Widespread suppression of intragenic transcription initiation by H-NS

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Widespread intragenic transcription initiation has been observed in many species. Here we show that the Escherichia coli ehxCABD operon contains numerous intragenic promoters in both sense and antisense orientations. Transcription from these promoters is silenced by the histone-like nucleoid structuring (H-NS) protein. On a genome-wide scale, we show that 46% of H-NS-suppressed transcripts in E. coli are intragenic in origin. Furthermore, many intergenic promoters repressed by H-NS are for noncoding RNAs (ncRNAs). Thus, a major overlooked function of H-NS is to prevent transcription of spurious RNA. Our data provide a molecular description for the toxicity of horizontally acquired DNA and explain how this is counteracted by H-NS.

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Pervasive transcription is a phenomenon ubiquitous across the three domains of life (Dornenburg et al. 2010). It is defined by an undercurrent of noncanonical transcripts within the primary transcriptome (Clark et al. 2011). In bacteria, such transcripts arise from normal promoter elements but are conspicuous because they are non-coding, not delineated by gene boundaries, and sometimes antisense (Peters et al. 2012). Sites of pervasive transcription are poorly conserved. Hence, even between closely related bacteria, patterns of pervasive transcription differ (Raghavan et al. 2012). This has led to speculation that pervasive transcription is a consequence of the evolution of protein-coding genes (Peters et al. 2012; Raghavan et al. 2012). It is likely that pervasive transcription is suppressed, as spurious RNA synthesis is potentially catastrophic. Synthesis, stability, and translation of mRNA may be perturbed (Dornenburg et al. 2010), and formation of R loops [DNA:RNA hybrids] can destabilize genome integrity (Gowrishankar and Harinarayan 2004).

In Escherichia coli, there are two major sources of pervasive transcription: inefficient termination of RNA synthesis at gene boundaries and spurious transcription initiation in genes (Dornenburg et al. 2010; Peters et al. 2012). Attention has turned to elucidating mechanisms that suppress these phenomena. As a first step, Peters et al. (2012) identified Rho and the cofactor NusG as suppressors of pervasive antisense transcription. In their model, Rho acts via two pathways. First, Rho ensures termination of transcripts at gene boundaries, preventing “leaky” transcription of downstream genes. Second, Rho terminates antisense transcripts arising from promoters in genes. Thus, on a global scale, noncanonical transcripts are targeted for termination. However, since most transcription is regulated at the level of initiation (Browning and Busby 2004), additional mechanisms to suppress pervasive transcription are likely.

The histone-like nucleoid structuring [H-NS] protein is a DNA folding factor. It is abundant in bacteria and influences transcription (Dillon and Dorman 2010). Nuclease-containing H-NS is structurally diverse; H-NS can form bridged or filamentous DNA complexes (Dame et al. 2006; Liu et al. 2010). Two mechanisms for transcriptional repression by H-NS have been proposed: (1) exclusion, in which H-NS masks RNA polymerase (RNAP)-binding sites (Lim et al. 2012), and (2) trapping, in which H-NS ensnares initiating RNAP in DNA loops (Dame et al. 2002). H-NS may also slow elongation, leading to increased Rho-dependent termination (Peters et al. 2012). Chromatin immunoprecipitation (ChIP) has been used to map binding of H-NS across bacterial chromosomes (Grainger et al. 2006; Lucchini et al. 2006; Navarre et al. 2006; Oshima et al. 2006; Kahramanoglou et al. 2011). These studies show that H-NS targets A/T-rich DNA, a distinguishing feature of horizontally acquired genes. Consequently, H-NS has been proposed to facilitate genome evolution by silencing transcription of foreign DNA (Navarre et al. 2006). A central tenet of this model is that foreign DNA is toxic. However, a molecular explanation for this toxicity and how H-NS counteracts this is lacking.

Here, we describe widespread suppression of intragenic transcription initiation in bacteria. We show that this is a major function of H-NS. First, we show that H-NS represses transcription from many intragenic promoters within the A/T-rich ehxCABD operon of E. coli O157:H7. Second, we show that most H-NS-repressed promoters genome-wide in E. coli K-12 are for noncoding RNAs (ncRNAs), with many promoters located inside genes. Third, we show that suppression of widespread intragenic transcription is a likely function of H-NS analogs in diverse bacterial species.

