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

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Transcription initiation by RNA polymerase (RNP) carrying the house-keeping σ subunit, σ^{70} ($E\sigma^{70}$), is repressed by H-NS at a number of promoters including *hdeAB*p in *Escherichia coli*, while initiation with RNP carrying the stationary phase σ, σ^{38} ($E\sigma^{38}$), 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 *hdeAB*p as a model promoter, we observed with purified components that H-NS, acting at a sequence centered at –118, selectively repressed transcription by $E\sigma^{70}$. This selective repression is attributed to the differences in the interactions between *hdeAB*p 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 (RP_O) at *hdeAB*p, but that $E\sigma^{70}$ failed to initiate transcription in the presence of H-NS. Interestingly, $KMnO_4$ assays and high-resolution atomic force microscopy (AFM) revealed that *hdeAB*p DNA wrapped around $E\sigma^{70}$ more tightly than around $E\sigma^{38}$, resulting in the potential crossing over of the DNA arms that project out of $E\sigma^{70} \cdot RP_O$ but not out of $E\sigma^{38} \cdot RP_O$. 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 $E\sigma^{70} \cdot RP_O$, resulting in effective sealing of the DNA loop and trapping of $E\sigma^{70}$. Such a ternary complex of H-NS · $E\sigma^{70}$ · *hdeAB*p was demonstrated by AFM. In this case, therefore, $E\sigma^{70}$ acts as a cofactor for DNA looping. Expression of this class of genes by $E\sigma^{38}$ in the stationary phase is not due to its promoter specificity but to the architecture of the promoter · $E\sigma^{38}$ complex.

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Bacterial RNA polymerase (RNP) consists of core subunits (2α, β, β′), and a σ subunit, which confers promoter specificity (Ishihama 2000). During the exponential phase of growth, σ^{70} is the predominant form that is responsible for expression of housekeeping genes in *Escherichia coli*. As the culture enters stationary phase, σ^{38} encoded by *rpoS*, is expressed (Mulvey and Loewen 1989; Lange and Hengge-Aronis 1991). RNP, loaded with σ^{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 σ^{70} ($E\sigma^{70}$) and that carrying σ^{38} ($E\sigma^{38}$) (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 $E\sigma^{38}$ 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-

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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 (Drlica 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 (K_d) to these preferred sites, therefore, is only in the range of micromolar concentration (Fried 1989; Sonnenfield et al. 2001), not vastly stronger than to the DNA with no curvature (Lucht et al. 1994). It has been suggested that initial binding of H-NS to a preferred site is followed by lateral extension along the DNA, by oligomerization of 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 oligomerization of H-NS is, therefore, essential for preferential binding and stabilization of the multimeric nucleoprotein complex (Spurio et al. 1997). A recent study of an *E. coli* ribosomal gene promoter *rrnB* P1 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 $E\sigma^{70}$ is repressed by H-NS while that with $E\sigma^{38}$ is unaffected. This class includes the *hdeAB*, *csgA*, *gadB/C*, and *csiD* promoters in *E. coli* and the *spvR* promoter in *Salmonella typhimurium* (Arnqvist 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 hdeAB*p 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 $E\sigma^{70}$ through H-NS-mediated repressive DNA looping, which requires an RP_O with $E\sigma^{70}$ 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 *hdeAB*p in *E. coli* during the course of bacterial growth in LB. The *hdeAB*p activity was determined using a λ lysogen carrying the *hdeAB*p–*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 A_{600} (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 *hdeAB*p increased as the culture entered stationary phase, reaching a maximum accumulation of ~35-fold (Fig. 1A). In the *rpoS* mutant background (triangles), *hdeAB*p activity was minimal. In the *hns* and *rpoS* double-mutant background (squares), however, the *hdeAB*p 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 *hdeAB*p in the *hns* and *rpoS* 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 *hdeAB*p belongs to the class of promoters normally transcribed by $E\sigma^{38}$, but also by $E\sigma^{70}$ in the absence of H-NS (Yoshida et al. 1993; Arnqvist et al. 1994; Robbe-Saule et al. 1997; Marschall et al. 1998; Waterman and Small 2003). H-NS selectively represses $E\sigma^{70}$-mediated transcription of this class of promoters.

