Effect of upstream curvature and transcription factors H-NS and LRP on the efficiency of *Escherichia coli* rRNA promoters P1 and P2 – a phasing analysis

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To study the influence of DNA curvature and DNA-binding proteins, which interact with curved DNA on bacterial promoters, we constructed two sets of promoter variants in which a synthetic DNA-bending module was fused at defined distances and angular orientations with respect to the transcription start sites. The distance between the synthetic binding site centre and the transcription start site of the different constructs varied by up to 20 bp, corresponding to almost two complete helical B-DNA turns. The rRNA promoters *rrnB* P1 and *rrnB* P2 were selected as target promoters. While in its natural context P1 depends on upstream curved DNA and several transcription factors that bind to this region, promoter P2 is not preceded by curved DNA, nor is it believed to be directly regulated by transcription factors. *In vitro* transcription measurements of both promoters in the absence of transcription factors varied with the phase of the curved upstream DNA element, underlining the importance of DNA conformation to promoter efficiency. Specific binding of H-NS and LRP to the curved DNA element was demonstrated by gel shift and footprint analysis. Binding affinity was not notably altered for the different distance variants. We demonstrated that the two proteins acted as repressors for both promoters. The extent of H-NS-mediated repression for both promoters did not vary strongly with the phasing of the upstream binding module. In contrast, LRP-dependent repression showed a clear dependence on the angular orientation of the constructs. Phasing-dependent repression is very distinct for P2 but only rudimentary for the P1 promoter.

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INTRODUCTION

The efficiency of bacterial promoters is determined by conserved sequence elements, such as the –35 and –10 hexamer sequences, which are recognized by the sigma factors responsible. Apart from several additional recognition elements that are characteristic of special promoters (Estrem *et al.*, 1998; Mitchell *et al.*, 2003; Shultzaberger *et al.*, 2007; Vicente *et al.*, 1991), the DNA geometry in the vicinity of the core promoter is generally of prime importance (Geiselmann, 1997). A number of bacterial proteins, termed nucleoid-associated proteins (NAPs) interact with DNA in a non-sequence-specific manner. NAPs bind preferentially to curved or flexible DNA, causing DNA compaction and structural rearrangements

during DNA transactions (Dame, 2005; Luijsterburg *et al.*, 2006). Some NAPs may recognize degenerated consensus sequences (Bouffartigues *et al.*, 2007; Shao *et al.*, 2008; Cui *et al.*, 1995). For many genes, these proteins can affect transcriptional efficiency directly through alteration of the DNA architecture in the vicinity of promoters (Rochman *et al.*, 2004). Often, DNA curvature and binding of regulatory proteins are synergistic elements in transcription regulation, and are strongly affected by the angular orientation with respect to the promoter core elements (Wu & Crothers, 1984). Hence, the DNA conformation and the exact geometry of bound transcription factors are crucial elements for the efficiency of many bacterial promoters (Jáuregui *et al.*, 2003; Olivares-Zavaleta *et al.*, 2006; Plaskon & Wartell, 1987).

In order to better understand the influence of DNA structure and protein-mediated regulation of promoters we have replaced the natural upstream sequences of the *Escherichia coli* rRNA promoters *rrnB* P1 and P2 by a synthetic curved DNA segment, containing sets of A:T tracts in helical phase, which results in a static curvature.

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Abbreviations: NAP, nucleoid-associated protein; UAS, upstream activation sequence.

A supplementary figure showing the sequences of the promoter constructs used in this study is available with the online version of this paper.

Such curved DNA structures are known to be important for the binding of NAPs, such as H-NS and LRP or FIS (Yamada *et al.*, 1990). Two sets of promoter variants were constructed, in which the distance of the curved DNA element was varied by about two helical turns. Our study shows that the basal transcriptional activity in the absence of proteins varies for both promoters as a function of the angular orientation of the curved DNA segment. As expected, all three proteins bind specifically to the curved DNA module. We also found, as has been shown previously, that LRP additionally binds to the P1 core promoter region, although with minor affinity.

To investigate the contribution of the distance and angular orientation to transcription factor-dependent regulation we performed *in vitro* transcriptional analysis in the presence of H-NS and LRP. We found that H-NS and LRP act as repressors for both promoters. For all constructs, H-NS-dependent repression did not vary substantially as a function of the distance and angular orientation of the synthetic binding site. In contrast, binding of LRP to the promoter variants caused repression in a helical-phase-dependent manner. Repression was maximal when LRP had been bound on the same face of the helix axis as the starting nucleotide. This effect was strong for the P2 promoter, and less pronounced for P1.

