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# Structural Basis for H-NS-mediated Trapping of RNA Polymerase in the Open Initiation Complex at the *rrnB* P1\*

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The *Escherichia coli* H-NS protein is a nucleoid-associated protein involved in both transcription regulation and DNA compaction. Each of these processes involves H-NS-mediated bridge formation between adjacent DNA helices. With respect to transcription regulation, preferential binding sites in the promoter regions of different genes have been reported, and generally these regions are curved. Often H-NS binding sites overlap with promoter core regions or with binding sites of other regulatory factors. Not in all cases, however, transcriptional repression is the result of preferential binding by H-NS to promoter regions leading to occlusion of the RNA polymerase. In the case of the *rrnB* P1, H-NS actually stimulates open complex formation by forming a ternary RNAP-H-NS-DNA complex, while simultaneously stabilizing it to such an extent that promoter clearance cannot occur. To define the mechanism by which H-NS interferes at this step in the initiation pathway, the architecture of the RNAP-H-NS-DNA complex was analyzed by scanning force microscopy (SFM). The SFM images show that the DNA flanking the RNA polymerase in open initiation complexes is bridged by H-NS. On the basis of these data, we present a model for the specific repression of transcription initiation at the *rrnB* P1 by H-NS.

The *Escherichia coli* nucleoid-associated protein H-NS, originally identified as a heat stable transcription factor (1), is one of the major components of the bacterial nucleoid (2, 3). It was therefore proposed to be involved in the structural organization of the *E. coli* chromosome. Overproduction of H-NS leads to extreme nucleoid condensation and is lethal (4). The absence of H-NS in an *hns* deletion mutant results in an increased degree of negative supercoiling of both plasmid and chromosomal DNA (5). *In vitro*, effects of H-NS on DNA topology and condensation have also been shown (6–8).

Mutations in H-NS not only affect nucleoid structure (9), but also the expression of a wide variety of genes (10), illustrating the second important role of H-NS as a pleiotropic regulator of transcription. Around 5% of the genes in *E. coli* are affected at

the level of transcription by changes in the intracellular levels of H-NS (11). Although other nucleoid-associated proteins that also function as transcription factors, such as IHF and Fis, recognize more or less specific sequences (for a recent review, see Ref. 12), H-NS binding does not occur with any obvious sequence specificity. Nevertheless, the protein is involved in specific regulation of transcription of a large number of genes such as *proU*, *hns*, *virF*, *fimB*, *rrnB*, *bgl*, and the genes involved in the early development of bacteriophage Mu (13–19).

To explain the role of H-NS in transcription regulation, two mechanisms, which are not necessarily mutually exclusive, have been proposed. First, H-NS might indirectly regulate initiation from supercoiling-sensitive promoters as a consequence of the *in vivo* effects of H-NS on DNA supercoiling (5, 20). Second, H-NS can also, as a classic prototype inhibitor (21), directly inhibit transcription by preferential binding to the promoter region. *In vitro* evidence for such preferential binding of H-NS to the promoter regions of *proU*, *hns*, *virF*, *clyA*, and *rrnB* has been obtained by footprinting experiments (13–15, 22, 23). In addition, indications that H-NS has a binding preference to these regions as well as the promoter region of *fimB* have come from competitive gel retardation studies (14, 16, 17, 23–25). However, the difference in affinity between a preferred H-NS binding site and a nonspecific site is not as large as for classical transcription regulators (often less than an order of magnitude).

Mutational studies have revealed the domain organization of H-NS. The protein (136 residues) consists of an oligomerization domain (residue 15–64) and a DNA binding domain (residue 90–121), connected by a flexible linker region (26). Whereas H-NS has long been thought to exist as a dimer or tetramer in solution (9, 27), recent studies point to the formation of a wide range of higher oligomeric forms dependent upon its concentration (28). The integrity of the oligomerization domain of H-NS has been shown to be essential both for preferential binding and DNA condensation (9).

