nature structural & molecular biology

H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing

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H-NS is a protein of the bacterial nucleoid involved in DNA compaction and transcription regulation. *In vivo,* H-NS selectively silences specific genes of the bacterial chromosome. However, many studies have concluded that H-NS binds sequence-independently to DNA, leaving the molecular basis for its selectivity unexplained. We show that the negative regulatory element (NRE) of the supercoiling-sensitive *Escherichia coli proU* gene contains two identical high-affinity binding sites for H-NS. Cooperative binding of H-NS is abrogated by changes in DNA superhelical density and temperature. We further demonstrate that the high-affinity sites nucleate cooperative binding and establish a nucleoprotein structure required for silencing. Mutations in these sites result in loss of repression by H-NS. In this model, silencing at *proU,* and by inference at other genes directly regulated by H-NS, is tightly controlled by the cooperativity between bound H-NS molecules.

H-NS is a small abundant protein that is key in bacterial gene regulation, acting as a global negative regulator and controlling the expression of a large number of genes (>200), whose products are mainly involved in bacterial adaptation to environmental changes. For instance, H-NS–induced repression is released in response to changes affecting either osmolarity or temperature, and in response to acidic shock[1]. H-NS is also involved in bacterial virulence, which is itself tightly regulated by environmental conditions[2–5].

In vivo, H-NS selectively silences particular genes or regions of the bacterial chromosome[6,7], which supports the existence of specific recognition sites; yet many studies have concluded that H-NS binds DNA in a relatively sequence-independent manner[8]. The H-NS–binding region varies greatly, ranging in size from 10 to ~100 base pairs (bp)[9–14]. This variability probably reflects a poorly defined type of nucleoprotein organization capable of reconciling the dual properties of this protein as a chromatin organizer and a modulator of gene expression. Both of these functions must be compatible with the high concentration of the protein in the cell (~20 μM)[15,16].

Although several studies have proposed that H-NS has a preference for intrinsically curved DNA[12,17,18], DNA footprinting of H-NS reveals precisely positioned binding sites[9,19,20]. Furthermore, even though the highest-affinity binding sites are located in curved sequences[9], medium- and low-affinity sites have also been found in the vicinity of the curved regions. A study conducted on artificial promoters led to the conclusion that the curved sequence constitutes a nucleation point that induces the binding of proteins to lower-affinity sites, leading eventually to the formation of a competent repression complex[9]. Here we address the mechanism of specific DNA recognition by the H-NS protein in the case of the natural *proU* promoter from *Escherichia coli*.

ProU is a binding protein–dependent transport system that mediates the accumulation of compatible solutes such as glycine-betaine, L-proline and related compounds during cell growth in media with elevated osmolarity[21]. This system needs to be active only in high-osmolarity media, where the expression of *proU* is induced several hundred times[22]. Regulation of *proU* is achieved primarily at the level of transcription: two start sites of transcription, P1 and P2, have been identified (**Fig. 1**), respectively 250 and 60 bp upstream from the first structural gene, *proV*. The P1 promoter is transcribed by the core RNA polymerase associated with the stationary-phase σ factor (σ^S) subunit[23,24], and P2 by RNA polymerase associated with σ^{70} (refs. 23,25). Various factors influence *proU* expression, including an increase in the intracellular potassium concentration[26], although the latter is insufficient to regulate *proU* expression[27]. At a molecular level, the *proU* promoter is regulated by changes in DNA topology[21]. Several *cis*-regulatory elements have been described. Two elements close to each promoter, P1R and P2R, confer a five- to eight-fold change in transcription in response to changes in osmotic tension, but these have not been further characterized[21]. In contrast, many studies have been carried out to characterize a third regulatory element, called the downstream regulatory element (DRE) in *Salmonella typhimurium* or the negative regulatory element (NRE) in *E. coli*. The NRE partially overlaps with *proV*[28,29] (**Fig. 1**). The *proU* promoter is induced between 50- and 100-fold by increased osmolarity, but only 15-fold in the absence of the NRE[30] and only 8-fold in an *hns* strain, although residual osmoregulation is retained[21]. The NRE thus regulates the promoter by modulating the binding of the H-NS protein, and

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Received 13 October 2006; accepted 14 March 2007; published online 15 April 2007; doi:10.1038/nsmb1233

Figure 1 Schematic representation of the *proU* promoter. Bars represent the beginning of the first structural gene of the operon *proV* and the NRE. The two transcription start points are designated P1 and P2; arrows indicate the sense direction.

no *trans*-acting factor other than H-NS seems to be required for the regulation of the *proU* P2 promoter. Therefore, we asked what the mechanism is for specific binding and subsequent regulation by H-NS at this promoter.

The model outlined above suggests that H-NS nucleates at curved sequences; however, the NRE region does not necessarily contain curved DNA. The only region that presents a slight curvature is located upstream of the promoter core region, between positions –81 and –360 with respect to the start site of transcription at P2. H-NS might be expected to interact with this curved region[25]; however, a detailed analysis of H-NS binding has shown that the NRE has a clearly stronger affinity for H-NS[20] than for this upstream element. Here we demonstrate that H-NS recognizes a short, specific high-affinity sequence and lower-affinity secondary sites. We propose a model in which these sites can be considered as modules of variable specificity used by the protein to obtain different levels of repression on different promoters.

