DNA looping-mediated repression by histone-like protein H-NS: specific requirement of $\sigma^{70}$ as a cofactor for looping

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Transcription initiation by RNA polymerase (RNP) carrying the house-keeping $\sigma^{70}$ ($\sigma^{70}$), is repressed by H-NS at a number of promoters including hdeABp in Escherichia coli, while initiation with RNP carrying the stationary phase $\sigma^{38}$ ($\sigma^{38}$), is not. We investigated the molecular mechanism of selective repression by H-NS to identify the differences in transcription initiation by the two forms of RNPs, which show indistinguishable promoter selectivities in vitro. Using hdeABp as a model promoter, we observed with purified components that H-NS, acting at a sequence centered at $-118$, selectively repressed transcription by $\sigma^{70}$. This selective repression is attributed to the differences in the interactions between hdeABp and the two forms of RNPs, since no other factor is required for the repression. We observed that the two forms of RNPs could form an open initiation complex (RPO) at hdeABp, but that $\sigma^{70}$ failed to initiate transcription in the presence of H-NS. Interestingly, KMnO$_4$ assays and high-resolution atomic force microscopy (AFM) revealed that hdeABp DNA wrapped around $\sigma^{70}$ more tightly than around $\sigma^{38}$, resulting in the potential crossing over of the DNA arms that project out of $\sigma^{38}$ · RPO, but not out of $\sigma^{70}$ · RPO. Based on these observations, we postulated that H-NS bound at $-118$ laterally extends by the cooperative recruitment of H-NS molecules to the promoter-downstream sequence joined by wrapping of the DNA around $\sigma^{70}$ · RPO, resulting in effective sealing of the DNA loop and trapping of $\sigma^{70}$. Such a ternary complex of H-NS · $\sigma^{70}$ · hdeABp was demonstrated by AFM. In this case, therefore, $\sigma^{70}$ acts as a cofactor for DNA looping. Expression of this class of genes by $\sigma^{38}$ in the stationary phase is not due to its promoter specificity but to the architecture of the promoter · $\sigma^{38}$ complex.

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Bacterial RNA polymerase (RNP) consists of core sub-units [2α, β, β'], and a σ subunit, which confers promoter specificity [Ishihama 2000]. During the exponential phase of growth, $\sigma^{70}$ is the predominant form that is responsible for expression of housekeeping genes in Escherichia coli. As the culture enters stationary phase, $\sigma^{38}$ encoded by rpoS, is expressed [Mulvey and Loewen 1989; Lange and Hengge-Aronis 1991]. RNP, loaded with $\sigma^{38}$ at the entry of stationary phase, then expresses a set of genes under its control [Hengge-Aronis 1996; Zambrano and Kolter 1996]. However, various in vitro studies with purified proteins have failed to differentiate the promoter specificity between the RNP carrying $\sigma^{38}$ ($\sigma^{70}$) and that carrying $\sigma^{38}$ ($\sigma^{38}$) [Tanaka et al. 1995; Espinosa-Urgel et al. 1996; Wise et al. 1996; Bordes et al. 2000; Lee and Gralla 2001]. The differences observed in vitro between the two forms of RNPs are too marginal to account for the selective expression of rpoS-dependent genes by $\sigma^{38}$ in vivo. It has been proposed that a second element, other than the promoter sequence, may be responsible for rpoS-dependent gene expression [Kim et al. 2004].

H-NS, one of the most abundant DNA-binding pro-
teins (20,000 molecules per cell) in *E. coli*, is implicated in global regulation of gene expression, as well as in the compact organization of the nucleoid structure [Drllica and Rouviere-Yaniv 1987, Atlung and Ingmer 1997, Deighan et al. 2003, Dorman 2004, Rimsky 2004]. A recent proteome analysis has revealed that up to 5% of the genes in *E. coli* are down-regulated by H-NS [Hommais et al. 2001]. Genes under the control of H-NS include those involved in bacterial adaptation to changes in the environment. For pathogenic bacteria, these changes are often related to the conditions encountered after invasion of mammalian hosts. H-NS has been referred to as a general transcription silencer, since it represses a large number of genes, which are not required during the exponential phase of growth under normal laboratory conditions. H-NS recognizes DNA with a certain configuration, such as curved DNA generated by stretches of AT-rich sequence [AT tracts] rather than a specific sequence [Rimsky et al. 2001, Rimsky 2004]. H-NS binding to DNA is different for preferential binding and stabilization of the multiple DNA fragments occurs as a result of the DNA around the binding site [Rimsky et al. 2001, Rimsky 2004]. [H-NS] through interactions between N-terminal domains of the protein [Dorman et al. 1999, Badaut et al. 2002, Schroder and Wagner 2002]. Scanning force microscopy has shown that the preferential binding to curved DNA fragments occurs as a result of the DNA around the curve being bridged by oligomeric H-NS, which leads to the formation of a hairpin-like structure [Dame et al. 2001]. The oligomization of H-NS is, therefore, essential for preferential binding and stabilization of the multimeric nucleoprotein complex [Sporio et al. 1997]. A recent study of an *E. coli* ribosomal gene promoter rnbP1 has suggested that repression involves DNA looping and that the loop is closed by the association of two patches of H-NS-bound DNA in which the RNP is trapped, instead of being excluded [Schroder and Wagner 2000; Dame et al. 2002, Dame 2005, Gralla 2005]. According to this model, expression from an H-NS-repressed promoter should require disruption of the nucleoprotein complex and the DNA loop by transcription factors. It has been noted that many of the genes repressed by H-NS require activation signals to overcome repression [Schroder and Wagner 2002, Yu and DiRita 2002].