Many of the preferred binding sites contain an A/T-rich region, suggesting that a sequence-induced curvature is causing the preferential binding. It has been shown *in vitro* that indeed H-NS binds with a higher affinity to strongly curved DNA when compared with noncurved or moderately curved DNA, independent of the base composition (29, 30). Footprints of H-NS on the most strongly curved DNA fragment examined show specific protection of the curved region (31). Scanning force microscopy has shown that preferential binding to this curved DNA fragment occurs as a result of the DNA around the curve being bridged by H-NS, which leads to the formation of a hairpin-like structure at the position of the curve (32).

Although evidence for promoter occlusion by specific binding of H-NS has been found in some cases, in other cases alterna-

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tive mechanisms are likely to be involved. It has recently been demonstrated that repression of transcription initiation at the *rrnB* P1 (33) is not due to occlusion of the RNA polymerase from its promoter region, even though preferential binding to this region in the absence of RNA polymerase had been shown (17). Instead, binding of RNAP together with H-NS occurs in a cooperative fashion. A ternary open initiation complex is formed, which is stabilized so strongly that it interferes with promoter clearance. Only short abortive transcripts are produced. To identify the molecular mechanism underlying this novel kind of H-NS-mediated repression, we have analyzed RNAP-H-NS-DNA complexes by SFM.<sup>1</sup>

#### MATERIALS AND METHODS

**Substrate for Scanning Force Microscopy**—The plasmid pGP1451 was constructed by inserting the 260-bp *EcoRI/BamHI* fragment of pUC18-1 (containing the complete *rrnB* P1 promoter region, including the upstream activating sequence (34)) in between the respective sites of pBR322. To obtain a plasmid on which no other promoters are present close to the *rrnB* P1, a DNA fragment had to be placed between the ampicillin gene and the *rrnB* P1 fragment. Insertion of the 1282-bp *EcoRI* fragment from pUC4KAPA (Amersham Biosciences, Inc.) in between the *EcoRI* sites of pGP1451 gave rise to pGP1452. The orientation of the insert was checked by restriction analysis. The DNA substrate for SFM was subsequently generated by PCR from pGP1452. One of the oligonucleotides was chosen within the pUC4KAPA insert, the second one on pBR322. Thus a fragment was obtained with a length of 1192 bp containing position +1 of the *rrnB* P1 close to its center (arms are 544 and 649 bp). One of the oligonucleotides used was biotinylated and also led to the introduction of a *SmaI* site in the PCR product. This allowed for purification of the DNA fragment using paramagnetic beads as described previously (32).

**Protein Purification**—RNA polymerase was purified as described previously (35, 36). The activity of the RNA polymerase was assessed by a quantitative assay (37). H-NS was purified from the overproducing strain KA1764 (38) as described previously (8).

**Scanning Force Microscopy**—The ~1200-bp DNA fragment (27 fmol/ $\mu$ l) and  $\sigma^{70}$ -saturated RNA polymerase (47.5 fmol/ $\mu$ l) were incubated at 37 °C for 15 min in incubation buffer (50 mM HEPES (pH 8.0), 60 mM KCl, 15 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM ATP, and 0.1 mM CTP), to allow stable open initiation complex formation. After open complex formation the mixture was further incubated at 24 °C for 10 min upon addition of an H-NS/heparin mixture (final concentration of 5.6 pmol/ $\mu$ l and 12.5 ng/ $\mu$ l, respectively). Samples were deposited on mica as described (8) after 20-fold dilution into dilution buffer (2.5 mM HEPES (pH 8.0), 8 mM MgCl<sub>2</sub>, 1 mM ATP, and 0.1 mM CTP) and subsequently imaged by SFM. The control samples without H-NS were prepared similarly, by adding only heparin after open complex formation.