RESULTS

Temperature effect on H-NS binding to the *proU* promoter

We have previously shown, using footprinting assays on the *proU* DNA fragment, that varying the temperature from 10 to 37 °C has a drastic effect on the H-NS binding process, decreasing the overall affinity of the protein for the promoter region[11]. Here we analyzed the affinity of binding at each site occupied by H-NS on this promoter at two temperatures, as a function of the DNA topology.

Binding of H-NS at 20 °C within the NRE region

DNase I footprints were obtained on a 372-bp fragment containing the *proU* promoter, incubated with H-NS over a wide range of protein concentrations (**Fig. 2**). At 20 °C, the region between +130 and +240

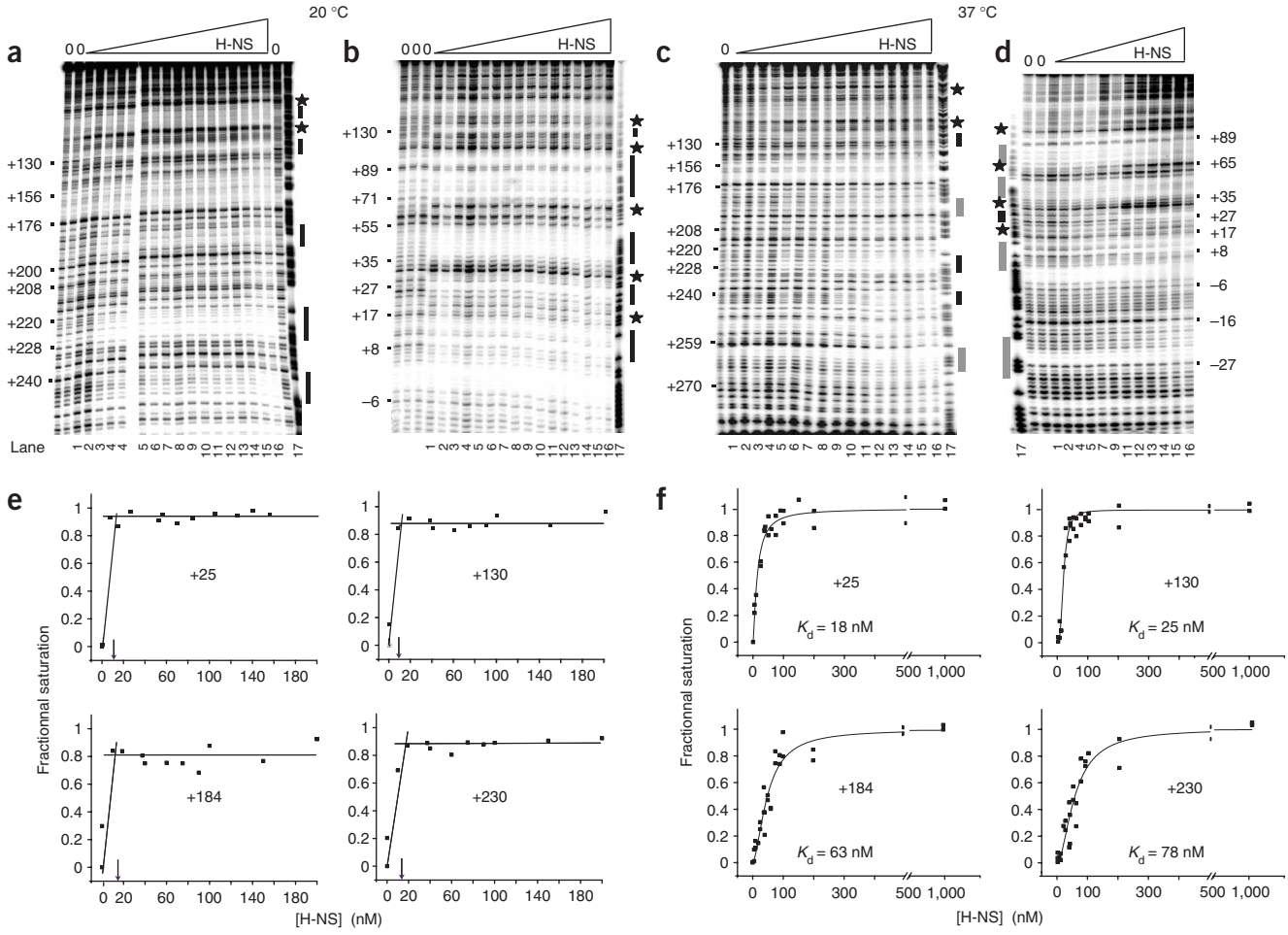

Figure 2 DNase I footprints and quantification of H-NS binding on the *proU* promoter. (**a**–**d**) DNase I digestion of *proU* promoter on coding strand (**a**,**c**) or noncoding strand (**b**,**d**) with increasing concentrations of H-NS, at 20 °C (**a**,**b**) or 37 °C (**c**,**d**). Lane 17 shows sequence ladder (G+A). Concentrations of H-NS are 2, 5, 10, 18.75, 25, 37.5, 40, 50, 60, 75, 90, 100, 150, 200, 500 and 1,000 nM for lanes 1–16, respectively. 0 at top of left lanes denotes reactions without H-NS. Black bars indicate positions of H-NS protection. Black stars indicate hypersensitive sites in the presence of H-NS. (**e**,**f**) Binding isotherms of fractional saturation at indicated sites as a function of total H-NS concentration, obtained from footprint titration experiments. Arrows indicate the H-NS concentration for half-saturation of binding.