In a number of promoters, transcription initiation with *Eo*70 is repressed by H-NS while that with *Eo*38 is unaffected. This class includes the *hdeAB*, *csgA*, *gadB/C*, and *cssID* promoters in *E. coli* and the *spvR* promoter in *Salmonella typhimurium* [Arnaqvist et al. 1994, Robbe-Saule et al. 1997, Marschall et al. 1998, Waterman and Small 2003]. These promoters contain AT tracts, creating an intrinsic DNA curvature immediately upstream of the promoter. Using the *E. coli hdeABp* as a model promoter, we investigated the molecular mechanism of selective repression by H-NS to identify differences in transcription initiation by the two forms of RNPs. We suggest that H-NS selectively represses transcription initiation by *Eo*70 through H-NS-mediated repressive DNA looping, which requires an RP70 with *Eo*70 acting as a looping factor. We propose here that the selective repression by H-NS is the result of differences in the degree of DNA wrapping around two forms of RNPs.

**Results**

**Regulation of hdeABp by H-NS**

We examined the expression profile of *hdeABp* in *E. coli* during the course of bacterial growth in LB. The *hdeABp* activity was determined using a λ lysogen carrying the *hdeABp–lacZYA* fusion [Yoshida et al. 1993] to eliminate possible changes in gene copy number. Overnight cultures were diluted 50-fold into fresh LB and grown until the cultures entered stationary phase. Bacterial growth was monitored by measuring the A600 (Fig. 1A, open symbols). The *lacZ* expression level was determined by assaying for β-galactosidase activity [Fig. 1A, closed symbols]. In the wild-type background [circles], the levels of β-galactosidase activity under the control of *hdeABp* increased as the culture entered stationary phase, reaching a maximum accumulation of ~35-fold [Fig. 1A]. In the *rpoS* mutant background [triangles], *hdeABp* activity was minimal. In the *hns* and *rpoD* double-mutant background [squares], however, the *hdeABp* activity in the exponential phase cultures was elevated at least 35-fold above the wild-type level at stationary phase and gradually increased about twofold in the stationary phase. The further increase in *hdeABp* in the *hns* and *rpoD* double mutant in the stationary phase was attributed to the *hns* homolog *stpa* [Supplementary Fig. 1; Sonden and Uhlin 1996, Zhang et al. 1996]. This observation suggested that *hdeABp* belongs to the class of promoters normally transcribed by *Eo*70, but also by *Eo*38 in the absence of H-NS [Yoshida et al. 1993, Arnaqvist et al. 1994; Robbe-Saule et al. 1997, Marschall et al. 1998, Waterman and Small 2003]. H-NS selectively represses *Eo*70-mediated transcription of this class of promoters.

An in vitro transcription assay using purified components with the reconstituted *Eo*70 or *Eo*38 holoenzyme was carried out to examine the selective repression of *hdeABp* by H-NS. Supercoiled plasmid carrying the *hdeAB* DNA sequence (~136 to +120 nucleotide [nt]), followed by a strong terminator, was used as the template (Choy and Adhya 1993). Figure 1B shows a gel pattern of multiple round transcription assay. Both *Eo*70 [Fig. 1B, panel 1] and *Eo*38 [Fig. 1B, panel 2] generated the same transcript from *hdeABp*, as is the case for most other *Eo*38 promoters tested in vitro [Kim et al. 2004]. In the presence of increasing concentrations of H-NS, the *hdeAB* transcript generated by *Eo*70 decreased, while the *rnlA* transcript from the origin of replication remained constant. The *lacUV5p* DNA transcript by *Eo*70 provided a control. The *lacUV5p* activity was unaffected by the presence of H-NS [Fig. 1B, panel 3]. Thus, the repression of *hdeABp* by H-NS was promoter specific under these in vitro conditions. Most notably, the *hdeAB* transcript...
with this set of constructed and tested. We conducted gel shift assays hdeAB/H11032 DNA constructs with truncated 5’ to identify the DNA site at which H-NS acts, a series of Action site of H-NS on hdeAB fators are present. promoter and the two forms of RNPs, since no other owing to a different mode of interaction between the RNPs could bind simultaneously to hdeABp DNA. Since it has been shown that H-NS molecules that bind upstream and downstream of the rnb1 promoter associate to form a repression complex [Dame et al. 2001], we also examined repression with hdeABp DNA truncated at +20 nt (with a fixed 5’ end at −136 nt). In the in vitro transcription assays with this template showed that the extent of repression was the same irrespective of the 5’ end of the template [Fig. 2B, panel 4]. It therefore appears that the upstream AT tract is sufficient for the observed repression by H-NS.