#### RESULTS

To study the structural basis of H-NS-mediated stabilization of RNA polymerase in the open initiation complex at the *rrnB* P1 promoter, we constructed a linear DNA fragment of around 1200 bp containing the transcription start site (+1) close to its center (see “Materials and Methods”). Incubation of this DNA fragment with RNA polymerase in the presence of the initiating nucleotides ATP and CTP, which have been shown to be essential for stable open complex formation at the *rrnB* P1 (39), followed by a challenge with heparin allows visualization of specific RNAP-DNA complexes (Fig. 1A). Nonspecific binding of RNA polymerase mostly occurs at the DNA ends, whereas nonspecific binding to internal positions on the fragment is negligible (not shown). In the observed specific open complexes an RNAP molecule is bound close to the center of the DNA molecule, and induces a strong “kink” in the DNA at that position. Quantitative analysis in the form of contour length measurements (see Table I) of these complexes reveals that around 50% of the RNAP-DNA complexes is considerably shortened when compared with naked DNA. The other complexes show no significant change in length. This is in agreement with

a recent SFM study, which showed that the apparent DNA contour length in open RNAP-DNA complexes is reduced as a result of the DNA being wrapped around the RNAP (40). The kink formed upon RNAP binding is a direct consequence of this DNA wrapping. The observed reduction in length (~30 nm) is in close agreement with the reduction observed by Rivetti *et al.* (40) and also with the size (around 80 bp) of the RNA polymerase footprint at the *rrnB* P1 (39). The other complexes show no significant change in length and probably correspond to a closed complex form, in which the DNA is not wrapped.

The structural mechanism underlying H-NS-mediated trapping of RNA polymerase in the open initiation complex (33) was investigated by simultaneous addition of H-NS and heparin to preformed RNAP-DNA complexes. The presence of heparin as a competitor is required both for specific binding of H-NS (41) and for limiting the amount of nonspecific RNAP-DNA complexes (39). Under these conditions we observe ternary complexes in which the DNA on both sides of the RNA polymerase is bridged by H-NS and held together over a large but variable part of the DNA molecule (Fig. 1B and Table II). Next to this, complexes are observed without bridging, which are similar to the open initiation complexes formed when no H-NS is present (Fig. 1A). The frequency with which complexes with specifically bound RNA polymerase are observed is around 10% (Table II). In close to 50% of these complexes the DNA aside of the bound RNAP is bridged. The binding of H-NS is evident from the increase in height relative to DNA alone in the bridged regions as can be seen in the tilt views of some typical ternary complexes (Fig. 1C). The increase in height in the DNA tracts bridged by H-NS is much larger than in the case of random DNA contacts, and is of the same order as observed in previous experiments, in which H-NS was shown to form bridges between two DNA helices on plasmid DNA (8) and around the apex of a curved sequence (32). The formation of such bridges is favored when two DNA helices are spatially close and is a consequence of the oligomeric nature of H-NS, which leads to the simultaneous availability of two or more DNA binding domains. Our observations indicate that RNAP can be trapped within a bridged H-NS-DNA complex, after an open complex has been formed. The wrapping of the DNA around RNAP apparently changes the conformation of the DNA in such a way that a “preferential binding site” is created on which a stable H-NS-DNA complex can be formed. Control reactions lacking RNAP (with or without heparin) did not show any specific H-NS-DNA structures in the region of the *rrnB* P1. Probably this kind of stable binding of H-NS encompassing the bound RNAP provides a physical barrier to promoter clearance. This would explain the H-NS-induced abortive initiation described previously (33).

#### DISCUSSION

The ternary RNAP-H-NS-DNA complexes that are observed by SFM strongly resemble the complexes formed with curved DNA (32), in which H-NS causes the DNA to fold back onto itself in a hairpin-like structure with the apex at the position of the curved sequence. It has been proposed that preferential binding to curved sequences stems from the fact that there is a higher probability of forming oligomers between DNA-bound H-NS proteins (32). The binding of H-NS to RNAP-DNA complexes is likely based on the same principle (Fig. 2). In the open initiation complex DNA is wrapped around the RNA polymerase (see “Results” and Refs. 40 and 42). Thus, after open complex formation the DNA upstream and downstream of the bound RNA polymerase is brought in close vicinity. In this situation H-NS-mediated bridging between these DNA regions will be favored, and the RNA polymerase becomes physically trapped (Fig. 2A). The parallel between preferential binding to

<sup>1</sup> The abbreviation used is: SFM, scanning force microscopy.