An in vitro transcription assay using purified components with the reconstituted $E\sigma^{70}$ or $E\sigma^{38}$ holoenzyme was carried out to examine the selective repression of *hdeAB*p by H-NS. Supercoiled plasmid carrying the *hdeAB*p 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 $E\sigma^{70}$ (Fig. 1B, panel 1) and $E\sigma^{38}$ (Fig. 1B, panel 2) generated the same transcript from *hdeAB*p, as is the case for most other σ^{38} promoters tested in vitro (Kim et al. 2004). In the presence of increasing concentrations of H-NS, the *hdeAB* transcript generated by $E\sigma^{70}$ decreased, while the *rna1* transcript from the origin of replication remained constant. The *lacUV5*p DNA transcribed by $E\sigma^{70}$ provided a control. The *lacUV5*p activity was unaffected by the presence of H-NS (Fig. 1B, panel 3). Thus, the repression of *hdeAB*p by H-NS was promoter specific under these in vitro conditions. Most notably, the *hdeAB* transcript

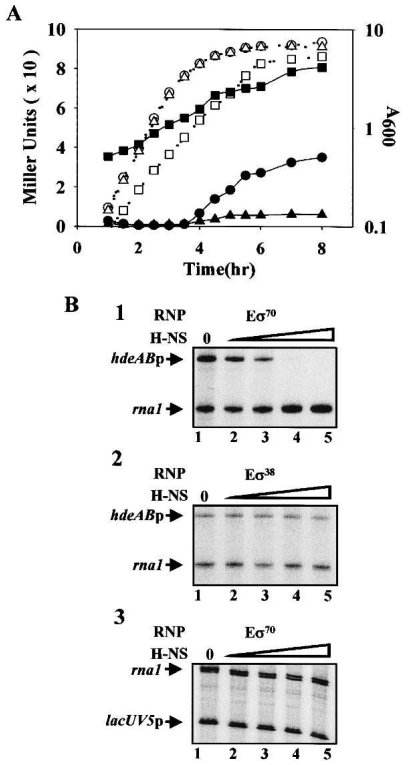


Figure 1. Regulatory effect of H-NS. (*A*) Expression from *hdeABp* (–136 to +120) fused to *lacZYA* was determined in vivo in the wild-type background (circles), in the RpoS$^-$ mutant background (triangles), and in the RpoS$^-$ and Hns$^-$ double-mutant background (squares). λ Lysogens carrying the *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 (A_{600}, *right* axis) and closed symbols represent β-galactosidase activity (A_{420}/min/mL/A_{600}, 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 Eσ^{70} (panels *1* and *3*) or Eσ^{38} (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 Eσ^{38} was not affected by the presence of H-NS. These data suggest that H-NS represses transcription from *hdeABp* by Eσ^{70}, but not by Eσ^{38}, 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 hdeAB*p*

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 Eσ^{70} (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 *rrnB*1 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 3′ 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 RP_O formation

Transcription initiation at *hdeABp* by Eσ^{70}, but not that by Eσ^{38}, is subject to repression by H-NS. The following experiments were conducted to identify which step during transcription initiation by Eσ^{70} 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 Eσ^{38} > H-NS > Eσ^{70}. A gel retardation assay was carried out with H-NS and/or RNP (Fig. 3). H-NS, Eσ^{70}, and Eσ^{38} 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 Eσ^{70} or Eσ^{38} 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