Transcription initiation is blocked by H-NS at a step subsequent to RPo formation

Transcription initiation at hdeABp by Eo70, but not that by Eo38, is subject to repression by H-NS. The following experiments were conducted to identify which step during transcription initiation by Eo70 is blocked. One model is a simple competition between H-NS and the two forms of RNPs for an overlapping site on hdeABp DNA. This model implies that the binding affinity of Eo38 > H-NS > Eo70. A gel retardation assay was carried out with H-NS and/or RNP [Fig. 3]. H-NS, Eo70, and Eo38 each bound to the hdeABp DNA −136 to +120 nt and migrated at distinct positions on the gel, as shown in Figure 3A. Next, we determined whether H-NS and RNPs could bind simultaneously to hdeABp DNA. Since H-NS, a structural component of the bacterial nucleoid, could induce structural alterations of the DNA that prevent RNP from binding to the promoter [Ussery et al. 1994], the competitive binding assay was carried out by adding RNPs to the preincubating H-NS and hdeABp DNA mix. The addition of Eo70 or Eo38 to the reaction mix containing hdeABp DNA and H-NS generated new bands that migrated with reduced mobility above the bands of the RNP-DNA binary complex, which indicated the possible formation of hdeABp DNA - RNP - H-NS ternary complexes [Fig. 3A, arrowheads in lanes 4,6]. To verify the composition of the supershifted bands, we

Figure 1. Regulatory effect of H-NS. (A) Expression from hdeABp (−136 to +120) fused to lacZYA was determined in vivo in the wild-type background (circles), in the RpoS− mutant background (triangles), and in the RpoS+ and Hns− double-mutant background (squares). λ Lysogens carrying the hdeABp::lacZYA were used for the assay. The expression from hdeABp was determined by β-galactosidase assay during bacterial growth into stationary phase. Open symbols represent the bacterial cell mass (A600, right axis) and closed symbols represent β-galactosidase activity (A420/min/mL/A600 Miller unit, left axis). (B) Regulatory effect of H-NS as determined by in vitro transcription assay using purified components. DNA templates were supercoiled plasmid DNA that carried hdeABp (−136 to +120, panels 1 and 2) or lacUV5p (−130 to +55, panel 3). Transcription was catalyzed by Eo70 (panels 1 and 3) or Eo38 (panel 2). H-NS concentrations in each reaction were 0 (lane 1), 8 nM (lane 2), 25 nM (lane 3), 76 nM (lane 4), and 228 nM (lane 5). The radioactive transcripts were analyzed on an 8 M urea/8% polyacrylamide gel. The major transcripts from the test promoters and rna1 are indicated.

generated by Eo38 was not affected by the presence of H-NS. These data suggest that H-NS represses transcription from hdeABp by Eo70, but not by Eo38, presumably owing to a different mode of interaction between the promoter and the two forms of RNPs, since no other factors are present.

Action site of H-NS on hdeABp

To identify the DNA site at which H-NS acts, a series of hdeABp DNA constructs with truncated 5’ ends were constructed and tested. We conducted gel shift assays with this set of hdeABp DNA fragments and purified H-NS protein in the presence of heparin (34 µg/mL) [Fig. 2A]. Protein–DNA complexes were separated on a 5% native polyacrylamide gel. A single shifted band of H-NS bound to DNA was detected. The gel shift with the hdeABp DNA fragment deleted to −77 nt showed a reduction in H-NS binding. H-NS was needed at approximately threefold higher concentration to obtain binding comparable to the full-length fragment. Further deletion to −52 nt completely abolished H-NS binding, indicating that H-NS binds primarily upstream of −77 nt. To verify the H-NS-binding site, an in vitro transcription assay was carried out with hdeABp DNA templates truncated at the 5’ ends at −136, −77, and −44 nt and the 3’ end fixed at +120 nt. Increasing concentrations of H-NS were added while transcription was catalyzed by Eo70 [Fig. 2B]. In agreement with the gel shift assay results, we observed no repression by H-NS with DNA truncated at −77 or −44 nt. These results demonstrate that an essential site for H-NS action is between −77 and −136 nt in hdeABp, most likely the AT tract centered at −118 nt [Fig. 2C].

