moreover, H-NS oligomerization has been reported to be higher at 37°C than 25°C [18] and uninfluenced by further temperature increases to 48°C or pH variation between 4.0 and 9.0. Similarly, while H-NS represses the transcription of a number of osmoregulated genes [14], many H-NS-silenced genes are unaffected by changes in osmolarity. These observations indicate that the relationship between H-NS and environmentally dependent alterations in gene expression is complex, and that H-NS should not be viewed as a simple temperature or osmolarity sensor.

Recognition of DNA by H-NS

H-NS was regarded for many years as a DNA binding protein without a specific consensus sequence for binding, and the well-known ability of H-NS to affect a wide range of genes was attributed to a preference for particular DNA structures [1]. H-NS recognition sites typically display planar curvature specified by AT-rich motifs, as commonly found at promoters. However, quantitative analysis of the H-NS binding site at the osmoregulated *E. coli proU* promoter, which regulates an osmoprotectant uptake locus, has demonstrated that H-NS specifically recognizes a 10bp sequence with a K_D of ~15 nM [19•]. This motif is also present at two locations downstream of the *proU* promoter. Synergy between these sites and lower affinity sites in the region favors the formation of a specific nucleoprotein complex that efficiently represses transcription. The 10bp high-affinity binding motif has been proposed as a nucleation site for H-NS binding. Upon binding to high affinity sites, H-NS spreads along DNA using sites of lower affinity to occupy the promoter region, allowing the formation of higher order structures. Moreover, insertion of a high-affinity site into a GC-rich sequence enhances H-NS binding, excluding the possibility that high affinity binding requires a more extensive structure for recognition. It has therefore been suggested that an H-NS binding region contains several sites with variable affinity, and that the organization of binding sites determines the formation of a repressive nucleoprotein complex.

Comparison of the 10bp high-affinity binding motif with a sequence inferred by footprinting experiments using the isolated DNA-binding domain of H-NS [20•] and with predicted and confirmed H-NS sites at the *fis* promoter allowed the identification of a putative consensus recognition sequence for H-NS. This experimentally deduced motif is presented in figure 1. The presence of this motif has been correlated with H-NS binding *in vivo* in chromatin immunoprecipitation on microarray (ChIP-on-chip) experiments [21•]. A statistically significant correlation was observed between the proposed consensus sequence and observed H-NS binding. Some promoters such as *bgl* display more than 10 potential binding sites for H-NS, while others like *nir* contain fewer binding motifs. This suggests that the regulation of gene expression by H-NS can be modulated by variations in the number and organization of binding sites. In summary, although DNA recognition by H-NS was long thought to be based on structure rather than sequence, the discovery of a specific high affinity H-NS-binding motif suggests that H-NS can bind to one or more high affinity sites and subsequently polymerize and bind adjacent sites to spread regionally along AT-rich DNA [20•]. Once bound, the predominant effect of H-NS on gene expression is repressive [22••].

Mechanism of Transcriptional Silencing by H-NS

Although some investigators did not find a correlation between H-NS and RNAP binding sites [23•], others have observed a significant correlation [21•,24] consistent with a model in which H-NS nucleoprotein complexes can trap RNAP to prevent transcription. Under conditions favoring repression, H-NS prevents open complex formation by RNAP at the *proU* promoter, and appears to exert similar actions at the *bgl* promoter [25••]. In other instances, such as the *hdeAB* promoter, H-NS appears to trap the open complex once it has formed [26•]. Thus, H-NS appears to be capable of silencing transcription by a variety of mechanisms.

The existence of specific binding sites for H-NS only partially explains the silencing effect of H-NS at various promoters. This question has been nicely approached by both experimental and computational work on the *bgl* promoter, and to a lesser extent, the *proU* promoter. The effect of H-NS binding on regions upstream (URE) and downstream (DRE) from the transcription start site on the activity of these promoters [27] and models derived from these results lead to the conclusion that repression by H-NS is inversely correlated to the transcription elongation rate across the H-NS binding region, suggesting that RNA polymerase engaged in elongation can disrupt the repressing nucleoprotein complex formed by H-NS. In the case of the *bgl* promoter, silencing requires synergy between H-NS bound at the URE and DRE sequences. H-NS bound to the DRE alone would not be predicted to impede RNA polymerase translocation. Thus, *bgl* transcriptional silencing by H-NS is intimately linked to protein-protein interactions between H-NS bound upstream and downstream of the promoter. The feedback loops created by H-NS repression of transcription initiation and downstream anti-termination mediated by the BglG protein in this model help to explain how small changes in *bgl* promoter activity can have large effects on overall operon expression [28•]. At the *proU* promoter, the mechanism by which a similar regulatory loop might be formed is not yet clear [25••,28•].