Table 1 Equilibrium dissociation constants for H-NS binding to various sites

DNA topology	Temperature	K_d (nM) at position					
		+25	+66	+90	+130	+184	+230
Supercoiled	20 °C	~15	23 ± 4	53 ± 9	~15	55 ± 9	50 ± 8
	37 °C	23 ± 4	40 ± 10	72 ± 11	15 ± 5	83 ± 12	83 ± 13
Linear	20 °C	~15	ND	ND	~15	~15	~15
	37 °C	18 ± 3	ND	ND	25 ± 2	63 ± 7	78 ± 8

Apparent K_d values were obtained by fitting isotherms for binding at the indicated individual positions on the *proU* promoter, in the context of linear or supercoiled DNA at 37 °C or 20 °C. Position is relative to +1 start site of transcription from the *proU* P2 promoter. ND, not determined. Errors are s.e.m.

(with respect to the start of transcription for the P2 promoter) was analyzed on the noncoding strand (**Fig. 2a**) and the region from +135 to –6 on the coding strand (**Fig. 2b**). Several H-NS–binding sites corresponding to those already published[11,20] were observed. The degree of protection by H-NS at four positions, +130, +184, +25 and +230, was quantified as a function of total protein concentration (expressed as monomer concentration). The resulting binding isotherms are shown in **Figure 2e**. At a concentration of 10–20 nM, there was a burst of binding at each site, corresponding to a stoichiometric interaction with the DNA; this suggests that at 20 °C the protein binds the linear fragment in a highly cooperative way, with an estimated apparent global equilibrium dissociation constant (K_d) of around 15 nM. Hyperactive sites, usually indicative of DNA bending, were present at several positions in the analyzed region of the promoter.

Binding of H-NS at 37 °C within the NRE region

DNase I footprinting experiments were repeated at 37 °C (**Fig. 2c,d**). The change in temperature clearly affects the affinity of the protein for the DNA, and footprints were more difficult to observe. However, the positions of the DNA footprints were the same as at 20 °C and we could determine the affinity of H-NS for each of the binding sites. Binding isotherms are shown in **Figure 2f**. Each single H-NS site on the *proU* promoter is characterized by its own affinity constant. This confirms that in natural promoters regulated by H-NS, sites exist with different affinities for the protein. It also indicates that the sites with the most avid binding affinity are at +130 and +25 (note that +25 is outside of the previously defined NRE). Notably, these two sites have identical sequences, 5′-TCGATATATT-3′, in the same relative orientation.

Binding of H-NS to *proU* in a supercoiled template

As a linear fragment is only an approximation of the natural substrate for the H-NS protein, we calculated the affinity of H-NS for these different sites on supercoiled DNA. The region between positions +1 and +180 was analyzed by DNase I footprinting and primer extension (data not shown). There was no change in the footprinting pattern on supercoiled templates. The relative affinity of H-NS as a function of temperature was measured, and apparent K_d values for different positions were estimated (**Table 1**). Although the use of a plasmid instead of a DNA fragment is equivalent to adding competitive DNA (consisting here of the whole non-*proU* sequence on the plasmid), at 20 °C the K_d values of the high-affinity sites are similar to those on the linear fragment, and those of the weaker sites are higher. However, the effect of cooperativity between the high- and low-affinity sites is diminished at 20 °C on supercoiled plasmids. The temperature effect was still observed on the plasmid, and this loss of cooperativity is somewhat accentuated at 37 °C, as the affinity at the low-affinity sites becomes even poorer. However, independently of the experimental conditions used, the +130 and +25 positions seem to always have a high affinity for the H-NS protein.

A short, specific high-affinity H-NS–binding site

To determine whether the identified sequence could still be recognized by H-NS outside of the NRE context, it was inserted into a DNA fragment that has an overall low binding affinity for H-NS. The latter was obtained by comparing the binding of H-NS on the restriction digestion fragments from pBR322 plasmid. Part of the coding region of the *tet* gene, a fragment rich in G+C (60%), has a very low affinity for the H-NS protein (data not shown), confirming that H-NS binds poorly to (G+C)-rich DNA[31–33]. A DNA fragment called U162 (162 bp) was produced from this sequence by PCR, and its interaction with H-NS was analyzed by DNase I footprinting. H-NS does not recognize any particular region on this fragment at 20 °C (**Fig. 3a**).