Since it has been shown that H-NS molecules that bind upstream and downstream of the rnb1 promoter associate to form a repression complex [Dame et al. 2001], we also examined repression with hdeABp DNA truncated at +20 nt (with a fixed 5’ end at −136 nt). The in vitro transcription assays with this template showed that the extent of repression was the same irrespective of the 5’ end of the template [Fig. 2B, panel 4]. It therefore appears that the upstream AT tract is sufficient for the observed repression by H-NS.
we examined whether or not the presence of H-NS prevented RNP, especially E70, from forming an RP0 at hdeABp. The presence of H-NS did not decrease the intensities of the KMnO4 hypersensitive bands created by E70 or E38. These data indicate that E70 forms an RP0 even in the presence of H-NS. We also examined whether H-NS blocks subsequent promoter clearance but found no accumulation of abortive transcripts [data not shown]. Thus, H-NS appears to block a step prior to first phosphodiester bond formation but subsequent to RP0 formation at the hdeABp by E70.

It should be emphasized that the DNA distortion induced by E70 binding was significantly different from that of E38, especially in the regions outside of the promoter [Fig. 4A,B, cf. lanes 2,3]. Figure 4E summarizes those bases that were hyper-reactive to KMnO4 in the presence of either one of the two forms of RNP. The different patterns of KMnO4 hypersensitivity generated by two forms of RNPs should reflect differences in the way the DNA wraps around these RNPs [see Discussion].

Mode of repression by H-NS

We investigated the mechanism of repression by H-NS by determining whether a direct protein–protein interaction between H-NS and E70 or an indirect effect such as a change in DNA topology, i.e., bending, looping, or supercoiling, was critical for repression. DNA fragments increasing by 5-base-pair [bp] increments [5–20 bp] were inserted at –44.5 nt in the hdeABp DNA (–136 to +30 nt) and these constructs were used to examine the extent of repression of E70-mediated transcription by H-NS in vitro [Fig. 5]. The addition of the DNA fragments [5–20 bp] only slightly reduced the repressive effect of H-NS in a stepwise manner. No obvious effect of changing the face of the DNA helix by insertion of the fragments was observed. This result indicates that the repression of hdeABp by H-NS is not through a direct interaction with E70 [Choy and Adhya 1996; Roy et al. 1998]. Therefore,
another mechanism, such as a structural change in the DNA induced by H-NS, would better account for the repression by H-NS. We favor a model in which E/o/H926870 is trapped in a DNA loop sealed by oligomeric H-NS spanning the upstream and downstream arms of hdeAB promoter DNA (see below).

**Atomic force microscopic images of hdeABp DNA–protein complexes**

Lastly, we employed high-resolution atomic force microscopy (AFM) to visualize the two forms of RNPs bound to hdeABp DNA (−216 to +580) in the presence or absence of H-NS. The DNA–protein complexes were formed using the in vitro reaction conditions and the complexes were deposited on mica and analyzed by AFM. Figure 6 shows hdeABp DNA bound by RNP, in the presence or absence of H-NS. Since E/o/H926870 should interact with the nucleotides approximately between −75 and +25 nt [Darst et al. 1989; Schickor et al. 1990; Craig et al. 1995; Rivetti et al. 1999], on any given promoter, the length of the upstream and downstream DNA arms leaving the RP(o) at hdeABp should be asymmetric [141 bp vs. 555 bp]. Thus, we considered only those complexes with asymmetric DNA arms with a ratio of 141:555 [roughly 1:4] projecting out of the RNP molecule to be the genuine RP(o) · hdeABp. These complexes constituted nearly 49% of the total binary complexes. Figure 6A and B shows the representative binary complexes formed with E/o/H926870 and E/o/H926838, respectively. The DNA bound by E/o/H926870 was sharply kinked at the position of the RNP, while DNA bound by E/o/H926838 was not. To determine

**Figure 3.** Simultaneous binding of H-NS and RNP to hdeABp DNA (−136 to +120), as assessed by gel mobility shift [A] and Western analysis [B for E/o/H926870 binding and C for E/o/H926838 binding]. See text for details. Proteins present in the assays are indicated above each lane. [A] A gel mobility shift assay with 228 nM H-NS and/or 20 nM RNP. For lanes 4 and 6, the DNA was first incubated with H-NS for 10 min and subsequently with RNP for 10 min. [B,C] The hdeABp DNA was incubated in the presence of increasing concentrations of H-NS: 0 [lane 1], 25 nM [lanes 2,5], 76 nM [lanes 3,6], 228 nM [lanes 4,7]. For lanes 5–7, 20 nM of RNP was added after incubation of hdeABp DNA and H-NS and the incubation continued an additional 10 min. Panels on the left show gel mobility shift assays on 5% native polyacrylamide gels. The gels were transferred to PVDF membranes and probed with antibody against the α subunit of RNP [middle panels] or H-NS [right panels]. Bound antibodies were detected by ECL. The closed arrows indicate ternary complexes of DNA · RNP · H-NS and open arrows indicate binary complexes of DNA · H-NS.