METHODS

Plasmid construction. First we constructed plasmids containing the P1 and P2 core promoters behind the multi-cloning site of pUC18. The complete P1 and P2 core promoters (positions -35 to +7 for P1 and -35 to +3 for P2) were obtained by hybridization of complementary single-stranded oligonucleotides (P1-1, 5'-CTT GTC AGG CCG GAA TAA CTC CCT ATA ATG CGC CAC CAC TGA CA-3'; P1-2, 5'-TGT CAG TGG TGG CGC ATT ATA GGG AGT TAT TCC GGC CTG ACA AG-3'; P2-1, 5'-CTT GAC TCT GTA GCG GGA AGG CGT ATT ATG CAC ACC CCG CGC CCC C-3'; P2-2, 5'-GGG GGC GCG GGG TGT GCA TAA TAC GCC TTC CCG CTA CAG AGT CAA GAG CT-3'). The P1 and P2 core promoter fragments were cloned into the *HindIII* site of pUC18 after treatment of the P2 insert with mung bean nuclease and Klenow filling of the linearized vector, yielding the new plasmids pUC18-P1 and pUC18-P2, respectively. The orientations and correct sequences of the inserts were verified by DNA sequencing.

The 92 bp synthetic curved DNA sequence CGA(GCCCGTTT-TT)₈GCCCTCTTG was obtained by *Hinfl* restriction of plasmid pMG4A, kindly provided by M. Gastens, Heinrich-Heine-Universität Düsseldorf, and blunt ends were generated by mung bean nuclease treatment. It was then ligated at different distances from the P2 promoter. For this, pUC18-P2 was linearized with the restriction enzymes *SphI*, *PstI*, *SalI*, *XbaI* or *HincII*, followed by mung bean nuclease treatment except for the *HincII*-cleaved DNA. The *SalI*-digested DNA was separately filled-in with Klenow polymerase (Promega). The different plasmids pUC18-P2-1, pUC18-P2-2, pUC18-P2-3, pUC18-P2-4, pUC18-P2-5 and pUC18-P2-6 were then obtained by blunt-end ligation of the 92 bp bending module to the different vector constructs, so that the sequence given above represents the non-template strand. Construction of the pUC18-P1 derivatives was accomplished similarly, starting with pUC18-P1, yielding plasmids pUC18-P1-1 to pUC18-P1-5. The control promoter fragments without upstream curvature P1-K (176 bp) and P2-K

(178 bp) were obtained by *PvuII/SmaI* restriction of pUC18-P1 and pUC18-P2, respectively. The sequences of all constructs were verified by sequencing and are shown in Supplementary Fig. S1.

Analysis of protein–DNA complexes by gel retardation. DNA fragments were obtained by *PvuII/XmaI* digestion of the pUC18-P1 and pUC18-P2 derivatives. These fragments were purified by agarose gel electrophoresis and end-labelled by Klenow polymerase incorporation of [α -³²P]dCTP. DNA-binding proteins were isolated and purified as described previously (Hillebrand *et al.*, 2005; Pul *et al.*, 2005). Protein–DNA complexes were formed in a buffer containing 50 mM Tris/HCl, pH 7.4, 70 mM KCl, 15 mM NaCl, 1 mM EDTA, 10 mM β -mercaptoethanol and 50 ng heparin μ l⁻¹. Standard reaction mixtures contained 2 nM DNA and the binding proteins LRP and/or H-NS at the concentrations indicated for the individual experiments.

***In vitro* transcription experiments.** *E. coli* RNA polymerase was isolated following published procedures (Burgess & Jendrisak, 1975; Gonzalez *et al.*, 1977). Transcription experiments were performed as pseudo-single-round reactions as described elsewhere (Hsu, 1996). NAPs were added at the indicated concentration together with the RNA polymerase. Briefly, initiation complexes were formed in a total volume of 50 μ l with 9 nM active RNA polymerase saturated with σ^{70} in the presence of 3 nM template DNA and the initiating nucleotides ATP (500 μ M) and CTP (50 μ M) in a buffer containing 50 mM Tris/acetate, pH 8.0, 10 mM magnesium acetate, 0.5 mM DTT, 0.1 mM EDTA, 100 μ g acetylated BSA ml⁻¹ and 80 mM potassium glutamate. [α -³²P]UTP [20 μ Ci (740 kBq)] [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹)] was added to the mixture, followed by 20 min incubation at 30 °C. Initiation was stopped by the addition of 50 ng heparin μ l⁻¹, and after 5 min incubation at 30 °C all four NTPs were added (500 μ M each), followed by a 5 min elongation reaction. A chase mixture (2 mM each NTP, 2 mg heparin ml⁻¹) was added and samples were incubated for another 5 min at 30 °C. The samples were put on ice and the reaction was stopped by adding formamide sample buffer, which contained a defined amount of a radiolabelled loading standard. Transcription products were separated on 10 % denaturing polyacrylamide gels and visualized by autoradiography.