Interactions between H-NS and other Nucleoid Proteins

In addition to forming homodimers and homooligomers, H-NS can form heteromeric complexes by interacting with other nucleoid-associated proteins such as Hha and the H-NS paralog StpA (figure 2). Phenotypes associated with Hha include altered plasmid supercoiling and insertion sequence transposition [29]. Hha is a member of the Hha/YmoA family of proteins, which by themselves do not bind DNA but structurally resemble the N-terminus of H-NS (containing the dimerization domain) and are capable of associating with H-NS to

enhance its repressive action at some but not all loci [30,31]. Hha/YmoA proteins are less abundant than H-NS, so heterodimers are the major form of Hha/YmoA in the cell [30].

Levels of StpA in wild-type cells are also low, because *stpA* expression is inhibited by H-NS. Furthermore, in its homodimeric form StpA is degraded by the Lon protease. Consequently the heterodimer form of StpA is predominant. A mutation in *stpA* has no discernable phenotype on its own in *E. coli* but derepresses a subset of genes in the absence of H-NS [32]. StpA can repress its own expression but cannot repress the *bgl* operon unless at least the N-terminus of H-NS is present to allow formation of heterodimers [33]. H-NS and StpA repress the expression of genes encoding the type II secretory system required for secretion of *E. coli* heat-labile enterotoxin (LT) [34] by inhibiting open complex formation. H-NS also represses *eltAB* encoding the enterotoxin itself [35]. H-NS can act synergistically as co-repressor with LRP, the leucine-responsive regulatory protein, to inhibit expression of the ribosomal RNA promoter [36], although no direct interaction between LRP and H-NS has been reported. Much remains to be learned about the physiological significance of H-NS interactions with other nucleoid proteins.

Counter-silencing of H-NS and its Functional Consequences

A striking correlation has been observed between AT content and H-NS binding [22•,23•]. High AT-content relative to the resident genome has long been recognized as a hallmark of horizontally transferred DNA [37]. An *hns* mutation in *Salmonella* is detrimental to cell viability unless accompanied by mutations in positive regulators of virulence gene expression (*rpoS*, *phoP*) or pathogenicity islands (SPI2), suggesting that overexpression of H-NS-repressed loci is harmful to the cell.

The ability of H-NS to recognize foreign DNA with higher AT content than the resident genome [22•,23•] has been referred to as “xenogeneic silencing” [38•]. The paradigm of xenogeneic silencing is providing a new framework for understanding the genomic evolution of pathogenic bacteria and specifically how alien genes can be integrated into existing regulatory networks [38•]. The recognition and transcriptional silencing of AT-rich DNA by H-NS simultaneously protects bacteria from adventitious effects of foreign gene expression [16,22•] and facilitates the incorporation of such genes. Moreover, xenogeneic silencing provides a rationale for the conservation of GC content by bacterial species, as a means of differentiating self and non-self DNA and an explanation for the strong tendency of foreign sequences to have higher AT content than the resident genome (allowing such sequences to be more readily harnessed). The inability of horizontally-transferred GC-rich DNA to be recognized and silenced by H-NS would make such sequences less likely to be tolerated by the recipient. Given the prominent role of horizontal gene transfer in the evolution of pathogenic bacteria [39], xenogeneic silencing is of particular importance in understanding the regulation of virulence gene expression [22•].