**Figure 4.** DNA kinks induced by RNP [20 nM, A,B] and/or H-NS binding (228 nM, C,D) to hdeABp DNA (−136 to +120) as probed by KMnO4 assay. Unpaired bases were revealed by primer extension since a supercoiled DNA template was used (see Materials and Methods). A and C show the result of analysis using the top strand, and B and D show the result of analysis using the primer with the bottom strand (see Materials and Methods). A and B show the entire sequencing gels, and C and D show those bases around the −10 hexamer. Asterisks indicate unpaired bases at or near the −10 element; open and closed arrowheads indicate those induced by E/o/H926870 and E/o/H926838 binding, respectively, and gray arrowheads indicate those induced by both RNPs. The first four lanes in each panel show the DNA sequencing ladder. E shows the top strand bases hyper-reactive to KMnO4 induced by E/o/H926870 binding [carets above bases] or by E/o/H926838 binding [carets below bases]. The −35, −10, and +1 elements are underlined.

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the DNA arms leaving Egr38-driven transcription initiation because the DNA arms leaving hdeABp are not close enough for the upstream bound H-NS to laterally oligomerize to the downstream DNA--no DNA looping.

Discussion

H-NS has generally been considered a nonspecific silencer of many genes. In this study, we investigated the biochemical mechanism of silencing using hdeABp as a model promoter to differentiate transcription initiation by Egr70 and Egr38, which share virtually identical promotor specificities [Kim et al. 2004]. There is a class of promoters that is regulated in a similar manner to that of the DNA template carrying a 20-bp insert. hdeAB

hdeAB

Figure 5. Effect of lengthening the interval between H-NS-binding site and hdeABp on H-NS-mediated regulation. DNA fragments increasing by 5-bp increments were inserted at ~44.5 nt of the hdeABp DNA (~136 to +30). Inserted DNA fragments were ATCGA (5 bp), ATGAGAACC (10 bp), ATGAGCTC GAGCGA (15 bp), and CTAGACCATGGCGCAGATCGA (20 bp). [A] Using the hdeABp carrying the above inserts, the repressive effect of H-NS was analyzed by in vitro transcription assay using the procedure described in Figure 1B. H-NS concentrations were 0 (lane 1), 25 nM (lane 2), 76 nM (lane 3), and 228 nM (lane 4). [B] The RNA transcripts were quantified with a β scanner [FLA3000], and the fraction [percent] of RNA in each lane relative to RNA made in the absence of H-NS was plotted as a function of H-NS concentration. [c] hdeABp DNA template carrying no insert; [●] hdeABp DNA template carrying a 5-bp insert; [▲] hdeABp DNA template carrying a 10-bp insert; [●] hdeABp DNA template carrying a 15-bp insert; and [●] hdeABp DNA template carrying a 20-bp insert.

Figure 6. Atomic force microscopy images of representative RNP (20 nM) bound to hdeABp DNA (~216 to +580) in the presence of absence of H-NS [228 nM]. A and B show representative montages of Egr70 and Egr38 stably bound to hdeABp DNA, respectively. RNP molecules are seen as bright dots. C and D show a montage of representative ternary complexes formed when H-NS bound to hdeABp DNA complexed with Egr70 [C] or Egr38 [D]. These images show thickening of the DNA arms by H-NS binding, which cross-bridged DNA in the presence of Egr70 [C]. No such DNA bridging was observed with Egr38 [D]. All images show a 300 × 300-nm surface area. Color represents height ranging from 0 to 5 nm from dark to bright. [E] Average contour length of free DNA and RNP-bound DNA. DNA contour length values are the average of at least 20 measurements for each condition. Figures in parentheses are standard deviations. [F] Schematic view of H-NS and two forms of RNPs bound to hdeABp DNA. [Left] Egr70 binding induces kinks into the target DNA, facilitating oligomerization of the bound H-NS molecules on the DNA arms project out of an Egr70 . RPO. [Right] H-NS bound to the upstream arm fails to extend to downstream DNA due to the steric distance between the two DNA arms leaving Egr38 · RPO.
hdeAB and that is transcribed by Eσ\textsuperscript{38}, but not by Eσ\textsuperscript{70} in the presence of H-NS (Arnyquist et al. 1994; Robbe-Saule et al. 1997, Marschall et al. 1998, Waterman and Small 2003). Thus, H-NS acts as a repressor of this class of promoters only when Eσ\textsuperscript{70} catalyzes its transcription. In this study, we showed that H-NS repressed transcription from hdeABp by Eσ\textsuperscript{70} but not by Eσ\textsuperscript{38}, in vitro with purified components (Fig. 1) and this activity required the sequence between –77 and –136 nt of hdeABp, most likely involving the AT tract centered at –118 nt, and no other specific sequence (Fig. 2). Eσ\textsuperscript{70} and Eσ\textsuperscript{38} could each bind to hdeABp DNA in the presence of H-NS (Fig. 3). H-NS repressed hdeABp transcription at a step subsequent to Rpo\textsubscript{70} formation by Eσ\textsuperscript{70}, but prior to the formation of the first phosphodiester bond (Fig. 4). The regulatory effect of H-NS was not through direct contact with Eσ\textsuperscript{70} (Fig. 5). AFM showed that Eσ\textsuperscript{70} binding to hdeABp induced a sharp kink in the DNA, but Eσ\textsuperscript{38} did not (Fig. 6). Most interestingly, the DNA arms projecting out of the Eσ\textsuperscript{70} hdeABp complex were joined in parallel with H-NS in a hairpin-like configuration, but not that of Eσ\textsuperscript{38} hdeABp. We propose here that H-NS bound to the AT tract centered at –118 nt (nucleation site) laterally extends by cooperatively recruiting H-NS molecules to the downstream sequence joined through DNA wrapping around Eσ\textsuperscript{70}, which results in trapping of Eσ\textsuperscript{70} in a DNA loop. Thus, the H-NS-mediated repression would depend on the configuration of DNA wrapping around the RNP.