DNase I footprinting. Limited hydrolysis of free DNA and protein–DNA complexes was performed with the corresponding P1 or P2 promoter fragments and different H-NS or LRP concentrations. DNA fragments were end-labelled by Klenow polymerase incorporation of [α -³²P]dCTP. Samples with or without protein were incubated in the presence of 0.5 mU DNase I μ l⁻¹ for 30 s at 25 °C. Hydrolysis was stopped by addition of 330 mM sodium acetate, pH 4.8, 10 mM EDTA and 10 ng glycogen μ l⁻¹, followed by phenol extraction. For the sequence assignment, G- and A-specific chemical cleavage reactions were performed as described elsewhere (Maxam & Gilbert, 1980). Cleavage products were separated on denaturing 8 % polyacrylamide gels and visualized by autoradiography.

RESULTS

Construction of rRNA promoter variants with differentially spaced AT-rich bending modules

It is known that NAPs such as H-NS and LRP bind to synthetic curved DNA consisting of repetitive AT-clusters, and specific binding to such bent DNA modules has been proven (Beloin *et al.*, 2003; Yamada *et al.*, 1990). Therefore, we constructed a synthetic NAP-binding domain consisting of eight repetitive AT-clusters that were separated by

GC sequences, placing the AT-tracts in helical register. The curved nature of the bending module was verified by gel electrophoresis at different temperatures and confirmed by *in silico* analysis, employing the DNA structure prediction program DIAMOD (Dlagic & Harrington, 1998). The DNA fragment exhibited notable curvature with a k-value of 1.73 and an approximate bending angle of 109° (Thompson & Landy, 1988). The 92 bp bending module was ligated upstream of the *rrnB* P1 and P2 promoters at different distances relative to the respective transcription start sites. The distances of the bending module centre and the transcription start sites varied between 87 and 107 bp, which corresponds to a phasing of the curved DNA module of almost two helical turns (Fig. 1). The resulting constructs were designated P1-1 to P1-5 and P2-1 to P2-6, respectively, depending on the particular promoter (P1 or P2), to which the modules were fused (Fig. 1).

The resulting constructs allowed the study of distance- and helical-phase-dependent effects of the upstream curved DNA module on the activity of promoters P1 and P2. Moreover, effects of transcription factors associating at different angular orientations and variable distances upstream from promoter core sequences could be investigated. Note that the synthetic binding modules and the constructs cloned did not contain sequences with notable

similarity to UP element consensus sequences (Estrem *et al.*, 1998). Hence, effects on promoter binding and transcriptional activity due to interaction of a potential UP element with the α -CTD of RNA polymerase can be excluded from this study.

Predicted DNA conformation of the different *rrnB* P1 and P2 promoter variants

As a reliable algorithm for the curvature prediction of the constructs employed in this study, we chose the program DIAMOD, which allows the application of different model calculations for curvature prediction and includes a versatile method for visualization of the calculated structures (Dlagic & Harrington, 1998).

Fig. 2(a, b) shows the predicted structures of the different phasing constructs, which were calculated according to the Gabrielian trinucleotide parameters (Gabrielian & Pongor, 1996). The spatial orientations of the different fragments are presented such that the promoter core regions and downstream sequences are arranged in the same plane and viewed from the same direction. It can immediately be recognized from the structure predictions that the different distances between the centres of the bending module (marked by the left-pointing arrows) to the transcription

Fig. 1. Schematic illustration of the different phasing constructs. (a) Constructs fused to the *rrnB* P1 promoter. (b) Constructs fused to the *rrnB* P2 promoter. The 92 bp bending module (red) consists of repetitive AT clusters separated by GC sequences, placing the AT tracts in helical register. Green and blue boxes depict the core P1 and P2 promoter elements, respectively. The distance in bp between the centre of the bending module (indicated by a dot) and the transcription start position (+1) of the promoter is indicated. Arrows indicate the direction of transcription. The number of complete right-handed helical turns between the transcription start site and the bending module centre is listed for each construct under the symbol depicting a circle with an arrowhead. The numbers listed under the symbol to the right of this indicate the angular orientation of the binding centre with respect to the transcription start site. The helical wheel on the far right summarizes the angular orientations of the different constructs as viewed along the helix axis. The view is from upstream in the direction of transcription; hence, the angles are shown counter-clockwise in accordance with the right-handed helical DNA twist.

Fig. 2. Predicted DNA structures of the P1 and P2 phasing constructs and the corresponding *in vitro* basal transcription activity of the different fragments. (a, b) Predictions of the DNA structures were calculated as described elsewhere (Dlakic & Harrington, 1998). All fragments are arranged identically showing the variation in the direction from the bending module as a function of distance relative to the promoter. The -35 and -10 promoter regions are in orange (P1) or magenta (P2), and the bending module is in red. The left-pointing arrows indicate the centre of the bending module, and the kinked arrows show the transcription start position. (c, d) Relative basal transcriptional activities for the different P1 (c) and P2 (d) constructs, as determined by *in vitro* transcription. The activities of P1-3 and P2-3 are both set at 100%. Error bars, SD.

start site (marked by the kinked arrows) lead to different angular orientations. From the visual inspection it appears that at both promoters the constructs P1-1 and P1-3 (Fig. 2a) and P2-1 and P2-3 (Fig. 2b) have nearly the same orientation of the two centres (shown by the arrows). This aspect of the helix orientation is also consistent with the arrangement depicted in Fig. 1, where B-DNA parameters have been used to indicate the angular orientation between the centre of the bending modules and the transcription start sites of the different constructs.