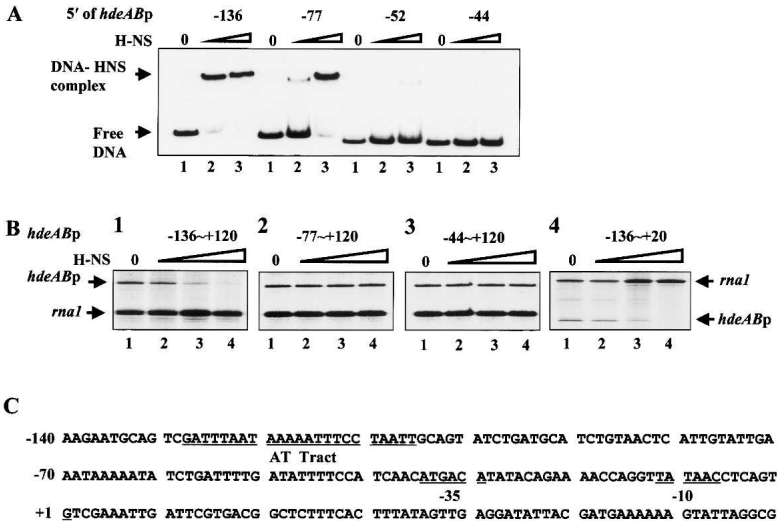


Figure 2. Identification of DNA-binding site on *hdeAB*p at which H-NS exerts its regulatory effect. (*A*) Gel mobility shift assay with *hdeAB*p DNA constructs with various 5′ ends and a fixed 3′ end (+120), and H-NS separated on a 5% native polyacrylamide gel. Heparin was added at 34μg/mL prior to loading the reaction mixes onto the gel. The H-NS added in the incubation mix was 0 (lane *1*), 76 nM (lane *2*), and 228 nM (lanes *3*). (*B*) H-NS titration of in vitro transcription, as described in Figure 1, using *hdeAB*p DNA templates with different 5′ ends and a fixed 3′ end at +120 (panels *1*–*3*) and *hdeAB*p DNA template carrying the sequence between –136 to +20 (panel *4*). H-NS added in the preincubation mix was 0 (lane *1*), 25 nM (lane *2*), 76 nM (lane *3*), and 228 nM (lane *4*). (*C*) The *hdeAB*p sequence. The AT tract centered at –118 nt at which H-NS is thought to bind and –35, –10, and +1 elements are underlined.

conducted gel shit assays in which the reaction mixes contained increasing amounts of H-NS alone (Fig. 3B,C, lanes 2–4) or together with 20 nM RNP (Fig. 3B,C, lanes 5–7). These gels were analyzed by Western blotting using antibodies specific for H-NS (Fig. 3B,C, right panels) or the α subunit of RNP (Fig. 3B,C, middle panels). The anti-α antibody detected the retarded band (Fig. 3B,C, closed arrow) and additional bands underneath that are unbound RNP for both $E\sigma^{70}$ and $E\sigma^{38}$ (see Supplementary Fig. 2). Importantly, the anti-H-NS antibody bound not only to the binary DNA–H-NS complexes (Fig. 3B,C, open arrow) but also to the supershifted band (Fig. 3B,C, closed arrow) with both $E\sigma^{70}$ and $E\sigma^{38}$. These data suggest that the two forms of RNPs do not compete with H-NS for *hdeAB*p DNA binding, but form a stable RNP–promoter complex, presumably an open promoter complex (see below), in the presence of H-NS.

The repression of transcription could occur at any step during transcription initiation: RP_C (closed promoter complex) formation, RP_O (open promoter complex) formation, or subsequent transition to transcription elongation (Choy and Adhya 1996). Thus, we determined which step during transcription initiation is blocked by H-NS using the $KMnO_4$ assay, which detects unpaired bases in the –10 region and also those unpaired bases generated by DNA distortion (Hayatsu et al. 1966; Sasse-Dwight and Gralla 1989; Rostoks et al. 2000). Figure 4 shows the $KMnO_4$ reactive bases generated by RNP binding in the absence and presence of H-NS (228 nM). Using supercoiled DNA, $KMnO_4$ reactive bases were detected by primer extension using primers that anneal to the upstream (Fig. 4A,C) or downstream (Fig. 4B,D) of *hdeAB*p in the vector, pSA508. Binding of RNPs induced the opening of several base pairs in the –10 region (asterisks in Fig. 4A,B) and also those outside of the promoter (see below). Figure 4C and D show the bases around the –10 region that were used to analyze the pattern of base-pair opening: specifically the bases between –8 and –11 for the top strand (Fig. 4C) and those between +2 and –13 for the bottom strand (Fig. 4D). Subsequently, we examined whether or not the presence of H-NS prevented RNP, especially $E\sigma^{70}$, from forming an RP_O at *hdeAB*p. The presence of H-NS did not decrease the intensities of the $KMnO_4$ hypersensitive bands created by $E\sigma^{70}$ or $E\sigma^{38}$. These data indicate that $E\sigma^{70}$ forms an RP_O 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 RP_O formation at the *hdeAB*p by $E\sigma^{70}$.

