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NOTE

Identification of High-Specificity H-NS Binding Site in *LEE5* Promoter of Enteropathogenic *Esherichia coli* (EPEC)

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Histone-like nucleoid structuring protein (H-NS) is a small but abundant protein present in enteric bacteria and is involved in compaction of the DNA and regulation of the transcription. Recent reports have suggested that H-NS binds to a specific AT rich DNA sequence than to intrinsically curved DNA in sequence independent manner. We detected two high-specificity H-NS binding sites in *LEE5* promoter of EPEC centered at -110 and -138, which were close to the proposed consensus H-NS binding motif. To identify H-NS binding sequence in *LEE5* promoter, we took a random mutagenesis approach and found the mutations at around -138 were specifically defective in the regulation by H-NS. It was concluded that H-NS exerts maximum repression via the specific sequence at around -138 and subsequently contacts α subunit of RNAP through oligomerization.

Keywords: *LEE5*, H-NS, nucleation site, consensus motif, transcriptional repression, gene regulation, transcription

Bacterial histone-like structuring protein (H-NS) is an abundant global regulator that is involved in organization of the bacterial chromosome and modulation of gene transcription. H-NS is implicated in selective gene silencing and can repress a large number of genes (up to 5% of the genes in *E. coli*) (Dorman, 2004; Rimsky, 2004; Noom *et al*., 2007). It has been suggested that the selective gene silencing could be established by blocking entry of RNA polymerase into specific loci in bacterial chromosome and/or by altering local DNA conformation (Blot *et al*., 2006). H-NS has recently been reported to play key role in silencing horizontally acquired genes that are AT-rich, including those genes housed in pathogenecity islands of many gram negative pathogens (Falconi *et al*., 1998; Falconi *et al*., 2001; Oshima *et al*., 2006; Dorman, 2007; Fang and Rimsky, 2008). H-NS was initially suggested to bind to intrinsically curved stretches of DNA in sequence independent manner. Lately, however, two high affinity binding motifs with identical sequence, 5′-TCGAT ATATT-3′, have been identified in *proU* gene of *E. coli* with a K_d of ~15 nM (Bouffartigues *et al*., 2007). Further, a consensus 10 bp H-NS binding motif 5′-tCG(a/t)T(a/t)AATT-3′ was proposed that is substantially conserved throughout the *E. coli* genome, particularly within the genes known to be regulated by H-NS (Lang *et al*., 2007). Using NMR spectroscopy and DNase I footprint analysis, it was demonstrated that carboxy terminal domain (CT)-H-NS binds to the 10 bp consensus sequence with high affinity through an electropositive surface involving Thr^{109}, Arg^{113}, Thr^{114}, and Ala^{116} within a protein loop, and neighboring Glu^{101} (Sette *et al*., 2009).

H-NS represses the transcription from a number of genes in response to multiple environmental signals and regulatory proteins (Martin *et al*., 2001; Umanski *et al*., 2002; Haack *et al*., 2003; Laaberki *et al*., 2006). *LEE5* operon, packaged in the *LEE* pathogenicity island of EPEC and EHEC, is repressed by H-NS along with other *LEE* operons, *LEE2* to *4*. We have previously reported the molecular mechanism underlying H-NS mediated repression of *LEE5* promoter (*LEE5*p), which is through a protein-protein interaction between the H-NS and E286 of carboxy terminal domain of the α subunit (αCTD) of RNAP that prevents processing of RNAP-promoter complex into initiation-competent open promoter complex (Shin *et al*., 2012). We found that H-NS represses *LEE5*p by binding to a cluster of AT tracks upstream of -114 (site II in Fig. 1), followed by spreading to a site at the promoter through the oligomerization of H-NS molecules.

Analysis of the nucleotide sequence in the proposed initial binding site revealed presence of two AT tracks centered at -110 and -138 (site I and II in Fig. 1). Both site I and II con-

Table 1. Sequence alignment of 10 bp H-NS binding motifs in *ProU* of *E. coli* and the two sites (site I and II) identified in *LEE5*p of *EPEC*. Centrally positioned T-A base step, an important feature of the H-NS are I in bold letters.