Effects of the upstream curved DNA regions on the intrinsic transcriptional activity of P1 and P2 promoter variants

We wished to know whether the different orientations of the bending module would influence the basal transcription activity of the P1 and P2 promoters fused to the

respective constructs. While the transcriptional activity of the *rrnB* P1 promoter has been shown to depend on an upstream curvature (Leirimo & Gourse, 1991; Zacharias *et al.*, 1992), no comparable observations have been made for the P2 promoter, which in its natural context completely lacks curved upstream sequences, although a weak UP element has been reported for *rrnB* P2 (Murray *et al.*, 2003). Fig. 2(c, d) shows the results of *in vitro* transcription analysis with the different P1 and P2 constructs. The orientations of the curved upstream regions had a strong effect on the basal transcriptional activity of the two promoters. Activity was highest when the inside of the curved DNA structure was in the same plane as the transcription start site (Fig. 2). The angular orientation of the curved sequence appeared to be even more important than its distance from the core promoter. This can be inferred by comparison of the transcriptional activity of P1-1 and P1-3 (and also P2-1 and P2-3), which

have different distances of the bending centre relative to the promoter, although the orientations are similar (see also Fig. 1).

In summary, it can be concluded that the influence of curved upstream sequences on the intrinsic promoter activity is phase-of-the-helix-dependent. One explanation for the different promoter activities may be the preferred orientation of upstream sequences, such that they wrap around RNA polymerase in a left-handed way, so that RNA polymerase is positioned at the inside of the curved DNA. This conclusion confirms the notion of the phase-of-the-helix dependence of upstream sequences at the *rrnB* P1 promoter (Meng *et al.*, 2001) and demonstrates that the observation is also valid for the P2 promoter constructs.

Binding of H-NS and LRP to promoter fragments without upstream curvature

At first, we had to verify that the curved DNA module functioned as an efficient binding site for the transcription factors H-NS and LRP, and determine whether or not the P1 and P2 promoter DNA fragments without the upstream bending module (P1-K and P2-K, respectively) were able to interact with the different proteins. The P1-activating protein FIS was used additionally as a control. Gel retardation analysis with the corresponding P1 and P2 promoter fragments, containing only the promoter elements without the curved DNA region, revealed, consistent with previous analyses, that LRP binds within the P1 core promoter element ($K_d > 2 \mu\text{M}$), while no complex formation occurred with P2-K (Fig. 3). Moreover, H-NS did not bind specifically to either of the two fragments without the bending module, supporting the recent finding that the curved DNA region of the P1 promoter acts as a nucleation site for H-NS binding. Only at sufficiently high concentrations ($> 3 \mu\text{M}$), after initial binding to a curved DNA target site, was H-NS able to polymerize into the core promoter region. The retardation analysis of promoter fragments without the bending module thus confirmed our previous results, namely that efficient H-NS binding requires a curved DNA region of the *rrnB* P1 promoter (Pul *et al.*, 2005, 2007). Moreover, we found that the transcription factor FIS binds to the genuine *rrnB* P2 core promoter region (data not shown), which implies repression and therefore indicates the potential for differential regulation of the two rRNA promoters (Liebig & Wagner, 1995).

Binding of H-NS and LRP to the promoter fragments with differentially spaced upstream curvature

Gel retardation analysis of H-NS and LRP binding to the constructs with the helically phased synthetic binding sites clearly demonstrated that all constructs formed specific complexes with H-NS and LRP (Fig. 4). Hence, the fusion of a synthetic curved AT-rich sequence leads to specific

Fig. 3. Analysis of H-NS and LRP binding to promoter fragments without bending modules. The left-hand panel shows binding analysis of the P1 promoter fragment P1-K in the presence of H-NS and LRP. Protein concentrations are given above the lanes. Two distinct complexes are visible for LRP alone, indicating a singly or doubly occupied promoter fragment. No H-NS complex is visible, even at $4 \mu\text{M}$ protein concentration. Right-hand panel: analysis for the P2 promoter fragment P2-K. No complex formation can be detected for either protein, even at the highest concentration. SS, single-stranded DNA contaminant present in the P2 fragment preparation.

binding of H-NS and LRP to promoter-containing target DNA fragments. The known binding cooperativity of H-NS (Pul *et al.*, 2005) is supported by the fact that no intermediate complex bands were observed in the binding studies. It can be seen that the mobilities of the H-NS–DNA complexes varied slightly for the different constructs, which reflects the different positions of the bending module with respect to the fragment ends. Moreover, the stability of the different complexes was not completely uniform, as can be inferred from some of the diffuse bands in Fig. 4.