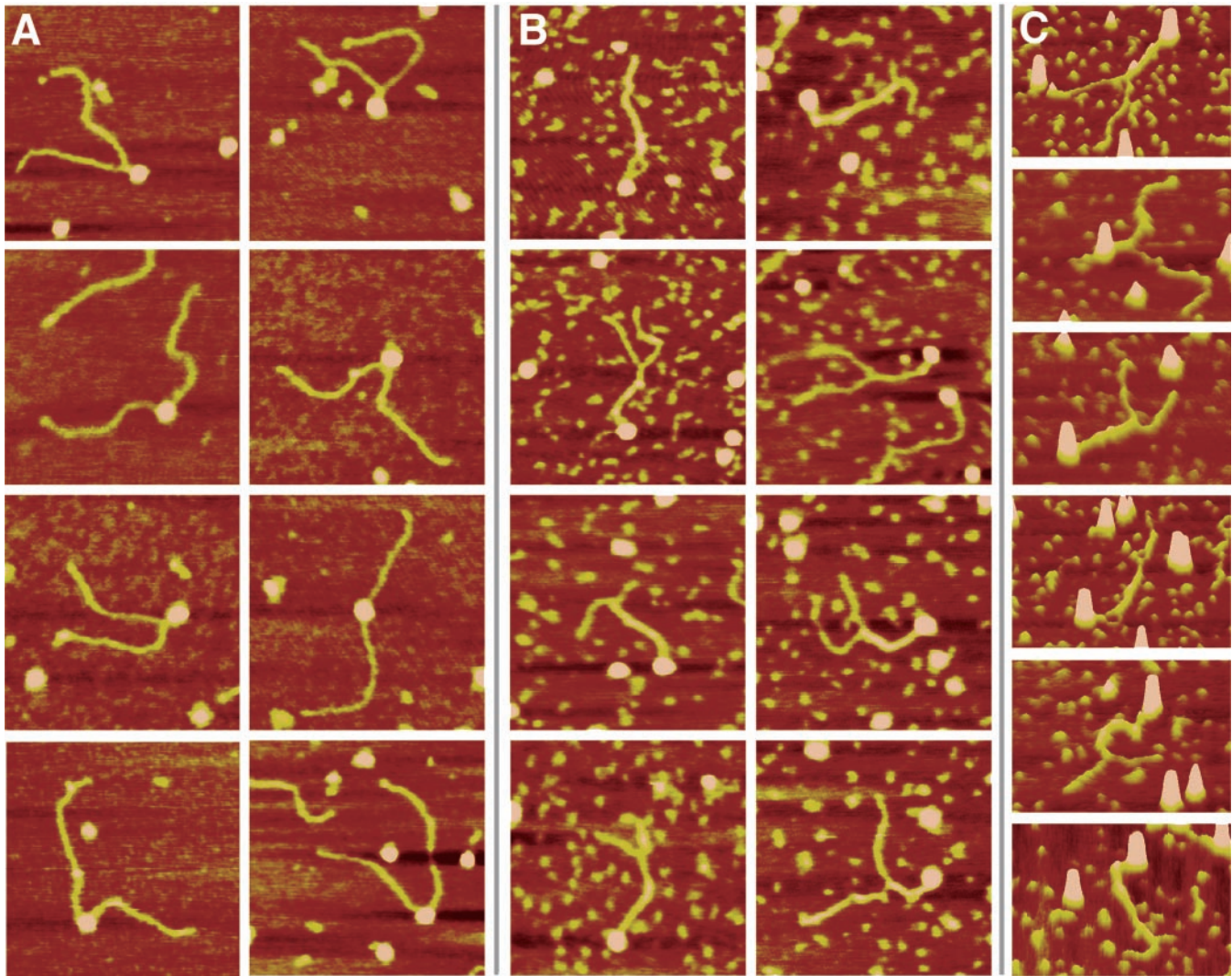


FIG. 1. *A*, SFM images of representative open complexes formed between RNAP and the *rnnB* P1. RNAP-DNA complexes are specifically formed at the position of the transcription start site (+1) of the *rnnB* P1 (at one half of the DNA fragment length) only. *B*, SFM images of representative ternary complexes formed when H-NS binds to open initiation complexes between RNAP and the *rnnB* P1. *C*, SFM images, presented as tilt views to emphasize topography, of representative ternary complexes formed when H-NS binds to open initiation complexes between RNAP and the *rnnB* P1. These images illustrate the considerable thickening caused by the H-NS-mediated DNA bridging. All images show a  $300 \times 300$  nm surface area. Color represents height ranging from 0.0 to 1.5 nm (from dark to bright).