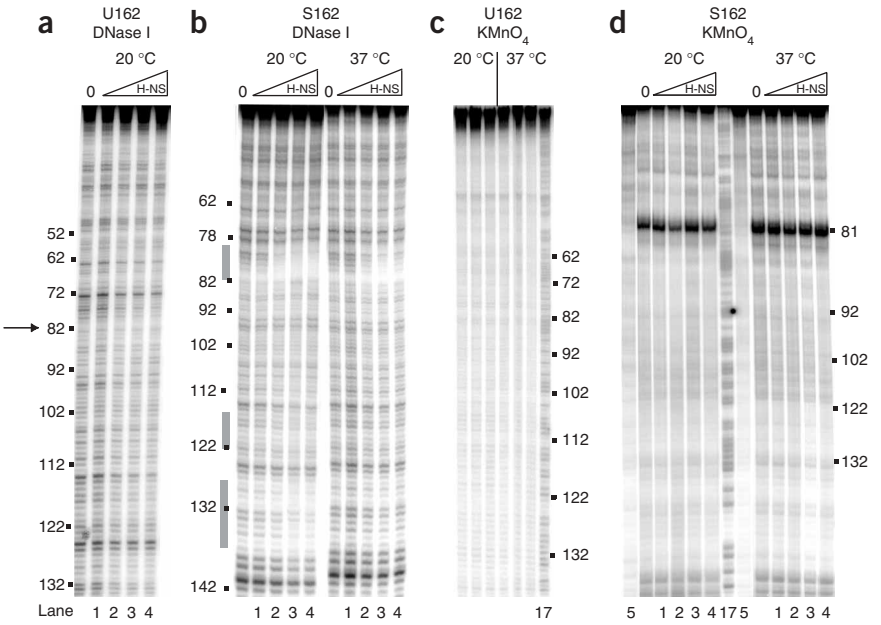

Figure 3 DNase I footprinting and $KMnO_4$ reactivity of H-NS binding on the U162 and S162 fragments. (**a**,**b**) DNase I footprints of H-NS at indicated temperatures. Grey vertical bars indicate position of H-NS DNase footprints. (**c**,**d**) $KMnO_4$ reactivity of the DNA at the indicated temperatures. H-NS concentrations are 5, 25, 50 and 100 nM for lanes 1–4, respectively. Bases are numbered from the first base of fragment U162; inserted sequence was not taken into account for S162. Arrow shows where 5′-TCGATATATT-3′ was inserted into U162 fragment to create S162. 0 indicates no H-NS and lane 17 contains a sequence ladder, as in **Figure 2**. Lane 5 shows a control experiment without $KMnO_4$.

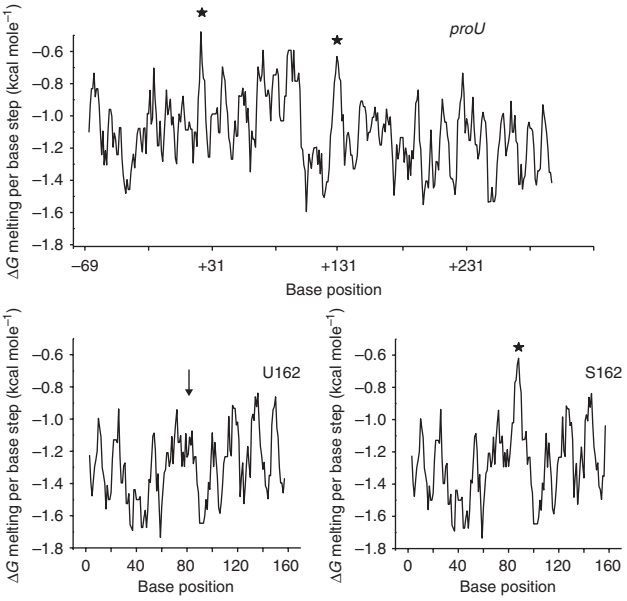

Figure 4 DNA stability plots. Δ*G* melting per base-step was calculated as a function of base position. Base position is relative to the +1 start site for transcription at the *proU* P2 promoter. For the U162 and S162 fragments, base position refers to the base's location along the whole fragment. Arrow shows where 5′-TCGATATATT-3′ was inserted into U162 to create S162. Stars indicate locations of highest instability.

The 10-bp sequence of the NRE +130 site (5′-TCGATATATT-3′) was introduced at position +82 (indicated by an arrow in **Fig. 3a**), near the center of the U162 fragment, to create a new fragment, which we called S162. DNase I footprinting of S162 in the presence of H-NS at 20 °C revealed an H-NS–binding site at the exact position of the insertion and secondary sites situated approximately at positions 128–137 and 116–121 (**Fig. 3b**). Quantification of binding at these sites gave apparent K_d values of 46 nM for the insert, and 48 nM and 50 nM for the secondary sites, at 20 °C, demonstrating the cooperativity between all of these sites. Increasing the temperature to 37 °C destabilized the H-NS–DNA interaction at the secondary sites, so that the resulting footprints were weaker (**Fig. 3b**) and the measured K_d values were higher than the measured K_d values for the inserted site (60 nM for the insert and 145 nM for the secondary sites). Thus, cooperativity with the secondary sites was lost at 37 °C. This clearly indicates that the introduction of the sequence 5′-TCGATA TATT-3′ creates a nucleation site in the fragment, allowing subsequent cooperative binding of H-NS. Mutations within this sequence in the *proU* promoter lead to the disappearance of the footprints at +130 and +25, although footprints at the other sites are still present (**Supplementary Fig. 1** online). These experiments constitute the first demonstration that H-NS can bind a short, specific sequence with high affinity.