The repression of hdeABp by H-NS is not due to direct occlusion of Eσ\textsuperscript{70}, since formation of an open promoter complex [Rpo\textsubscript{70}] was not affected in the presence of H-NS. Therefore, the classical steric hindrance model of transcription repression does not explain the H-NS-mediated repression of hdeABp. Rather, it is reminiscent of repression of rrrB P1 by H-NS, in which an open complex, formed in the presence of H-NS, may be too stable to go into the elongation mode (Schroder and Wagner 2000). It has been suggested that H-NS bridges the DNA arms projecting out of the complex, thereby creating a DNA loop that traps RNP (Dame et al. 2002). H-NS has been proposed to form oligomers by intermolecular pairings at the N-terminal domain that allows cross-bridging of DNA through interactions with two or more binding sites (Falconi et al. 1988; Friedrich et al. 1988; Ueguchi et al. 1996, Spurio et al. 1997; Dorman et al. 1999; Ceschini et al. 2000; Smyth et al. 2000; Badau et al. 2002; Schroder and Wagner 2002). Alternatively, it has been suggested that H-NS-mediated action requires first the building up of a substructure resulting from the binding of H-NS at a curved sequence (the nucleation step) and then recruitment by a cooperative process of H-NS molecules bound at other strategic sites, in particular at the –10 region of a promoter for transcription repression (Badau et al. 2002). A similar mechanism could account for the repression of hdeABp by H-NS.

DNA looping has been implicated in transcription repression by repressors that bind to multipartite DNA-binding sites (Choy and Adhya 1996). Association of repressors bound to two separate DNA sites results in looping of the intervening DNA, in which the promoter element is encompassed. The consequence of DNA looping is a perturbation of the DNA helix through torsional inflexibility in short stretches of DNA (Le Bret 1979). Perturbation of the DNA structure within the promoter region then creates a kinetic or energetic barrier for processional movement of the RNP along the DNA. Thus, DNA looping is an energetically costly process. In principle, there should be two classes of DNA looping depending on the nature of the repressor that binds to such multipartite operators. Repressors such as the tetrameric LacI simultaneously bind to a bipartite operator separated by nine helical turns on supercoiled DNA by itself, resulting in the looping out of the intervening DNA (Riggs et al. 1970; Oehler et al. 1990). In contrast, dimeric GalR molecules bound to bipartite operators separated by 11 helical turns associate to form DNA loops on supercoiled DNA only in the presence of HU (Choy et al. 1995; Aki et al. 1996). Therefore, DNA looping should be classified according to the requirement for cofactors that may either bend DNA to facilitate association of DNA-bound repressors or stabilize the looped DNA-protein complex.

RNP has not previously been considered a looping factor, even though the DNA is almost completely wrapped around the open initiation complex. Various experiments have suggested that –30 nm of DNA, corresponding roughly to –75 to –25 nt position of a given promoter, lies in an extensive groove on the surface of Eσ\textsuperscript{70}, and wraps 300° around the RNP in the RP\textsubscript{c} (Darst et al. 1989; Schickor et al. 1990; Craig et al. 1995; Rivetti et al. 1999). No such DNA wrapping has been observed with the RP\textsubscript{p} (Rivetti et al. 1999; Dame et al. 2002). We propose here that DNA looping by H-NS requires an open promoter complex with Eσ\textsuperscript{70} as a cofactor, that is, H-NS forms DNA loops only when the upstream and downstream DNA arms project out of the Eσ\textsuperscript{70}. This is because the DNA wrapping in the Eσ\textsuperscript{70} groove puts the DNA arms into such close proximity that oligomeric H-NS extends to the downstream arm of DNA (Fig. 6). Apparently, weak DNA-binding proteins such as H-NS, with a binding constant in the micromolar range (Fried 1989), would never be able to bend short stretches of the DNA helix to create looping by itself, and thus require an aid, the Eσ\textsuperscript{70}. AFM images of Eσ\textsuperscript{70} bound to hdeABp DNA in the presence or absence of H-NS convincingly suggested such a mechanism of DNA looping (Fig. 6).