TABLE I

Contour length of free DNA molecules and RNAP-DNA complexes

RP<sub>c</sub> and RP<sub>o</sub> indicate closed and open RNAP-DNA complexes, respectively. The DNA contour length values are the mean of a Gaussian fit to the measured values  $\pm$  the S.D. from the mean. The theoretical contour length was calculated assuming an ideal B DNA structure. Only a minor difference is observed between the theoretical and the experimental value. The total number of molecules/complexes analyzed is 650.

	Contour length	No. of molecules
	<i>nm</i>	
DNA <sub>theoretical</sub>	399	
DNA <sub>experimental</sub>	392 $\pm$ 21	54
RP <sub>c</sub>	395 $\pm$ 14	36
RP <sub>o</sub>	366 $\pm$ 11	32

curved DNA, which could lead to promoter occlusion when involving the promoter region, and RNA polymerase trapping is illustrated by the structural similarity schematized in Fig. 2.

The binding of H-NS does not have apparent boundaries (*i.e.* the length of the DNA tracts involved in the bridging events is variable, see Table II), which agrees with a model in which bridge formation results from an increased probability of oli-

TABLE II

Length of bridged tracts of RNAP-H-NS-DNA complexes

RP<sub>o/c</sub> indicates RNAP-DNA complexes. RP<sub>o</sub>-H-NS indicates RNAP-H-NS-DNA complexes. The length of the bridged tracts is the mean of the measured values  $\pm$  the S.D. from the mean. The total number of molecules/complexes analyzed is 700.

	Length of bridged tracts	No. of molecules
	<i>nm</i>	
RP <sub>o/c</sub>	0	45
RP <sub>o</sub> -H-NS	79 $\pm$ 39	42

gomerization. Upon formation of the first bridges at a nucleation point, which is likely to be close to the RNA polymerase, lateral extension will occur in a cooperative fashion (8). Similarly, also on curved DNA bridge formation by H-NS is often not limited to the curved region only (32).

The process of transcription initiation is a multistep process. Regulation of transcription initiation (either activation or repression) by transcription factors can occur at any of the steps in the initiation process. In general, the step affected is the rate-limiting step for factor-independent transcription initiation on a specific promoter (21). The mechanism of H-NS-