Structure and stability of the high-affinity binding site

What is the molecular basis for the specific recognition of this short fragment by H-NS? Could it involve some local, specific property of the DNA? To investigate this, we decided to carry out potassium permanganate ($KMnO_4$) assays. This reagent is a sensitive probe to detect structural changes in DNA. It is highly reactive toward single-stranded DNA, attacking the 5,6 double bond of a pyrimidine ring when it is accessible—that is, when bases (especially thymidine) are unpaired or distorted in duplex DNA. Upon attack by $KMnO_4$ on the U162 fragment, no reactivity was observed anywhere on the sequence (**Fig. 3c**). On the S162 fragment, containing the 10-bp insert, position +80 reacted strongly (**Fig. 3d**). This position corresponds to the thymine base immediately upstream of the insertion. The observed reactivity was slightly enhanced by an increase in temperature from 20 °C to 37 °C but was quite insensitive to the incubation of the fragment in the presence of H-NS. The distortion in the DNA structure is thus due to an intrinsic property of the inserted sequence. In the context of the *proU* sequence, the whole promoter region was reactive to $KMnO_4$ and the sequence at +130 did not present stronger reactivity than the rest of the fragment (data not shown).

Another way of assessing the stability of the H-NS–binding sequence is to evaluate its melting energy. We summed melting energy, Δ*G*, over 6-bp steps for the *proU* promoter, the U162 and the S162 sequences (**Fig. 4**). The *proU* profiles show two major peaks at +25 and +130, corresponding to relatively unstable regions, each flanked on either side by two relatively stable regions. The U162 fragment has a generally lower Δ*G* profile and no major peaks. Notably, on the S162 fragment, the peak with the largest amplitude corresponds to the position of the insertion, +81 to +90, and indeed has the same Δ*G* as the peaks in *proU*. The insertion of the 10-bp sequence thus creates a single high spot of instability surrounded by a very stable region (owing to the 60% G+C content).

In vivo effect of mutations on H-NS binding

To ascertain whether the specific H-NS–binding sites are sufficient to repress transcription, we constructed a series of plasmids containing a segment of the *proU* promoter, extending from –187 to +1160 (that is, containing the *proU* P2 promoter and the *proV* structural gene), fused to the green fluorescent protein (GFP) gene. Mutations at +25, +130 or both were introduced into this construct. The resultant plasmids were transformed into wild-type *Escherichia coli* or *hns* strains, and GFP fluorescence was monitored at 37 °C. In an *hns* background, the expression from the *proU* P2 promoter is not affected by modification of the sequence (**Fig. 5**). In wild-type strains, however, the elimination of the H-NS–binding site at either +25 or +130, as observed by DNase I footprinting (see **Supplementary Fig. 1**), results

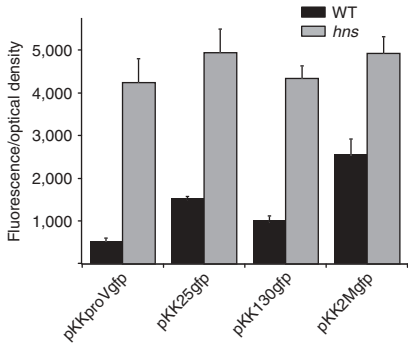

Figure 5 Relative expression from the various *proU* constructs measured by fluorescence analysis. Plasmids contain the *proU* P2 promoter upstream of the *proV* gene fused to the GFP gene. pKK*proVgfp* contains the wild-type promoter; pKKM25*gfp* contains the promoter with the sequence 5′-TCGATGGGCT-3′ instead of 5′-TCGATATATT-3′ at position +25; pKKM130*gfp* contains the same modification but at position +130; pKK2M*gfp* contains the modification at both +130 and +25. GFP fluorescence was measured in midexponential growth phase for wild-type and *hns* strains. Bars represent mean fluorescence intensities of all bacteria in a population; error bars show s.e.m.

Table 2 Sequences of potential H-NS–binding sites on promoters known to be regulated by H-NS

Gene	Sequence	Position (first/last)
proU	AATATATCGA	–39/–30 +68/+77
kdpD (refs. 41,43)	AATATATCGg	–266/–257
caiF (ref. 41)	AgTATATCGA	–284/–275
csgD (refs. 41,42)	AATATAaCGA	–218/–208
ebgC (ref. 41)	AAaATATCGA	+83/+92
gltF (refs. 2,41)	AATATATaGA	–237/–228
flgG (ref. 41)	AATtTATCGA	–102/–95
yfiR (ref. 41)	AAaATATCGA	–112/–103
nirB (ref. 44)	AATATAcCcA	–105/–96
hdeA (refs. 2,41)	AtTAaATCGA	–172/–163
hdeD (ref. 2)	AtTAaATCGA	–82/–73

We searched genes known to be controlled by H-NS from positions +100 to –300 relative to the ATG start site, for close matches to 5′-AATATATCGA-3′. Position is defined relative to the ATG on the corresponding gene.

in a 2.8- or 1.9-fold increase, respectively, in *proU* P2 promoter expression. The effect is thus dependent on the presence of H-NS. Moreover, the double mutation increases expression by a factor of 4.8, which strongly indicates an additive effect of the two mutations. The same experiments conducted at 20 °C gave essentially similar results (data not shown). Although we did not observe complete derepression for the double mutants, we can conclude that the specific sites for H-NS have a major role in H-NS binding in a repression-competent complex *in vivo*.