We speculate that DNA wraps around Eσ\textsuperscript{70} through a series of DNA kinks. The unpaired bases on hdeABp DNA created by Eσ\textsuperscript{70} binding detected by KMinO\textsubscript{4} treatment, which should reflect these DNA kinks, appeared at those bases around –70, +50, and +63 nt positions, in addition to those around –10 region (Fig. 4). It is noteworthy that DNA kinks produced by Eσ\textsuperscript{70} at these positions are located far from the boundary of Eσ\textsuperscript{70} on the promoter DNA. These DNA kinks may be induced specifically by RP\textsubscript{c} formation with Eσ\textsuperscript{70}. We observed that Eσ\textsuperscript{38} binding induced little DNA kinks, especially downstream of the promoter, suggesting that the promoter DNA wraps around the two forms of RNP in a different
manner. AFM images revealed that the *hdeA*Bp DNA bound by *Er*° was not as sharply kinked or shortened as those bound by *Er*70 (Fig. 6). We obtained the same results with *lacUV5*·5p DNA bound to the two forms of RNPs: A sharp kink was observed with *Er*70 but not with *Er*° (Supplementary Fig. 3). A similar reduction of ~32 nm in the DNA was measured after *Er*70 binding but only ~19 nm after *Er*° binding. Thus, we suggest that DNA is more loosely wrapped around *Er*° than *Er*70 (far less than 300°), which would result in the DNA arms leaving the open *Er*° promoter complex being too far apart for the upstream-bound H-NS to extend to the downstream arm. Thus, such a mechanism would provide an explanation for the observed differential regulatory effect of H-NS on *Er*70- and *Er*°-driven transcription initiation, as shown schematically in Figure 6F. A similar mechanism may account for the selective expression of *osmY* by *Er*°. Its expression by *Er*70 is efficiently repressed by CRP, IHF, and rpoS transcription factors (Collet et al. 2000).

We propose here that two forms RNPs may be distinguished not by the promoter specificity, but by a difference in the promoter DNA wrapping around the RNP molecule that could provide a clue for the selective repression by H-NS. It is speculated that this difference may be ascribed to the conformational changes that occur in the core RNP upon σ binding.

Materials and methods

**Strains and plasmids**

All *E. coli* strains used in this study are derived from the MG1655 background. The bacterial strains constructed by P1 transduction and plasmids are listed in Table 1. Bacteriophage λ carrying *hdeA*Bp·*lacZYA* was obtained from the lysogenic strain TY001, by induction (Yoshida et al. 1993).

The plasmids used for in vitro transcription assays were constructed by cloning DNA fragments carrying the promoter sequences between the EcoRI and PstI sites of the transcriptional vector pSA508 (Choy and Adhya 1993). Plasmids carrying various *hdeA*Bp segments were obtained by cloning PCR-amplified fragments into the restriction sites in pSA508 (see Results). Mutants carrying insertions at ~44.5 nt were generated by cloning the synthetic DNA oligomers (see Results).

**Table 1. Strains and plasmids**

| Strains       | Description                                   | References          |
|---------------|----------------------------------------------|---------------------|
| *Escherichia coli* |                                              |                     |
| MG1655        | Wild type                                    |                     |
| CH 1018       | Δ*arg-lac*J169                               | Kim et al. 2004     |
| CH 1282       | CH1018, ΔpoS                                 | Kim et al. 2004     |
| CH 1281       | CH1018, *hdeA*Bp·*lacZYA*                    | Ueguchi et al. 1996, Kim et al. 2004 |
| CH 1291       | CH1282, *hdeA*Bp·*lacZYA*                    | Ueguchi et al. 1996, Kim et al. 2004 |
| CH 1333       | CH 1291, Δhns                                | Ueguchi et al. 1996, Kim et al. 2004 |
| Plasmids      |                                              |                     |
| pRS415        | *LacZ* fusion vector, Amp'                    | Simons et al. 1987  |
| pSA508        | Transcriptional vector, Amp'                  | Choy and Adhya 1993 |

**Growth conditions**

*E. coli* carrying λ·*hdeA*Bp·*lacZYA* were grown in LB medium (Difco Laboratories, Becton Dickinson) containing 1% NaCl with vigorous aeration at 37°C. For solid support medium, 1.5% agar (Difco Laboratories) was included. Antibiotics (Sigma) were added at the following concentrations: ampicillin, 50 µg/mL; tetracycline, 15 µg/mL; kanamycin, 30 µg/mL. X-gal (Sigma) was used at 20µg/mL.