Source	Sequence	Fit
Consensus	TCGA**TA**AATT	10/10
NRE of *ProU*	TCGA**TA**TATT	9/10
Site I of *LEE5*	TCGA**TA**ATTA	8/10
Site II of *LEE5*	TGCA**TA**TAAA	5/10

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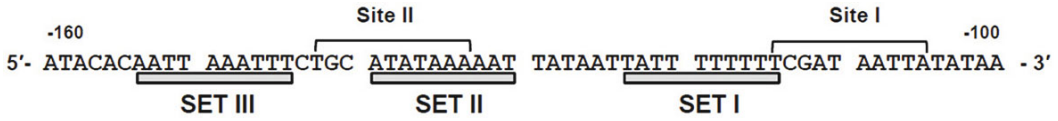

Fig. 1. Strategy for random mutational analysis around site II. The figure shows nucleotide sequence of *LEE5p* coding strand. The base positions are numbered with respect to transcription start site (+1). Boxed 10 bp segments represent wobbled bases from positions -124 to -115, -140 to -131, and from -154 to -145, that are named as Set-I, Set-II, and Set-III respectively. Sequences under the half bracket represent H-NS high-specificity binding sites (Site I and Site II) that are close to the proposed consensus binding motif (Bouffertrgues *et al*., 2007).

tain centrally positioned T-A base step that has been reported to be highly conserved within the individual H-NS binding DNA motifs (Table 1) (Lang *et al*., 2007). In the previous reports we have shown that H-NS exerts majority of the repression through the site upstream of -114 and that no other factors are required to achieve full repression by H-NS on *LEE5*p (Shin *et al*., 2012). In this study, we isolated *cis* mutants of *LEE5*p that were defective in transcriptional repression mediated by full-length H-NS of EPEC at the upstream of -114. We used random mutagenesis approach to mutate the AT rich regions of site II and its neighboring sequences, using degenerative mega-primers (Fig. 1). *LEE5*p DNA fragments from positions -161 to +88 were PCR amplified using normal primers and three sets of 5′ degenerative primers wobbled from positions, -124 to -115, -140 to -131, and -154 to -145 (as set-I, set-II and set-III respectively), and ensured that the sequence maintained AT richness by inserting A or T only. Amplified PCR fragments from each set were inserted at *Eco*RI-*Bam*HI sites of pRS415 (Simons *et al*., 1987) and transformants were grown on MacConkey Lac agar indicator plates containing 100 µg/ml Ampicillin. Approximately 1500 individual transformants were obtained for each set. As expected (Shin *et al*., 2012), all of the intense red color transformants were seen in the set-II. Ten transformants with most intense red color were selected for sequence analysis, which consequently revealed several of these transformants had the same set of mutations indicating that the mutagenesis was to saturation. At last, five mutants were chosen for further studies (mutA to E in Table 2).

Five *cis* mutants from set-II were subsequently subjected to homologous recombination with bacteriophage λRS45 under the MG1655 strain background (Simons *et al*., 1987). The β-galactosidase assay was performed as described earlier (Miller, 1972), using cells that were permeabilized with Koch's lysis solution (Putnam and Koch, 1975). The cultures were grown at 37°C in LB using appropriate antibiotics (100 µg/ml of Kennamycin for *hns::Km* strains) under aeration and at least five samples were taken for the enzyme assay during balanced growth stage. The differential rate of enzyme synthesis was estimated from the slope of A_{420} min^{-1} plotted as a function of A_{600} and the fold repression by H-NS were calculated by dividing the values obtained from the Hns^+ and Hns^- background strains (Table 2). As reported earlier (Shin *et al*., 2012), we observed ~ 6.5-fold repression with -304 and -161 constructs, while only ~ 2-fold repression with -114 construct. However, the mutA, mutB, and mutC showed major loss of H-NS mediated repression with 1.5 folds, 2.2 folds and 3 folds respectively that was comparable to that of -114 construct. The mutD showed 4.1 folds repression, whereas the mutE showed 6.5 folds which is similar to the wild type constructs. It should be noticed that mutA, mutB, and mutC had the same set of substitutions at positions indicated in Table 2. The T substitutions at -136 and -134 seems to be less significant since the same substitutions at identical positions in mutE did not had any effect on H-NS mediated repression.