DISCUSSION

Analysis of the binding of H-NS to the *proU* NRE leads to two main conclusions. First, the NRE contains two essentially identical high-affinity binding sites for H-NS, and second, the cooperativity of H-NS binding to NRE depends on both temperature and DNA superhelicity.

For more than 10 years, the H-NS protein was thought to recognize curved DNA and not a primary sequence[8]. Multiple binding sites were described either on a synthetic curved promoter or on the *proU* promoter[9,11]. The multiple binding sites identified here are approximately 10 bp in size, in agreement with observations of H-NS binding to λ DNA[34]. However, we found that on a specific H-NS–regulated promoter, each is characterized by its own binding affinity for H-NS, ranging from 15 nM to 100 nM, and only the higher-affinity sites are involved in nucleation. In *proU*, two strong binding sites, of identical sequence, were notable for their stability over the range of temperature and topological constraints we used. Once isolated and introduced into a sequence having a generally low affinity for H-NS, this site conferred a higher affinity for H-NS to the overall sequence. This directly demonstrates that this motif contains sufficient information for recognition by H-NS. Furthermore, the insertion induces the presence of secondary sites—that is, it acts as a nucleation site—even when the sequence into which it is inserted does not have any obvious site for H-NS binding. These experiments thus demonstrate that a single specific sequence suffices for H-NS to nucleate and oligomerize on DNA.

Our work suggests that once a nucleation site is introduced into a DNA sequence, new binding sites may appear. These new sites have previously been grouped with the high-specificity sites, masking the existence of the latter. Thus, an H-NS–binding region can be described as a series of small binding sites forming modules for a final architecture that is organized around one or several nucleation sites. In the case of the *proU* promoter, which is strongly repressed by H-NS, nucleation sites can actually be found at two positions. A key factor for silencing could be the general organization of the different sites in the silenced regions. This brings to mind the DnaA protein, for which the number and position of boxes on the DNA sequence determines the architecture and composition of initiator complexes, and successive binding to sites with different affinities generates a particular order of assembly[35,36]. We postulate that for H-NS, depending on the promoter, the spatial arrangement of the binding sites could allow the protein to inhibit transcription, with its effect ranging from a factor of 2 up to total silencing. This could partly explain the *in vivo* data, where the absence of the two nucleation sites does not completely alleviate repression. There are two extraneous factors that could potentially be involved: first, local superhelicity, which has been shown to control *proU* expression[18]—indeed, *hns* strains are known to have altered superhelical density[37]—and second, the bridging of DNA strands under conditions in which H-NS binds nonspecifically to DNA[34], possibly involving secondary sites. *In vivo*, the effect of mutating the high-affinity sites is additive, which suggests that the cooperativity observed for the binding of the protein may derive as much if not more from interactions between secondary sites and nucleation sites as from interactions between nucleation sites themselves.

We note that the cooperativity *in vitro* is less efficient at 37 °C and on a supercoiled template, both parameters that are close to physiological conditions. This suggests that a determining factor *in vivo* for specific repression by H-NS (as is the case for *proU*) will be the presence of one or more high-affinity binding sites.

The specific binding site has an unusual property. When the 10-bp site was introduced into a (G+C)-rich fragment, base opening was observed and the affinity of H-NS for this fragment increased. Studies[31,32,38] have shown that *in vivo* binding of H-NS proteins is essentially restricted to (A+T)-rich DNA. This is consistent with the base composition of the high-affinity sites in the *proU* NRE. Notably, base opening does not occur within the small inserted sequence itself, but rather very close to it. Insertion of the small sequence may modify the properties of the neighboring DNA sequence and thus nucleate a destabilization of double-strand stacking that propagates to a position where base pair unstacking could occur. This agrees with the observation that the amplitude of permanganate reactivity of the sites in *proU* (data not shown) is similar to that seen in the S162 fragment but that the overall higher reactivity of all the bases in *proU* somewhat masks this differential effect. In the S162 context, any untwisting would be concentrated at this site; the (A+T)-rich sequence of *proU* may dissipate the effects of an induced instability. The relatively low stability of the high-affinity H-NS sites is a consequence of the two adjacent TpA base-steps.

General implications

Our results directly address the question of how H-NS can selectively silence genes. We suggest that silencing requires the presence of one or more high-affinity sites for H-NS and that nucleation at these sites induces cooperative H-NS binding, which in turn is responsible for silencing. *In vitro*, the K_d values for these high-affinity sites range between <15 nM and 25 nM. In contrast, the overall *in vivo* concentration of H-NS is $\sim$20–50 μM. A similar discrepancy has been observed for FIS binding to the upstream activator sequence of the *tyrT* gene, where the K_d *in vitro* is $\sim$10 nM, whereas the *in vivo* concentration of the protein in exponential phase is $\sim$40 μM[16]. Both H-NS and FIS have dual roles in gene regulation and chromatin structure and similar affinities, shared by another abundant nucleoid-associated protein, HU, for nonspecific binding; therefore, they

probably compete with each other for nonspecific sites *in vivo*. Consequently, silencing by H-NS would be favored only when cooperative binding was allowed, producing a local structure with different (presumably lower) affinities for other nucleoid-associated proteins. Removal of this cooperativity would then allow other nucleoid-associated proteins access to hitherto restricted low-affinity sites.