Regulated expression of H-NS-silenced genes requires specific mechanisms of counter-silencing, such as activation by an alternative sigma factor or competition from sequence-specific DNA-binding proteins and H-NS homologs (figure 3). Direction of transcription by the alternative sigma factor σ^S appears to be an important mechanism of counter-silencing. The specific ability of H-NS to assemble nucleoprotein complexes with $E\sigma^{70}$ but not $E\sigma^S$ RNAP holoenzyme may account in part for the selectivity of σ^S promoters [26•,40•]. In the absence of H-NS, it appears that many σ^S -activated promoters can be transcribed by either form of RNAP. For example, the *csgBA* and *hdeAB* loci of *E. coli* require $E\sigma^S$ for expression in the presence of H-NS, but can be transcribed by $E\sigma^{70}$ in an *hns rpoS* mutant background [41]. Hsp31, encoded by *hchA*, is regulated by both $E\sigma^{70}$ and $E\sigma^S$. H-NS silences *hchA* expression during exponential growth, when expression is driven by $E\sigma^{70}$, but does not effectively repress

hchA expression in stationary phase when $E\sigma^S$ becomes dominant [42]. Interestingly, H-NS also appears to be required for the normal proteolytic turnover of σ^S [43]. Circumvention of H-NS silencing provides an explanation for σ^S involvement in the regulation of horizontally-acquired virulence genes [44,45], and complex interactions between σ^S and H-NS are responsible for their co-involvement in the expression of certain osmoregulated genes [41, 46,47].

The ability of other DNA-binding proteins to act as counter-silencers by antagonizing the effects of H-NS on transcription and the ability of H-NS to polymerize along AT-rich sequence tracts can rationalize the location of transcription factor binding sites downstream of translational start sites, which is difficult to reconcile with classical activation mechanisms but consistent with an ability of H-NS to mediate interactions between upstream and downstream binding sites [25•]. Many examples of counter-silencing by DNA-binding proteins have been recently described. The AraC-like activators GadX and GadW promote *gadA* (glutamate decarboxylase) expression, important for acid stress resistance, by antagonizing H-NS [48]. Interestingly, the RNAP-associated protein SspA has been proposed to enhance cell survival at acid pH by reducing *hns* expression during stationary phase [49]. The MarR family regulator SlyA counters H-NS silencing at sites both upstream and downstream of the hemolysin gene *hlyE* transcriptional start site in *E. coli* [50] by competing with H-NS for binding. Interestingly, SlyA appears to counter H-NS silencing at the *E. coli* K5 capsule biosynthetic gene cluster by remodeling the nucleoprotein complex rather than by inhibiting H-NS binding [51]. Thus, a single protein may counter silencing by more than one mechanism. The LysR family regulator LeuO counters H-NS silencing of the putative virulence gene *yjjQ* in avian pathogenic *E. coli* [52] and H-NS/StpA silencing of the *ompSI* porin gene in *Salmonella* [53]. The importance of H-NS in regulating horizontal gene transfer is not limited to pathogenesis. The regulatory protein TraJ counter-silences H-NS repression of the *tra* genes in the F plasmid of *E. coli* but is dispensable for conjugal transfer in an *hns* mutant [54], demonstrating that H-NS can limit plasmid mobilization. Some plasmids such as the *Salmonella* plasmid R27 carry their own H-NS-like protein (Sfh, in the case of R27) that appears to prevent detrimental consequences of H-NS titration by AT-rich sequences [55•] and thereby allow the recipient cell to tolerate the plasmid.

H-NS homologs might act as counter-silencers by competing for binding sites or interfering with multimerization. Ler is an H-NS homolog (figure 2) encoded by the LEE (Locus of Enterocyte Effacement) pathogenicity island found in EHEC (enterohemorrhagic *E. coli*), EPEC (enteropathogenic *E. coli*) and *Citrobacter rodentium*. Ler can antagonize H-NS repression of LEE genes but also antagonizes the expression of Lpf long polar fimbriae [56]. EPEC and UPEC (uropathogenic *E. coli*) genomic islands carry additional truncated H-NS homologs called H-NST that can antagonize H-NS silencing, but with less specificity than Ler [57].

H-NS silencing and counter-silencing of virulence genes is important in other pathogenic γ -proteobacteria as well. ToxT, a transcriptional activator belonging to the AraC family, promotes the production of cholera toxin and other virulence factors of *Vibrio cholerae* by both activating transcription and countering H-NS silencing [58,59]. The VirB protein activates virulence gene expression in *Shigella* but resembles a plasmid partitioning protein rather than a classical transcriptional activator. VirB was found not to promote *icsB* transcription in an *in vitro* assay but rather to antagonize H-NS [60]. Binding of VirB upstream of the *icsB* promoter to a heptameric motif related to plasmid partitioning *cis* elements appears to result in derepression. Nearly all RovA (SlyA)-activated genes in *Yersinia* spp. are repressed by H-NS, and most RovA-bound promoters are also bound by H-NS [61]. The *Y. enterocolitica* *inv* gene encoding invasins is repressed by H-NS in conjunction with Hha (called YmoA in *Y. enterocolitica*) [62] and derepressed by RovA. Interestingly, removal of the last four amino

acid residues of RovA abolishes its ability to displace H-NS even though dimerization and DNA-binding *in vitro* are retained, leading to the suggestion that the C-terminus of RovA might interact with H-NS directly (or stabilize interactions with RNAP or other RovA dimers) [63].