LRP, in comparison with H-NS, binds generally with higher affinity to the DNA constructs with apparent dissociation constants of $< 0.5 \mu\text{M}$. The binding affinity of H-NS ($K_d \sim 4 \mu\text{M}$) and LRP ($K_d < 0.5 \mu\text{M}$) for each individual fragment did not vary noticeably, however, which indicates that the affinity for the initial binding sites of a given fragment is independent of the relative position of the binding module. Analysis of the binding of LRP to the different P1 constructs revealed, however, that the number of individual complexes, their cooperativity and the mobility of the different LRP–DNA complexes varied for the different fragments. Hence, for the P1 constructs the position of the curvature with respect to the core promoter affects the cooperativity of LRP binding. This can be seen when complexes from the constructs P1-1 and P1-3 are

Fig. 4. Binding of H-NS and LRP to the different P1 and P2 phasing constructs. Gel retardation analyses of transcription factor binding to the synthetic bending module fused at different distances from *rrnB* P1 (a) or *rrnB* P2 promoters (b) are shown. The different phasing constructs P1-1 to P1-5 (a) and P2-1 to P2-6 (b) were incubated with H-NS or different amounts of LRP, or a combination of both proteins. Lane 1 of each panel contains the free DNA in the absence of protein. The following protein concentrations were employed: lane 2 of each panel, 8 μM H-NS; lanes 3–6, increasing LRP concentrations of 0.5, 1, 2 and 3 μM. In lane 7 of each panel, 8 μM H-NS together with 0.5 μM LRP was used. The positions of the free DNA and the DNA–protein complexes are indicated. Each experiment was performed with 2 nM radiolabelled DNA fragment and 50 ng heparin μl⁻¹ as competitor.

compared with the other DNA fragments (Fig. 4). For these constructs, the formation of a second complex with lower mobility, indicative of looping or wrapping, could be observed at low LRP concentrations. Interestingly, constructs P1-1 and P1-3 had very similar helical phasing geometry, while the distance between the bending module and the transcription start site for these two fragments differed by 10 bp, corresponding almost exactly to one full helical turn (Fig. 1a). Formation of such a higher-order complex is obviously enhanced by the spatial orientation of the core promoter and the synthetic bending module, which appears to be optimal for the constructs P1-1 and P1-3. The centre of curvature and the core promoter region are on the same face of the DNA helix, facilitating cooperative protein interactions. Such a steric arrangement is also supported by the structure predicted in Fig. 2(a).

The LRP-binding studies with the P2 constructs, which do not have a second protein-binding site within the core

promoter structure, did not reveal evidence for higher-order complexes, supporting the conclusion that cooperativity likely results from protein contacts at a distance when placed at the same face of the DNA helix. Our results support the view that the helical phase matters more than the lateral distance, because neither a shorter nor a larger distance revealed comparable binding properties. As will be seen below, the same is true for the transcriptional activity of the different constructs.

Interestingly, the simultaneous presence of H-NS and LRP results in supershifted complexes on retardation gels (Fig. 4a, b; lanes 7), indicating that binding of both proteins is not mutually exclusive but rather occurs synergistically. Such synergism between H-NS and LRP has been observed in previous studies (Pul *et al.*, 2005).

In summary, our results are consistent with the notion that efficient H-NS binding requires a natural AT-rich

curved sequence element, such as that present upstream of all rRNA P1 promoters (upstream activation sequence; UAS) (Lindahl & Zengel, 1986). Note that this curved AT-rich UAS region should not be mistaken by the so-called UP element, which is known to interact directly with the α -CTD of RNA polymerase. The natural UAS region can effectively be replaced by a synthetic bending module, as shown in this study. Likewise, any high-affinity binding of LRP requires a potential UAS region or a bending module, even if the P1 core promoter apparently contains a low-affinity LRP-binding site ($K_d > 2 \mu\text{M}$), which only exhibits LRP binding at high concentrations. The fusion of the synthetic bending module converts the two DNA fragments with the *rrnB* promoters P1 and P2 to primary LRP target sites with K_d values of about $0.5 \mu\text{M}$.