It should be emphasized that the DNA distortion induced by $E\sigma^{70}$ binding was significantly different from that of $E\sigma^{38}$, 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 $KMnO_4$ in the presence of either one of the two forms of RNP. The different patterns of $KMnO_4$ 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 $E\sigma^{70}$ 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 *hdeAB*p DNA (–136 to +30 nt) and these constructs were used to examine the extent of repression of $E\sigma^{70}$-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 *hdeAB*p by H-NS is not through a direct interaction with $E\sigma^{70}$ (Choy and Adhya 1996; Roy et al. 1998). Therefore,

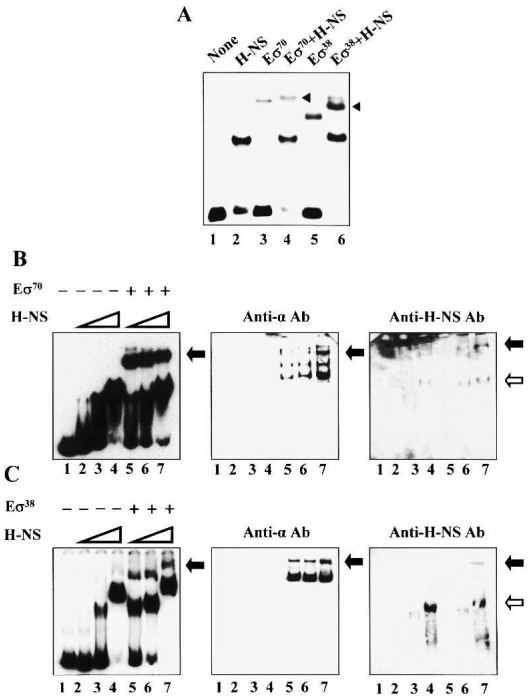


Figure 3. Simultaneous binding of H-NS and RNP to *hdeAB*p DNA (–136 to +120), as assessed by gel mobility shift (*A*) and Western analysis (*B* for Eσ^{70} binding and *C* for Eσ^{38} binding). See text for details. Proteins present in the assays are indicated *above* each lane. (*A*) A gel mobility 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 *hdeAB*p 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 *hdeAB*p 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.

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 Eσ^{70} 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 hdeAB*p DNA–protein complexes*

Lastly, we employed high-resolution atomic force microscopy (AFM) to visualize the two forms of RNPs bound to *hdeAB*p 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 *hdeAB*p DNA bound by RNP, in the presence or absence of H-NS. Since Eσ^{70} 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 *hdeAB*p 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 · *hdeAB*p. These complexes constituted nearly 49% of the total binary complexes. Figure 6A and B, shows the representative binary complexes formed with Eσ^{70} and Eσ^{38}, respectively. The DNA bound by Eσ^{70} was sharply kinked at the position of the RNP, while DNA bound by Eσ^{38} was not. To determine

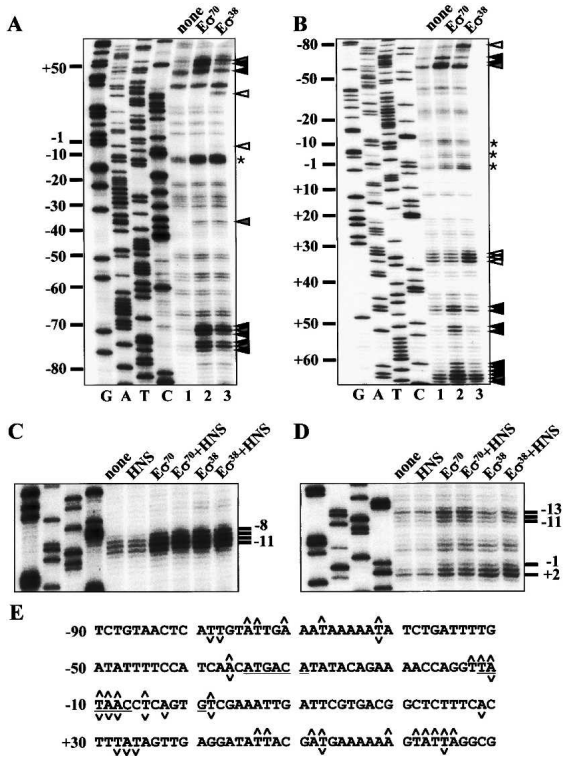


Figure 4. DNA kinks induced by RNP (20 nM, *A*,*B*) and/or H-NS binding (228 nM, *C*,*D*) to *hdeAB*p DNA (–136 to +120) as probed by $KMnO_4$ assay. Unpaired bases were revealed by primer extension since a supercoiled DNA template was used (see Materials and Methods). *A* and *C* show the result of analysis using the primer with the *top* sequence, 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 Eσ^{70} and Eσ^{38} 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 hyperreactive to $KMnO_4$ induced by Eσ^{70} binding (carets *above* bases) or by Eσ^{38} binding (carets *below* bases). The –35, –10, and +1 elements are underlined.