Does the 10-bp sequence with high affinity for H-NS occur in or near other H-NS–regulated genes? We found no exact fits (**Table 2**) but did find sites with one mismatch with respect to *proU*. Whereas the genes listed in **Table 2** have been identified by transcriptome studies as being *hns* dependent[39], the regulation of the promoters for the genes *csgD*[40] and *kdpD*[41] has already been described by other studies. Extending the search to encompass two mismatches identified two promoter regions, for the genes *nirB*[42] and *hdeA*[14], for which the footprint coincides with a match to the *proU* sequence.

Our results demonstrate that highly negative DNA superhelicity and higher temperatures abrogate cooperativity *in vitro*. The former result is fully consistent with previous reports that osmotic shock both increases negative supercoiling and induces *proU* expression, and the latter is consistent with the induction of *virF*. How might DNA supercoiling affect cooperativity? Cooperative binding implies that the proteins bound in the complex are oriented toward each other on the DNA molecule. A model has been proposed in which apparent cooperativity can be explained by bridging of H-NS *in trans*[43]. We observed DNase I hyper-reactivity between the two high-affinity sites. This would be expected upon bending of the DNA, presumably induced by a protein-protein interaction between two bound H-NS entities followed by the binding (and perhaps bridging, although by this we imply H-NS bridging *in cis*) of H-NS proteins that had previously bound cooperatively to the lower-affinity sites. Increasing the negative superhelicity results in a change in the length of the helical repeat from ~10.5 bp in relaxed DNA to ~11.1 bp in plectonemic supercoils[44]. The two high-affinity sites in the NRE have a center-to-center distance of 107 bp (approximately 10 double-helical turns). An increase in the helical repeat from 10.5 to 11.1 bp would change the angular relation of the two sites by ~198°, shifting the relative locations of the sites from the same face of the double helix to opposite faces. Although increasing temperature also changes the relative rotational orientation of the two sites, the effect is much smaller (~20° over 10 double-helical turns) and possibly insufficient to account for the loss of cooperativity.

Our observations relate to the binding of H-NS to a single regulatory element, the NRE. However, a full osmoresponse of *proU* requires additional elements[21,25,28]. To achieve full silencing, we suggest that cooperative binding of H-NS to the NRE facilitates interactions *in cis* with H-NS bound at other sites. This process, equivalent to oligomerization[10,11,45] or bridging[34], would result in the formation of a fiber constraining two duplexes[46,47], possibly in the form of a high-pitch plectoneme. The two intertwined strands would be connected by a loop. The high pitch of the plectoneme would result in a high intrinsic twist, which could lead to modification of the local helix architecture. This might repress *proU* transcription by antagonizing formation of the open complex[27]. Osmotic shock results in an abrupt increase in DNA's negative superhelicity[48], which in turn disrupts the cooperative binding of H-NS at the NRE. This change would concomitantly disrupt the fiber, allowing transcription to proceed. The primary basis of regulation at the *proU* NRE is thus not differences among H-NS's affinities for the nucleation sites but rather direct control of the cooperativity of binding.

More generally, H-NS's role in differential silencing and its ability to act as a nucleoid organizer would both be consistent with the same principle. Indeed, H-NS is one of the *E. coli* domainin proteins involved in the global organization of chromosomal topological loop domains as a domain barrier[49]. In our model, a combination of H-NS–binding sites could be optimized to form a domain barrier, and this combination may be different for silencing purposes.

METHODS

Protein purification. The H-NS protein was purified as described[50].

Oligomers and fragments. Oligonucleotides were from Eurogentec. PCRs were done with the Taq platinum polymerase (Invitrogen). The *proU* DNA fragment was obtained by PCR using the specific primers FPF (5′-GCATCAATATTCATGCC-3′) and FPR (5′-GGCGAGCATCCACAGCGA-3′) on the pKK*proU* plasmid[51]. The U162 and the S162 fragments were amplified by PCR using the primers 200u3. (5′-ATAGTTCCCGGGCTCATAGCGCTTGTTTC-3′) and 313d (5′-ATAGTTCCCGGGACGCTCTCCCTTATGCGACT-3′) on the pBR322 plasmid.

Mutagenesis. The S162 DNA fragment was generated by the overlapping PCR method described[51]. Mutagenic reactions were designed in which two separate PCR products had partially overlapping sequences containing the mutation. The 162-bp U162 fragment was amplified by PCR using the primers 200u3 and 313d on the pBR322 plasmid. This DNA fragment starts at position 86 on pBR322. The 10-bp fragment, corresponding to the H-NS–binding site, was introduced in the U162 DNA fragment via the other two mutagenic primers, 200u3a (5′-TGAGCACCGCCGCCGCAAGGAAAATATATCGATGGTGCATGCAAGGAGATGGCGCCC-3′) and 313da (5′-CCATCTCCTTGCATGCACCATCGATATATTTTCCTTGCGGTGCTGCTCAACGG-3′). The two PCR products, one with primers 200u3 and 200u3a and the second with 313d and 313da, were gel-purified. These products were then used for overlapped extension PCR. The *proU* was mutated as above to introduce the motif 5′-TCGATGGGCT-3′ to replace 5′-TCGATATATT-3′ at position +25 or +130, or both.