DNase I footprint analysis

To verify the locations and intensities of H-NS and LRP binding to the fusion constructs, we performed DNase I footprinting analysis at various protein concentrations. A representative example for the non-template strands of the constructs P1-2 and P2-2 is shown in Fig. 5. Note that different protein concentrations were applied for LRP and H-NS, in accordance with the different binding affinities of the two proteins. Both fragments showed clear protection throughout the sequence of the bending module. Although this sequence is identical in both constructs, a significantly stronger protection in the presence of H-NS could be detected for the P1 fragment. This is in line with the stronger H-NS-dependent repression of transcription observed for the P1 compared with the P2 fusion constructs shown below (Figs 7b and 8b). Interestingly, significant binding of H-NS to the P1 promoter core region was also apparent from the corresponding DNase I protection pattern, suggesting that, in case of the P1 promoter fragment, increased inhibition results from the cooperative interaction of two distant H-NS-binding sites. Note that no pattern of comparable H-NS-dependent protection was apparent for the promoter core region of the P2 fragment. A single hyperreactive site, which is rarely detected in H-NS footprints, might indicate an H-NS-dependent widening of the small groove on the outside of the curved bending module, giving rise to better accessibility for the nuclease.

The presence of LRP protects against DNase I digestion, which is predominantly restricted to the bending module region. No significant difference in the protection patterns existed between P1 and P2 promoter fragments, except at the highest LRP concentration, at which slight protection of the promoter core region occurred for the P1 fragment. The footprinting results for the P1 fragment obtained in this study are in very good agreement with work published previously, in which the interaction of H-NS and LRP with DNA was analysed in greater detail (Pul *et al.*, 2007).

Fig. 5. DNase I footprint analysis of P1-2 and P2-2 fragments with LRP and H-NS. Footprint gels of the non-template strands for the P1-2 (left-hand panel) and P2-2 (right-hand panel) fragments are shown. The different proteins and their respective concentrations are indicated above the lanes. Regions of protection are marked by a vertical line (H-NS) or a dashed line (LRP). A hypersensitive site in the presence of H-NS is indicated by an arrow. Numbers at the left-hand margin denote base positions relative to the transcription start site determined by sequencing. The position of the core promoter is indicated.

Effects of H-NS and LRP on the transcriptional activity of the different promoter fragments without upstream curvature

The effects of LRP and H-NS on the two promoter DNA fragments P1 K and P2 K without curved upstream regions were also analysed under *in vitro* transcription conditions (Fig. 6). These experiments showed that the P1 core promoter was strongly inhibited in the presence of LRP (nearly 90 % inhibition at $2 \mu\text{M}$ LRP), but only weak

Fig. 6. *In vitro* transcription assays with P1-K and P2-K containing the core promoter elements without upstream curved sequences. The *in vitro* transcription reactions were performed with 3 nM DNA fragments P1-K (a) or P2-K (b) and 9 nM active RNA polymerase in the absence and presence of LRP or H-NS. The protein concentrations are given above the lanes. Transcription products are indicated (P1 runoff or P2 runoff). A radioactive loading standard for normalization is included. In the right-hand panels of (a) and (b), the quantitative evaluation of several experiments is shown according to densitometry. Results were normalized to the intensity of the loading standard. The transcript signal of the protein-free lane was set to 100 % and the inhibition in the presence of the protein factors is plotted relative to this value. Error bars, SD.

inhibition was observed for P2 (Fig. 6a). The results support the findings of previous binding and DNase I footprint analyses, which demonstrate that LRP can bind at the *rrnB* P1 promoter between the -35 and -10 regions (Fig. 3; Pul *et al.*, 2007). In the P2 construct, which lacks a binding site upstream of the promoter core region, inhibition at 2 μ M LRP was reduced to 40 % (Fig. 6b). Transcription in the presence of H-NS was inhibited for both promoters to an identical extent (40 % inhibition at 2 μ M H-NS). Apparently, there is transient interaction of H-NS with the core promoter DNA, but this interaction is not stable enough to survive gel electrophoresis during the binding assays.

Importance of H-NS and LRP for the transcriptional activity of the phasing constructs

Next, we wanted to determine whether and how the distance from the transcription start site and the orientation of the bending module would affect transcription from the different P1 and P2 phasing constructs, and what effects such an architectural alteration of the DNA structural element would have on the activities of the rRNA promoters P1 and P2 in the presence of the regulatory proteins H-NS and LRP.

In vitro transcription reactions were performed in the presence of different concentrations of the two transcription

Fig. 7. Effect of H-NS and LRP on *in vitro* transcription of the P1 constructs. (a) The gel electrophoretic separation of products from *in vitro* transcription reactions with the different P1 phasing constructs in the absence of H-NS and at two different H-NS concentrations is shown. Transcription products are indicated (P1 run-off). H-NS concentrations are given above the lanes. A radioactive loading standard for normalization is included. The reactions were performed with 3 nM DNA fragment and 9 nM active RNA polymerase, as described in Methods. (b) Quantitative evaluation of transcripts shown in (a). Results were normalized to the intensity of the loading standard. The transcription signal in the absence of protein (lane 1 for P1-1; lane 4 for P1-2; lane 7 for P1-3; lane 10 for P1-4; lane 13 for P1-5) was set to 100 % for each construct. The relative inhibition for the different fragments at 1 or 2 μ M H-NS is presented in the upper or lower diagram, respectively. (c) Transcription reactions as in (a) in the absence of LRP and at two different LRP concentrations. (d) Quantitative evaluation of several experiments shown in (c). Each protein-free lane was set to 100 % and the relative inhibition is given for the different constructs in the presence of 1 μ M (upper diagram) or 2 μ M (lower diagram) LRP relative to the basal transcription activity in the absence of protein. Error bars, SD.