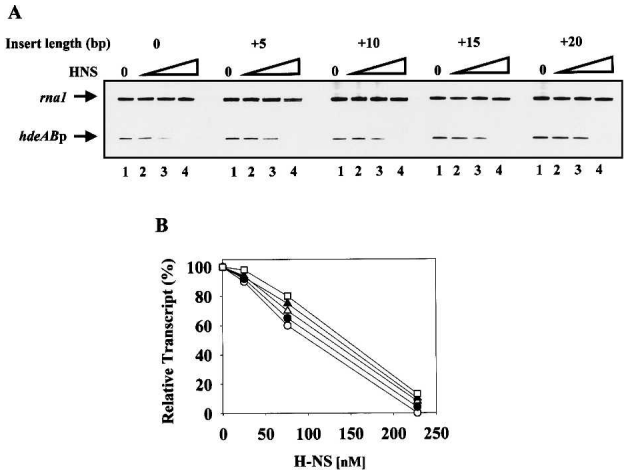


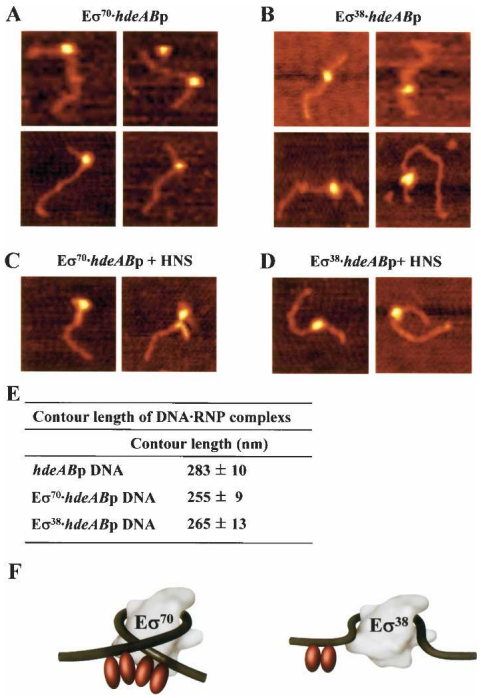
Figure 5. Effect of lengthening the interval between H-NS-binding site and *hdeAB*p on H-NS-mediated regulation. DNA fragments increasing by 5-bp increments were inserted at –44.5 nt of the *hdeAB*p DNA (–136 to +30). Inserted DNA fragments were ATCGA (5 bp), CTAGAAACGA (10 bp), CTAGAGCTC GAGCGA (15 bp), and CTAGACCATGGCTCGATCGA (20 bp). (*A*) Using the *hdeAB*p 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. (○) *hdeAB*p DNA template carrying no insert; (●) *hdeAB*p DNA template carrying a 5-bp insert; (△) *hdeAB*p DNA template carrying a 10-bp insert; (▲) *hdeAB*p DNA template carrying a 15-bp insert; and (□) *hdeAB*p DNA template carrying a 20-bp insert.

the degree of DNA wrapping in the complexes, we measured the DNA contour length of the RNP · *hdeAB*p DNA by tracing the DNA backbone (Rivetti et al. 1999). In agreement with Rivetti et al. (1999), we found the contour length of $E\sigma^{70}$ · *hdeAB*p DNA was shortened by ~80–90 bp (~28 nm) when compared with the naked DNA. But, most interestingly, the contour length of $E\sigma^{38}$ · *hdeAB*p DNA was reduced only by ~18 nm. These data indicate that the nature of the interaction between promoter DNA and the two forms of RNPs is architecturally different.