All five *Salmonella enterica* serovar Typhimurium pathogenicity islands (SPI1-5) are bound by H-NS. Activation of the SPI1 *rtsA* gene only requires the AraC family activators HilC and HilD if both H-NS and Hha are present [64,65]. The SPI1-encoded transcriptional activator HilA not only promotes expression of SPI1 genes but also appears to counter H-NS silencing of the SPI4 pathogenicity island [66], which acts in concert with SPI1 during *Salmonella* interactions with the host intestinal mucosa [67]. A requirement for the transcriptional activator SsrB for SPI2 expression is reduced in the absence of H-NS, suggesting that SsrB both activates transcription and relieves H-NS-mediated repression [68]. This rationalizes the presence of SsrB binding sites both within the promoter region and downstream of the translational start site. An AT-rich gene from *H. pylori* introduced into *S. Typhimurium* was silenced by H-NS [22•], suggesting that H-NS inactivation may facilitate the overexpression of foreign genes for biotechnological applications.

Conclusions

A great deal has been learned about H-NS since its original description as a DNA-binding protein able to stimulate bacteriophage gene transcription [69]. Biophysical studies have revealed how H-NS dimers bridge DNA. Separate studies have demonstrated how cooperative binding and polymerization can impede transcription initiation. Global analyses of H-NS binding and effects on gene expression demonstrate that H-NS recognizes and preferentially binds to sequences with AT-content higher than the resident genome. A high-affinity H-NS binding site has been identified which can nucleate H-NS at a promoter region and allow subsequent spreading along adjacent lower affinity sites to silence transcription. Transcriptional silencing of sequences bound by H-NS can in turn be countered by a variety of mechanisms including activation by an alternative sigma factor, antagonism by other DNA-binding proteins, and interactions with H-NS paralogs. This provides a richer paradigm by which to understand the regulation of bacterial gene expression, in which silencing and counter-silencing complement transcriptional activation and repression. The ability of H-NS to recognize and silence the transcription of AT-rich DNA has important implications for the evolution of bacteria by horizontal gene transfer. Thus, the view of H-NS as a modulator of gene expression in response to environmental conditions is now being augmented by the appreciation that xenogeneic silencing by H-NS facilitates the integration of foreign DNA into regulatory networks.

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Figure 1. Logo representation of the high-affinity H-NS binding motif

Figure 2. Sequence alignment of the four *E. coli* H-NS like proteins cited in the text

Structural modularity is evident in this family of gene-regulating proteins. For clarity, sequences have been aligned with the known structures of *E. coli* H-NS amino- and carboxyl-terminal domains. Models are based on RMN studies of the N- terminal dimer (a) coiled coil interaction and the C-terminal (b) domains of H-NS [3]. A schematic representation of the domain organization of H-NS (c) is aligned with the primary sequence of H-NS, StpA, Hha and Ler. The N-terminal domain contains three alpha helices ($\alpha 1$, $\alpha 2$ and $\alpha 3$). A linker region separating the N and C termini consists of two β strands intercalated between loops 1 and 1' and loops 1' and 2 terminating in an α helix and a 3_{10} α helix that form a nucleic acid binding domain.

Figure 3. Mechanisms of Counter-silencing

Potential mechanisms of counter-silencing include: (A) disruption of H-NS complexes by multimerization antagonists such as H-NST; (B) competition for DNA binding by a high-affinity sequence specific DNA binding protein such as ToxT or RovA; (C) activation of certain promoters such as the *E. coli hdeAB* promoter by the alternative sigma factor σ^S ; (D) changes in promoter geometry due to protein binding or environmental changes that disrupt H-NS complex formation such as may occur at the *virF* promoter in *Shigella*. These mechanisms are not mutually exclusive, and it is possible that more than one mechanism may be operating at any given promoter. Reproduced with permission from (38).