factors. Results for the P1 promoter constructs are summarized in Fig. 7. Representative gels for the analysis in the presence of H-NS and LRP are presented in Fig. 7(a, c), respectively. In Fig. 7(b, and d) the quantification of three independent experiments is summarized as bar diagrams. It can be seen that the presence of H-NS caused considerable inhibition of the basal transcription (40–60 % at 1 μ M and about 90 % at 2 μ M). The degree of inhibition did not deviate significantly, however, for the different phasing constructs (Fig. 7b). This observation is consistent with the mechanism often proposed for H-NS-dependent transcriptional inhibition, which is based on oligomerization of the protein along the DNA, thereby preventing RNA polymerase from binding to the promoter.

The effect of LRP on the P1 constructs is shown in Fig. 7 (c, d). The inhibition in the presence of LRP was stronger than that observed for H-NS (60–90 % at 1 μ M and > 90 % at 2 μ M), which is in line with the stronger binding affinity of LRP and with the existence of a low-affinity LRP-binding site within the core promoter region. A slight effect of the phasing of the binding site was apparent at the lower LRP concentration. Construct P1-3, which has its centre of curvature opposite the transcription start site, showed maximal inhibition. This might indicate a low degree of synergism between the two LRP-binding sites.

The same analysis was performed with the P2 phasing constructs. Results are shown in Fig. 8. Compared with the curvature-free P2 promoter fragment (P2 K), inhibition by both proteins was stronger for fragments that contained the curvature (compare Fig. 8 with Fig. 6 at 1 and 2 μ M protein). Quantitative evaluation of H-NS-dependent inhibition revealed that it was independent of the location of the binding sites. In the presence of 1 μ M H-NS the relative inhibition reached an average of 30 %, and 70 % in the presence of 2 μ M H-NS (Fig. 8a, b). These results support the view that H-NS-mediated inhibition occurs by occlusion of the promoter region through oligomerization of H-NS proteins along the promoter region, irrespective of angular effects.

While inhibition in the presence of LRP was likewise enhanced compared with the curvature-free P2 DNA fragment, the extent of inhibition clearly depended on the position and angular orientation of the protein-binding site (Fig. 8d). The degree of inhibition for the different P2 constructs varied periodically with increasing distance between the protein-binding site and the core promoter. It was maximal for constructs P2-2 and P2-5, and approached a minimum for constructs P2-1, P2-3 and P2-6. Comparison of the angular orientation of the centre of the binding site with respect to the transcription start site for the different constructs revealed that P2-2 and P2-5 were located at the same face of the DNA helix as the start position, while P2-1 and P2-6 were further apart, and P2-3 had almost the opposite orientation (Fig. 1b). This oscillation in promoter activity for the different P2 promoter constructs was visible at both transcription

factor concentrations (1 and 2 μ M), although it was more pronounced at 1 μ M. The results clearly underline the importance of the angular orientation of regulatory LRP-binding sites to the relative promoter efficiency.

DISCUSSION

The NAPs FIS, H-NS and LRP have been described previously as acting as transcription factors for rRNA synthesis (Hillebrand *et al.*, 2005; Hirvonen *et al.*, 2001; Pul *et al.*, 2005, 2007). In *E. coli* all rRNA P1 promoters are preceded by a curved regulatory DNA region responsible for transcription factor binding. The binding sites of these proteins are located in the AT-rich sequences found upstream of all *rrn* P1 promoters. These regions are known as UASs, because they enhance P1 activities both *in vivo* and *in vitro* (Gosink *et al.*, 1993; Leirimo & Gourse, 1991; Ross *et al.*, 1990; Zacharias *et al.*, 1992). The seven *E. coli* rRNA operon UAS regions differ in structure and transcription factor-binding efficiencies (Hillebrand *et al.*, 2005; Pul *et al.*, 2005), and contribute to the differential regulation of rRNA transcription, in both the presence and the absence of transcription factors. The mechanisms of regulation mediated by the NAPs are not unique but include alterations of DNA structure and topology, protein–protein interaction and competition with RNA polymerase binding (Afflerbach *et al.*, 1999; Aiyar *et al.*, 2002; Bokal *et al.*, 1997; Dame *et al.*, 2002; Muskhelishvili *et al.*, 1995; Pul *et al.*, 2005; Schröder & Wagner, 2000). Although topological effects cannot be excluded (Travers & Muskhelishvili, 1998), FIS activates the *rrnB* P1 promoters by binding upstream of the promoter and interacting with the α subunit of RNA polymerase (Bokal *et al.*, 1997). In contrast, LRP binding inhibits transcription from P1 promoters via the formation of higher-order looped nucleoprotein complexes, including the core promoter region. H-NS binds to a nucleation site located in the UAS region and polymerizes along the DNA, reaching into the promoter region. In the case of *rrnB* P1, a trapping mechanism has been demonstrated that locks RNA polymerase in the apex of a DNA loop formed by promoter-flanking regions bridged by H-NS oligomers (Dame *et al.*, 2002; Schröder & Wagner, 2000).