In order to confirm the above result and show that *LEE5*p transcription repression was repressor-specific, *in vitro* transcription assay was performed using purified H-NS. DNA templates were constructed by inserting PCR amplified DNAs from -161 to +40 carrying the same set of mutations into *Eco*RI and *Pst*I sites of pSA508 (Squires *et al*., 1981; Choy and Adhya, 1993). The *Pst*1 site was located immediately upstream of the 54 bp Rho-independent transcription terminator of the *rpoC* gene of *E. coli* so that transcript with distinct length would be generated from the wild type and mutant *LEE5* promoters. [α-^{32}P] UTP was included in the reaction to detect nascent RNA. Transcription was initiated by the addition of 20 nM RNA polymerase in a total volume of 10 µl containing 20 mM Tris-acetate, pH 7.8, 10 mM magnesium acetate, 200 mM potassium glutamate, ATP (1 mM), GTP and CTP (0.1 mM), UTP (0.01 mM) at 37°C and terminated after 10 min by addition of an equal volume of RNA loading buffer consisting of 80% (v/v) deionized formamide, 1× TBE (89 mM tris, 89 mM boric acid, 2 mM EDTA), 0.025% bromophenol blue, and 0.025% xylene cyanole. The mixture was separated by electrophoresis in an 8 M urea/8% polyacrylamide sequencing gel (40 cm × 0.4 mm). RNA polymerase holoenzyme from strain BL21 was

Table 2. Differential rates of β-galactosidase synthesis by λ lysogens containing the various *LEE5*p fused to *lacZYA* in the presence or absence of functional *hns*. Fold repression was calculated by dividing activity in the presence (hns^+) or absence (hns^-) of H-NS. The wild type and the substituted sequences from positions -140 to -131 were indicated. Individual substituted bases are shown in bold letters. Identical set of substitutions at the same positions among the mutants with highest loss of H-NS mediated repression are boxed.

Strain	Sequence[a]	hns^+	hns^-	Fold repression
-304 to +171	ATATAAAAAT	0.1442	0.8511	6
-161 to +88	ATATAAAAAT	0.1281	0.85	6.6
-114 to +88	–	0.356	0.75	2.1
mutA	**TATA**A**T**A**TT**T	0.5739	0.8617	1.5
mutB	**TATA**A**T**A**AA**	0.2853	0.6303	2.2
mutC	**TATA**A**TTTT**T	0.352	1.055	3
mutD	A**TTAT**AA**TTA**	0.1817	0.7515	4.1
mutE	**T**TAT**TTTTT**T	0.1295	0.8501	6.5

[a] Indicates sequence from position -140 to -131 of *LEE5*p.