**β-Galactosidase assay**

β-Galactosidase assays were performed as described by Miller (1972), using cells permeabilized with Koch’s lysis solution (Putnam and Koch 1975). β-Galactosidase-specific activity was expressed as Miller units [A1550/min/A600 = 1000).

To measure β-galactosidase levels in bacteria at different stages of growth, overnight cultures were diluted 1:50 into LB and grown at 37°C until the cultures reached stationary phase. Samples were taken for enzyme assays at regular time intervals. Each strain was assayed in triplicate and average enzyme activities were plotted as a function of time.

**In vitro DNA–protein interaction assays**

Transcription reactions were carried out using the procedure described by Choy and Adhya (1993). Briefly, 2 nM DNA template, 1 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP, and 10–20 µCi of [α-32P] UTP were preincubated in buffer (20 mM Tris-acetate at pH 7.8, 10 mM magnesium acetate, 100 mM potassium glutamate, 1 mM dithiothreitol) for 5 min at 37°C. H-NS was always included in the preincubation mix. Transcription was initiated by the addition of RNP (20 nM) in a total volume of 20 µL and was terminated after 10 min at 37°C by the addition of an equal volume (20 µL) of RNA loading buffer (80% [v/v] deionized formamide, 1 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanole). The mixture was electrophoresed in an 8 M urea/8% polyacrylamide sequencing gel (40 cm × 0.4 mm) for analysis. The RNA transcripts were quantified by determining counts per minute with a β scanner (FLA3000, Fuji Instrument).

**KMM04 reactions**

Following the protocol described by Rostoks et al. (2000). The reaction conditions were the same as those of in vitro transcription reactions except that nucleotides were omitted. Bases modified by KMnO4 were analyzed by primer extension analysis using the alkaline denaturation procedure described in Rostoks et al. (2000). The primers were 5’-GGCTTCACCCGAGCTCGTCGACCCGGGTACCGA-3’ for the top strand sequence or 5’-GGCGGGTTTTTACGTTATTTGC-3’ for the bottom strand sequence of the sequences flanking *hdeA*Bp.

**Gel mobility shift assay**

Gel mobility shift assays were carried out as described in Shin et al. (2001). Assays were performed using various PCR fragments of the *hdeA*B promoter DNA end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega). Reaction mixtures contained 2 nM end-labeled *hdeA*Bp DNA fragment in transcription buffer and different concentrations of H-NS and/or RNP (see Figs. 2, 3). The mixture was incubated for 10 min at
37°C and then loaded onto a 5% native polyacrylamide gel (50:1) and electrophoresed at 100 V for 1.5 h.

**Western blot analysis**

A monoclonal antibody against the α subunit of RNP was purchased from Neoclon. Anti-H-NS serum was prepared from a New Zealand White rabbit following the procedure described in Lee et al. [2000]. DNA-protein complexes on native polyacrylamide gel were transferred to polyvinylidene difluoride (PVDF) membrane and probed with antibody against the α subunit of RNP (Neoclon). Bound antibodies were detected by ECL (Amersham) and exposure to X-ray film. The membrane was stripped with Western blotting strip buffer (Pierce) and probed again with anti-H-NS and visualized by ECL.

**Atomic force microscopy and analysis**

DNA-protein complexes were prepared by incubating RNP [20 nM] and/or H-NS [228 nM] together with 2 nM of the double-stranded 796-bp hdeAB DNA fragment (~216 to +580) obtained by PCR. The mixture was incubated in transcription buffer at 37°C for 10 min, then 10 µl of reaction mix was deposited onto freshly cleaved mica and incubated for 15 min at room temperature. The mica disk was rinsed with distilled water and dried under nitrogen gas. Atomic force microscopic images of DNA-protein complexes under air were obtained with a Nanoscope IIIa [Digital Instruments] in the tapping mode with silicon tips [Digital Instruments]. The microscope was equipped with a type J scanner (125 × 125 µm). Images (512 × 512 pixels) were collected with a scan size of 2 µm at a scan rate of 1 scan line/sec.

The DNA contour length of the RNP-promoter DNA complex was measured by tracing the DNA in the AFM images [Rivetti et al. 1999].

**Proteins**

α-Free RNP core enzyme from the BL21 strain was purchased from Epicentre. α70 and α48 were purified using the IMPACT system (New England Biolabs). Briefly, the respective α factor gene was cloned in frame to the 5′ end of intein in the pTYB2 plasmid. Pure α was eluted from chitin beads after 1 mM DTT treatment. RNP holoenzymes were prepared by incubating the purified core enzyme and fourfold molar excess of the respective α subunit at 37°C for 30 min [Rusano et al. 1996]. H-NS was purified from the BL21 strain carrying a plasmid in which hns was cloned under the T7 promoter [pPFD3, a gift from E. Bremer, FRG] using the procedure of Dersch et al. [1993].

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DNA looping-mediated repression by histone-like protein H-NS: specific requirement of Escherichia coli 70 as a cofactor for looping

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