We have shown here that the intrinsic basal activity of the natural P1 and P2 promoters of the *rrnB* operon depends on the presence of an upstream bending module. Variations of the distance of this curved DNA from the core promoter result in different orientations of the centre of the bending module with respect to the transcription start site. Comparison of the activities of the constructs P1-1 and P1-3 shows that the angular orientation of the bending module is obviously of a similar importance as the distance to the promoter region. This seems to be valid even for a promoter that, in its natural context, is not preceded by a curved upstream region, as shown here for P2. The increase in promoter activity likely results from extended RNA polymerase–DNA contacts due to DNA

Fig. 8. Effect of H-NS and LRP on *in vitro* transcription of the P2 constructs. (a) Gel electrophoretic separation of products from *in vitro* transcription reactions employing the different P1 constructs at different H-NS concentrations. Concentrations are given above the lanes. Lanes 1, 4, 7, 10, 13 and 16: no protein present. (b) Diagrams showing the inhibition of transcription with the different fragments in the presence of 1 or 2 μ M H-NS. (c) Same analysis as in (a) in the presence of 1 or 2 μ M LRP. (d) Quantitative evaluation of experiments as shown in (c). Each protein-free lane was set to 100 % and the relative inhibition is given for the different constructs in the presence of 1 μ M (upper diagram) or 2 μ M (lower diagram) LRP relative to the basal transcription activity in the absence of protein. Error bars, SD.

wrapping and concomitant facilitated open complex formation (Cellai *et al.*, 2007; Davis *et al.*, 2005).

We have deliberately constructed the promoter variants without UP element sequences in order not to complicate the analysis by additional stabilization of RNA polymerase

initiation complexes via the C-terminal domain of the α -subunits (α -CTD). While such an interaction is of known significance for the activity of rRNA P1 promoters, the P2 promoters in their natural context do not share such an interaction. Because we were only interested in the effects of curvature and position relative to the core promoter,

and because neither H-NS nor LRP is known to bind to UP element sequences, none of the linker sequences between the core promoters and the bending module of the constructs employed in this study contains a sequence similar to the published UP element consensus: $^{-59}\text{nnAAA}(\text{AyT})(\text{AyT})\text{T}-(\text{AyT})\text{TTTTnnAAAAAnnn}^{-38}$ (Estrem *et al.*, 1998).

Basal transcription activities were repressed in the presence of H-NS for both promoter constructs. This argues for the existence of a silencing mechanism whereby H-NS initially binds to the upstream bending modules and polymerizes towards the core promoter region. H-NS-dependent repression is more pronounced for P1 constructs due to additional weak interaction of H-NS with the P1 promoter core region, as shown by the *in vitro* transcription and DNase I experiments. Binding of H-NS to the promoter core alone in the absence of the bending module does not cause significant inhibition, however. In contrast, LRP shows a distinct mode of repression. Our study shows that LRP binds directly to the P1 core promoter in the absence of the curved DNA module. No comparable interaction can be observed with the P2 core promoter. This argues for a differential regulation of the two *rrn* promoters. In contrast to H-NS, LRP-dependent inhibition shows a clear phasing characteristic for the P2 constructs with maximal inhibition at an angular orientation of the binding centre on the same face of the helix as the transcription start site. This phasing is less clear for the P1 constructs. We assume that a mechanism exists whereby the orientation of the LRP–nucleoprotein structure differentially interferes with RNAP binding to the promoter region. The phasing dependence of the LRP repression is impaired for the P1 constructs due to additional, though weak, direct binding of LRP to the core promoter region. Overall, this leads to a stronger inhibition of the P1 constructs in the *in vitro* transcription assays.

In summary, both H-NS and LRP require curved or flexible upstream DNA elements in order to bind with high affinity to the *rrnB* promoters. Their effects on transcription are not identical, however. The two proteins have different modes of action: whereas H-NS leads to silencing of promoter activity by coating the DNA, LRP causes looped or wrapped DNA structure, attenuating RNA polymerase binding to the promoter. The latter depends on the orientation of the upstream sequence relative to the core promoter region.

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