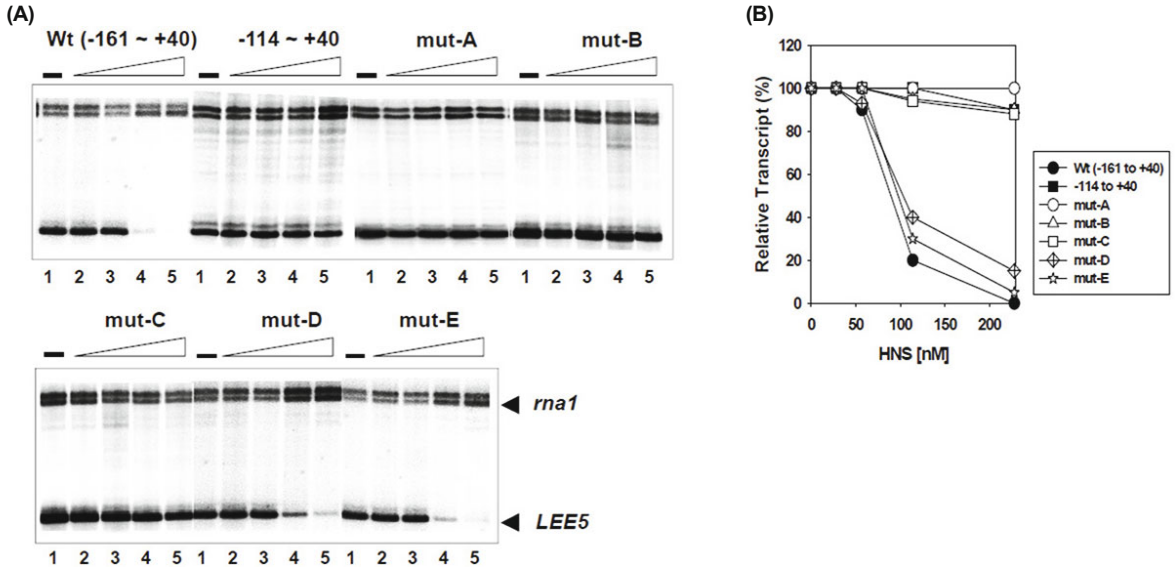

Fig. 2. H-NS mediated repression of various *LEE5*p *cis* mutants. *In vitro* transcription assay was performed using pSA508 carrying the wild type or mutant *LEE5*p as DNA templates. (A) The reactions were carried out using 0, 28, 57, 114, and 228 nM H-NS (lanes 1–5) and the transcripts were separated on an 8% denaturing gel. (B) A plot showing the relative amounts of transcripts generated in the absence of H-NS. The amounts were averaged and plotted as a function of H-NS concentration.

purchased from Epicentre (USA). Multiple round transcription assays were performed in the presence of increasing concentrations of H-NS. With the wild type DNA construct, two transcripts were detected in the absence of H-NS; a 94 nucleotide RNA corresponding to *LEE5*p and a 105 nucleotide *rna1* transcript from the origin of plasmid replication (Fig. 2A). The transcripts were quantified using FLA3000 β scanner and plotted as a function of H-NS concentration (Fig. 2B). With the increasing concentration of H-NS the *LEE5*p transcript decreased to undetectable levels in the wild type construct. H-NS showed virtually no effect on the -114 construct. Whereas, mutA, mutB, and mutC constructs showed complete loss of repression even at highest concentration of H-NS (228 nM). mutD construct showed 40% repression at half-maximal concentrations of H-NS while mutE construct was repressed similarly as the wild type construct with full repression at 228 nM H-NS. Thus, we concluded that site II present within the set-II region is the primary binding site that initializes H-NS oligomerization.

Taking both *in vivo* and *in vitro* results into account, we suggest that the H-NS represses *LEE5*p by binding to a sequence similar to consensus sequence (Bouffartigues *et al.*, 2007) with a high degree specificity which may collaborate with similar downstream sequence (site I) by oligomerization to achieve stable repression. We couldn't identify any other high-affinity binding site upstream of -161 that may synchronize with site II to establish oligomerization of H-NS. This conclusion was further corroborated by the *in vivo* result (Table 2). Both -304 and -161 constructs displayed almost the same fold-repression by H-NS. Nevertheless, any secondary sites couldn't be ignored since our model suggested extension of H-NS from the nucleation site (site II) to promoter proximal site (Shin *et al.*, 2012). Although site I has *hns* target sequence, with better fit (8/10, Table 1) than site II (5/10) to the consensus H-NS-binding motif, we observed that H-NS exerted repression primarily via site II, while the -114 construct comprising of site I, exhibits only 2-fold repression which indicates the latter site as the possible high-affinity binding site when compared to the site I. Secondly, the differences in AT-rich sequences of site I and II confer changes in thermal stability of the high-affinity H-NS binding sites (Lingbeck *et al.*, 1996; Bouffartigues *et al.*, 2007). Importantly, as in the case of *proU* NRE of *E. coli*, the site II of *LEE5*p contains centrally located *two* adjacent T-A base steps that may result in relatively low stability (Table 1). We have seen that the *LEE5*p *cis* mutants with highest loss of repression (mutA, mutB, and mutC) have identical sequence TATA from position -140 to -137 that disrupts centrally positioned T-A base step to A-T within the site II (Table 2). Further, intact T-A base step in mutE retains full length repression by H-NS. Interestingly, in the recent NMR study, CT-HNS showed large dissociation constant of 102 μM when TATA was positioned 3 base pairs downstream of a 10-mer DNA duplex (Cordeiro *et al.*, 2011). Yet another NMR study using CT-HNS and the consensus sequence showed that a structural anomaly of having reduced width of DNA minor groove, associated with T-A base step is crucial for sequence recognition by H-NS (Sette *et al.*, 2009). Thus, by combining the current and our previous study together (Shin *et al.*, 2012), we have identified high-specificity, primary H-NS binding site through which H-NS oligomerize to reach the αCTD contact site to achieve H-NS-αCTD repression complex on the *LEE5*p DNA.

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