Subsequently, we analyzed the ternary complexes consisting of *hdeAB*p DNA, either form of RNP, and H-NS, by AFM. The AFM image revealed that H-NS bridges the DNA arms flanking $E\sigma^{70}$ and this traps $E\sigma^{70}$ within a hairpin-like configuration of DNA (Fig. 6C). We observed no such hairpin-like configuration with H-NS alone or with the *hdeAB*p DNA lacking the sequence upstream of –77 in the presence of H-NS and $E\sigma^{70}$ (data not shown). Thus, we suggest that the H-NS bound to the upstream site would laterally extend to the downstream sequence joined by DNA wrapping around $E\sigma^{70}$ that seals off the loop. Consistently, H-NS was found on only one side of the DNA arms flanking $E\sigma^{38}$ (Fig. 6D). Thus, H-NS fails to repress $E\sigma^{38}$-driven transcription initiation because the DNA arms leaving $E\sigma^{38}$ · *hdeAB*p 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 *hdeAB*p as a model promoter to differentiate transcription initiation by $E\sigma^{70}$ and $E\sigma^{38}$, which share virtually identical promoter specificities (Kim et al. 2004). There is a class of promoters that is regulated in a similar manner to that of



Contour length of DNA·RNP complexes	
	Contour length (nm)
*hdeAB*p DNA	283 ± 10
$E\sigma^{70}$·*hdeAB*p DNA	255 ± 9
$E\sigma^{38}$·*hdeAB*p DNA	265 ± 13

Figure 6. Atomic force microscopy images of representative RNP (20 nM) bound to *hdeAB*p DNA (–216 to +580) in the presence or absence of H-NS (228 nM). *A* and *B* show representative montages of $E\sigma^{70}$ and $E\sigma^{38}$ stably bound to *hdeAB*p 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 *hdeAB*p DNA complexed with $E\sigma^{70}$ (*C*) or $E\sigma^{38}$ (*D*). These images show thickening of the DNA arms by H-NS binding, which cross-bridged DNA in the presence of $E\sigma^{70}$ (*C*). No such DNA bridging was observed with $E\sigma^{38}$ (*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 *hdeAB*p DNA. (*Left*) $E\sigma^{70}$ binding induces kinks into the target DNA, facilitating oligomerization of the bound H-NS molecules on the DNA arms project out of an $E\sigma^{70} \cdot RP_O$. (*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 $E\sigma^{38} \cdot RP_O$.

hdeAB and that is transcribed by $E\sigma^{38}$, but not by $E\sigma^{70}$ in the presence of H-NS (Arnqvist 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 $E\sigma^{70}$ catalyzes its transcription. In this study, we showed that H-NS repressed transcription from *hdeAB*p by $E\sigma^{70}$, but not by $E\sigma^{38}$, in vitro with purified components (Fig. 1) and this activity required the sequence between –77 and –136 nt of *hdeAB*p, most likely involving the AT tract centered at –118 nt, and no other specific sequence (Fig. 2). $E\sigma^{70}$ and $E\sigma^{38}$ could each bind to *hdeAB*p DNA in the presence of H-NS (Fig. 3). H-NS repressed *hdeAB*p transcription at a step subsequent to RP_O formation by $E\sigma^{70}$, but prior to the formation of the first phosphodiester bond (Fig. 4). The regulatory effect of H-NS was not through direct contact with $E\sigma^{70}$ (Fig. 5). AFM showed that $E\sigma^{70}$ binding to *hdeAB*p induced a sharp kink in the DNA, but $E\sigma^{38}$ did not (Fig. 6). Most interestingly, the DNA arms projecting out of the $E\sigma^{70} \cdot$ *hdeAB*p complex were joined in parallel with H-NS in a hairpin-like configuration, but not that of $E\sigma^{38} \cdot$ *hdeAB*p. 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 $E\sigma^{70}$, which results in trapping of $E\sigma^{70}$ in a DNA loop. Thus, the H-NS-mediated repression would depend on the configuration of DNA wrapping around the RNP.