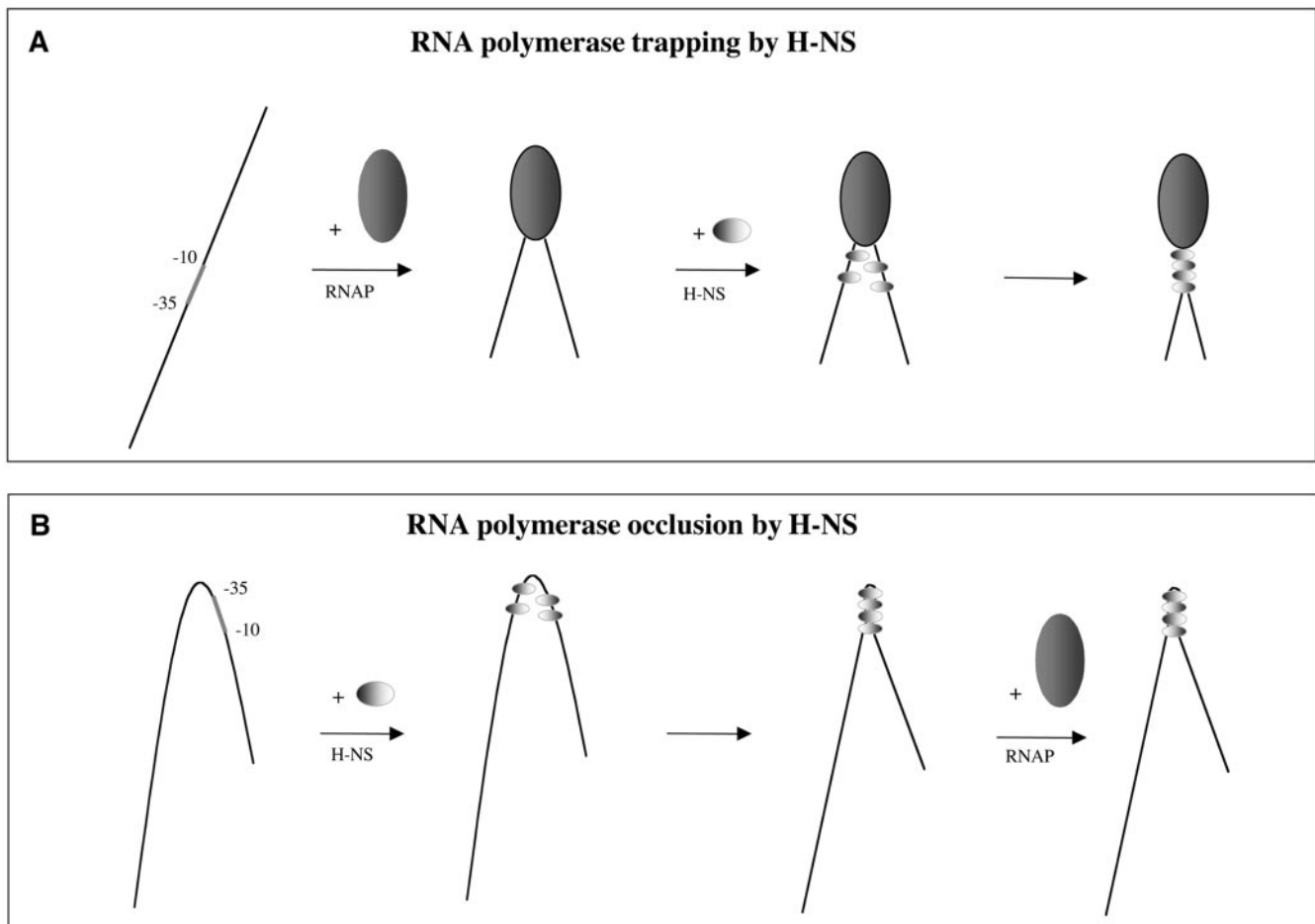


FIG. 2. *A*, model for H-NS-mediated trapping of RNA polymerase in the open initiation complex. When the DNA strands are close enough (as is the case for the regions flanking the RNA polymerase upon wrapping of the DNA), the bound protein can form intramolecular bridges. Split oval represents an H-NS oligomer exposing at least two binding domains. *B*, model for preferential binding of H-NS to curved DNA, which can lead to promoter occlusion when the promoter region is close to or part of the region of preferential binding (32). This figure illustrates the mechanistic parallel between preferential binding of H-NS to curved DNA and trapping of RNA polymerase in an open initiation complex.

mediated repression as described here exhibits a structural parallel with repression by loop formation, *e.g.* as occurs in the case of the AraC protein (44). There is to our knowledge, however, no evidence that, in any of the systems for which regulation by loop formation has been described, trapping of RNAP causes repression. A mechanistic parallel exists with several other transcription factors, which have been shown to cause repression of transcription initiation by stabilization of either the closed complex (GalR at P1 in the *gal* operon (45, 46)) or the open complex (p4 at the  $\phi$ 29 A2C promoter (47)). The observed stabilization in those cases, however, is due to specific contacts between the repressor and the  $\alpha$ -CTD of RNA polymerase. The mechanism of H-NS-mediated repression as proposed here differs from these previously described mechanisms as it does not necessarily involve specific H-NS-RNAP contacts. Such contacts cannot be excluded on the basis of the data presented here. However, the binding of H-NS will be different from binding of GalR or p4, which interact with a more specific DNA target site as defined dimers or tetramers, respectively, and direct interaction of H-NS with the  $\alpha$ -CTD has not been shown. An obvious advantage of mechanisms in which RNA polymerase is trapped on the promoter is that it needs not be recruited from "solution" once repression is relieved, which allows a rapid response to changes in environmental conditions.