DNase I footprinting on DNA fragments. Fragments containing the *proU* promoter were obtained by PCR using a combination of one unlabeled primer and a second primer end-labeled with [γ^{32}P]ATP (3,000 Ci mmol^{-1}) using phage T4 polynucleotide kinase. This fragment was purified on a glass fiber column (Roche Molecular Biochemicals). The labeled fragment (2–5 nM) was incubated for 20 min at the indicated temperature and concentration of H-NS, in 10 µl of 40 mM HEPES (pH 8.0), 8 mM magnesium aspartate, 60 mM potassium glutamate, 5 mM DTT, 0.05% (v/v) Nonidet P-40 and 0.1 mg ml^{-1} acetylated BSA (Biolabs). The DNA was attacked by 0.05 µg ml^{-1} of DNase I (Worthington Biochemicals) for 30 s or 25 s (in the absence of protein). The reaction was stopped by the addition of 90 µl of phenol-chloroform (5:1, pH 8.0) and after extraction the DNA was precipitated by 90 µl of a solution of 0.4 M sodium acetate, 100 µg ml^{-1} glycogen, 2.5 mM EDTA and 0.7 ml of ethanol. The samples were resuspended in 5 µl of formamide blue (90% (w/v) formamide, TBE 1×, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue) and loaded on 7.5% (w/v) denaturing polyacrylamide gels.

DNase I footprinting on supercoiled DNA. DNase I footprinting was done in 10 µl of the binding buffer containing 2 nM of pKK*proU* plasmid[52]. H-NS was added and each sample incubated at the indicated temperature. DNase I attack was done as described above. Sites of DNase I cleavage were detected by primer extension after addition of buffer (400 µM dNTPs, 4 mM $MgCl_2$, 2× Taq platinum polymerase buffer, 50 nM labeled primer, 20 µg ml^{-1} calf thymus DNA, 0.5 U µl^{-1} Taq polymerase). Samples were amplified for 20 cycles, precipitated with ethanol and resuspended in 5 µl formamide blue before electrophoresis on denaturing polyacrylamide gels. DNase I attack positions were identified using the dideoxy sequence ladders generated using the same primer (Sequencing Kit, USB).

Potassium permanganate reactivity in vitro using linear DNA template. $KMnO_4$ (80 mM, 1 µl) was added to the same reaction mix as used in the DNase I experiments for 30 s. The reaction was stopped by 2 µl of 14 M β-mercaptoethanol, 88 µl of 0.3 M ammonium acetate and 16 µg ml^{-1} calf thymus DNA. After phenol-chloroform extraction and precipitation with

ethanol, DNA pellets were resuspended in 100 µl of 1 M piperidine and incubated for 30 min at 90 °C. After evaporation, the DNA pellets were rinsed twice with 100 µl distilled water, redissolved in 5 µl formamide blue and separated by electrophoresis in denaturing polyacrylamide gels.

Quantitative gel analysis. Digital images of gels were quantitatively analyzed using ImageQuant (Molecular Dynamics)[53]. We determined the fractional saturation of sites and fit this data by the nonlinear least-squares method[53]. Apparent dissociation equilibrium constants (K_d) for H-NS binding to the different constructs were calculated from curves shown in **Figure 2** using the expression

$$\text{Fractional saturation} = y = \frac{nK_d[\text{H-NS}]^n}{1+n(K_d)^n[\text{H-NS}]^n}$$

where n is the Hill coefficient.

DNA stability plots. Plots of DNA stability were generated by a moving window summing the average melting energy of six successive base-steps. Values for the melting energy of individual base-steps were taken from ref. 54 and processed by a custom program (see Acknowledgments).

***In vivo proU* expression.** The GFP gene was obtained from the pQBIT7GFP (QBIOGENE) plasmid by digestion with BglII and HindIII restriction enzymes. This fragment was inserted in pKK*232-8* (GE Healthcare). The resulting plasmid was called pKK*gfp*. A 1,348-bp fragment was obtained by PCR with 5′-ATAGTTCCCGGGGCATTATTCGCCTGAAACCAC-3′ and 5′-ATAGTTTCT AGAGGGTGCCTGTCCGACATG-3′ primers on the *proU* promoter. After restriction by XmaI and XbaI, this fragment was inserted at the same sites in the pKK*gfp* plasmid. The plasmid harbors a translation stop site between *proV* and the GFP gene to prevent protein fusion.

E. coli FB8 (wild-type) and isogenic *hns* (1001) strains were used (see Acknowledgments). Three independent colonies of each strain transformed with each plasmid of interest were grown in LB medium to midexponential phase and GFP fluorescence was measured at 507 nm after excitation at 477 nm on a fluorimeter (Varian). The absorbance was simultaneously measured at 600 nm on a spectrophotometer (Kontron). The values reported represent means from three independent determinations.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

This work was supported by the Centre National de la Recherche Scientifique and the Agence Nationale pour la Recherche (projet MASTRIT). E.B. was funded by the Fondation pour La Recherche Médicale and the Ministère délégué à l'Enseignement supérieur et à la Recherche. We thank B. Robert and J. Gowrishankar for comments on the manuscript, I. Pemberton for his advice with the various constructions, G. Mitchison (University of Cambridge) for writing the DNA stability plot program and P. Bertin for kindly providing the strains.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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