The repression of *hdeAB*p by H-NS is not due to direct occlusion of $E\sigma^{70}$, since formation of an open promoter complex (RP_O) 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 *hdeAB*p. Rather, it is reminiscent of repression of *rrnB* 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; Badaut 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 (Badaut et al. 2002). A similar mechanism could account for the repression of *hdeAB*p 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 $E\sigma^{70}$, and wraps 300° around the RNP in the RP_O (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_C (Rivetti et al. 1999; Dame et al. 2002). We propose here that DNA looping by H-NS requires an open promoter complex with $E\sigma^{70}$ as a cofactor; that is, H-NS forms DNA loops only when the upstream and downstream DNA arms project out of the $E\sigma^{70}$. This is because the DNA wrapping in the $E\sigma^{70}$ 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 $E\sigma^{70}$. AFM images of $E\sigma^{70}$ bound to *hdeAB*p 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 $E\sigma^{70}$ through a series of DNA kinks. The unpaired bases on *hdeAB*p DNA created by $E\sigma^{70}$ binding detected by $KMnO_4$ 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 $E\sigma^{70}$ at these positions are located far from the boundary of $E\sigma^{70}$ on the promoter DNA. These DNA kinks may be induced specifically by RP_O formation with $E\sigma^{70}$. We observed that $E\sigma^{38}$ binding induced little DNA kinks, especially downstream of the promoter, suggesting that the promoter DNA wraps around the two forms of RNPs in a different

manner. AFM images revealed that the *hdeAB*p DNA bound by $E\sigma^{38}$ was not as sharply kinked or shortened as those bound by $E\sigma^{70}$ (Fig. 6). We obtained the same results with *lacUV5*p DNA bound to the two forms of RNPs: A sharp kink was observed with $E\sigma^{70}$ but not with $E\sigma^{38}$ (Supplementary Fig. 3). A similar reduction of ~32 nm in the DNA was measured after $E\sigma^{70}$ binding but only ~19 nm after $E\sigma^{38}$ binding. Thus, we suggest that DNA is more loosely wrapped around $E\sigma^{38}$ than $E\sigma^{70}$ (far less than 300°), which would result in the DNA arms leaving the open $E\sigma^{38}$ 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 $E\sigma^{70}$- and $E\sigma^{38}$-driven transcription initiation, as shown schematically in Figure 6F. A similar mechanism may account for the selective expression of *osm*Y by $E\sigma^{38}$: Its expression by $E\sigma^{70}$ is efficiently repressed by CRP, IHF, and lrp transcription factors (Colland 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 *hdeAB*p::*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 EcoR1 and Pst1 sites of the transcriptional vector pSA508 (Choy and Adhya 1993). Plasmids carrying various *hdeAB*p 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)U169*	Kim et al. 2004
CH 1282	CH1018, Δ*rpoS*	Kim et al. 2004
CH 1281	CH1018, Φ *hdeAB*p::*lacZYA*	Ueguchi et al. 1996; Kim et al. 2004
CH 1291	CH1282, Φ *hdeAB*p::*lacZYA*	Ueguchi et al. 1996; Kim et al. 2004
CH 1333	CH 1291, Δ*hns*	Ueguchi et al. 1996; Kim et al. 2004
Plasmids		
pRS415	*LacZ* fusion vector, Amp^r	Simons et al. 1987
pSA508	Transcriptional vector, Amp^r	Choy and Adhya 1993

Growth conditions

E. coli carrying λ[Φ*hdeAB*p-*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 (A_{420}/min/A_{600} × 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-^{32}P] 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× TBE [89 mM Tris, 89 mM boric acid, 2 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).

$KMnO_4$ reactions followed 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 $KMnO_4$ were analyzed by primer extension analysis using the alkaline denaturation procedure described in Rostoks et al. (2000). The primers were 5′-GGCTTCAACCGAGCTCGTCGACCCGGGTACCGA-3′ for the top strand sequence or 5′-GCGGGTTTTTACGTTATTT GC-3′ for the bottom strand sequence of the sequences flanking *hdeAB*p.

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 *hdeAB* promoter DNA end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Reaction mixtures contained 2 nM end-labeled *hdeAB*p 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 Neoclone. 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 (Neoclone). 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*p 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

σ-Free RNP core enzyme from the BL21 strain was purchased from Epicentre. σ^{70} and σ^{38} were purified using the IMPACT system (New England Biolabs). Briefly, the respective σ factor gene was cloned in frame to the 5′ end of intein in the pTYB2 plasmid. Pure σ 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 σ subunit at 37°C for 30 min (Kusano et al. 1996). H-NS was purified from the BL21 strain carrying a plasmid in which *hns* was cloned under the T7 promoter (pPD3, a gift from E. Bremer, FRG) using the procedure of Dersch et al. (1993).

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