The repression of transcription initiation at the *rrnB* P1 can be alleviated by Fis (17, 43), which has three binding sites (-70, -100, and -140) in the upstream region of this pro-

moter. These binding sites are located within the region where bridging of the DNA by H-NS occurs. Sequence-specific binding of Fis may interfere with H-NS binding and thus with bridging.

The conditions under which the *in vitro* experiments (both the SFM and the structural and functional analyses of Schröder and Wagner (33)) were conducted basically reflect the physiological situation bacterial cells encounter when growth ceases or during stationary phase. Under those conditions rRNA synthesis is effectively shut off mainly as a result of specific inhibition of rRNA P1 promoters. Although several additional mechanisms are responsible for this rapid shut down (*e.g.* the global effector ppGpp) the transcriptional activator FIS and the repressor protein H-NS contribute to a great extent to this down-regulation. FIS and H-NS act as antagonists, and their regulatory effects on rRNA transcription have been demonstrated both in *in vitro* and *in vivo* (43, 48). The antagonistic properties of both regulators largely correlate with their cellular concentrations, which at stationary growth are significant for H-NS (about 20,000 copies per cell) and negligible for FIS (<100 copies per cell). Both factors bind to overlapping sites upstream of all seven rRNA P1 promoters (49),<sup>2</sup> and mechanistic models for their antagonism based on binding competition studies have been proposed (22). The RNA polymerase-trapping mechanism, which has been documented biochemically

<sup>2</sup> A. Hillebrand and R. Wagner, unpublished data.

(33) and for which the SFM images provide strong structural support, not only explains the physiological situation at rRNA synthesis shut off, but also gives an immediate explanation for the rapid increase in rRNA synthesis after nutritional upshift when growth resumes. The cellular FIS concentration shows a strong transient increase (50), relieving H-NS-mediated repression. Transcription can then be resumed from promoters at which RNAP has been trapped immediately without the requirement of *de novo* initiation after the balance between the transcription factors has been changed in favor of FIS. This may be of special importance under conditions in which the cellular RNA polymerase concentration is low, and rRNA promoters, which have a low affinity are not saturated.

It is very likely that the mechanism we propose here for transcriptional repression by H-NS at the *rrnB* P1 is also responsible for H-NS-mediated repression of other genes. The fact that DNA wrapping by RNA polymerase in the open complex seems to be a general phenomenon (42) does, however, pose a number of new questions as to how this type of repression may affect only some genes in an *in vivo* situation. Partly this may be explained by differences in the extent of DNA wrapping around RNAP or by differences in the spatial orientation of the "arms," leaving the RNAP in the open initiation complex. In the case of the *rrnB* P1, the upstream UP element, which is involved in the extended RNAP-DNA interactions (51), could play a role in changing the extent of wrapping and thus the relative orientation of the DNA arms in the open complex. Furthermore, a preferential binding of H-NS within the upstream activating sequence, close to the RNAP initiation site, could constitute a "nucleation site" for H-NS and thereby contribute significantly to directing the bridging events. Finally, trapping of RNA polymerase by H-NS is expected to be affected by the rate with which the transition occurs from the open complex into the phase of productive elongation. This rate differs for different promoters and is determined by the ease with which RNAP-DNA contacts can be broken. Especially if the rate-limiting step of transcription initiation is promoter clearance, a gene may be more susceptible to repression by H-NS. A favorable combination of the factors mentioned above will determine whether a promoter is regulated by H-NS through the described trapping mechanism. Identification of the promoter-specific factors that are involved in providing susceptibility for the H-NS-mediated regulation described in this paper will require further systematic study.

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## Structural Basis for H-NS-mediated Trapping of RNA Polymerase in the Open Initiation Complex at the *rrnB* P1

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