[Keywords: H-NS; nucleoid; pervasive transcription]

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Results and Discussion

The enterohemorrhagic E. coli O157:H7 ehxCABD operon contains many intragenic promoters

The ehxCABD operon is a well-characterized H-NS target (Li et al. 2008; Rogers et al. 2009; Iyoda et al. 2011). Our previous analysis of ehxCABD suggested the existence of intragenic promoters [Singh and Grainger 2013]. We began here by scanning the operon for sequences that resemble promoter –10 elements. As a control, we applied the same analysis to the E. coli O157:H7 and K-12 genomes. For ehxCABD, our analysis identified 95 putative intragenic –10 elements, an approximately fourfold enrichment over the genome averages for both E. coli strains. To test for function, we cloned the 95 sequences, each on a 62-base-pair [bp] DNA fragment, upstream of lacZ in the reporter plasmid pRW50 [Supplemental Fig. S1]. In some cases, predicted –10 elements overlapped. Hence, we were able to clone the 95 sequences on 83 DNA fragments. Twenty of the fragments stimulated β-galactosidase activity twofold or more above background [Fig. 1]. We selected five of the 20 active constructs representing sense and antisense promoters with a wide range of activities [labeled a–e in Fig. 1] and examined the effects of disrupting the predicted –10 element. In all cases, this resulted in a significant decrease in β-galactosidase activity [Supplemental Fig. S2].

Direct repression of intragenic promoters in the ehxCABD operon by H-NS

Transcription of ehxCABD is well characterized as being repressed by H-NS [Li et al. 2008; Rogers et al. 2009; Iyoda et al. 2011]. Despite this, H-NS is unable to block transcription from the canonical ehxCABD promoter [Singh and Grainger 2013]. We hypothesized that H-NS acts via the intragenic ehxCABD promoters. To test this, we measured β-galactosidase activity in wild-type and Δhns cells containing different sections of ehxCABD fused to lacZ in plasmid pRW50. First, we examined a large, 5429-bp section of ehxCABD lacking the canonical upstream promoter. Transcription initiating in this coding DNA, which is repressed fivefold by H-NS, must be due to intragenic promoters [Fig. 2A, panel i]. Sequential removal of upstream sequences reduces repression by H-NS [Fig. 2A, panel ii]. Similarly, transcription from promoters a–e was unaffected by H-NS in the context of the 62-bp fragments [Fig. 2A, panel iii]. We conclude that H-NS represses transcription from intragenic ehxCABD promoters, but repression requires extensive flanking DNA. Note that the sequence of the flanking DNA is also crucial because, when flanked by plasmid DNA, the 62-bp ehxCABD fragments were not subject to repression by H-NS.

Given that H-NS repressed intragenic ehxCABD promoters only in the context of larger ehxCABD fragments, we predicted that H-NS would bind tightly throughout the operon but weakly to small fragments in pRW50. Consistent with this, H-NS forms bridged and filamentous complexes with DNA that are more stable than isolated H-NS-binding events [Bouffartigues et al. 2007]. Hence, we measured binding of H-NS to promoters a–e in the context of the large (5429-bp) and short (62-bp) fragments of ehxCABD cloned in pRW50. ChIP was used to measure binding of H-NS to the different targets in vivo. Figure 2B shows PCR analysis of DNA immunoprecipitated with anti-H-NS. Control experiments analyzing DNA recovered from mock immunoprecipitations are also shown. As predicted, loci a–e bind H-NS in the context of the large ehxCABD fragment [Fig. 2B, top panel]. Conversely, H-NS did not bind the same loci when they were isolated from the rest of the operon [Fig. 2B, bottom panel]. No enrichment of the control chromosomal yabN locus, which is not bound by H-NS, was detected in any immunoprecipitate [Supplemental Fig. S3]. In complementary experiments, we measured binding of H-NS to the same DNA fragments in vitro using electrophoretic mobility shift assays [EMSA]. H-NS bound to the 5429-bp ehxCABD fragment but not the short intragenic promoter fragments [Fig. 2C].

To confirm direct H-NS-mediated repression of intragenic ehxCABD promoters, we recapitulated the phenomenon in vitro. Two DNA fragments were used as templates for in vitro transcription: a 7435-bp DNA fragment containing the entire ehxCABD operon and upstream region [Fig. 2D, lanes 3–9] and a 6435-bp fragment lacking the upstream region and canonical promoter [Fig. 2D, lanes 10–16]. RNA polymerase catalyzed the synthesis of multiple transcripts of varying size from each template. Consistent with a previous report [Singh and Grainger 2013], transcription from the canonical ehxCABD promoter was unaffected by the addition of H-NS, even at high concentrations [7312-nucleotides [nt] RNA] [Fig. 2D, lanes 3–9]. Conversely, synthesis of shorter transcripts, ranging from <100 nt to thousands of nucleotides in length, was dramatically reduced by increasing concentrations of H-NS [Fig. 2D, lanes 4–9, 11–16]. The similarity of transcript profiles synthesized from the two templates confirms that the short RNAs arise from internal initiation events and not from stalled transcription from the canonical promoter.

Widespread repression of intragenic promoters by H-NS

To determine whether H-NS suppresses intragenic transcription at other loci, we investigated the effect of H-NS

Figure 1. The E. coli ehxCABD operon contains many intragenic promoters. β-Galactosidase activity driven by short intragenic ehxCABD promoter fragments in strain JC857. The top chart shows the activity of promoters that drive sense transcription, and the bottom chart shows the activity of promoters that drive antisense transcription. Each data point is aligned with the corresponding section of the ehxCABD operon. Data points labeled a–e correspond to promoters used in further experiments. The red bar represents the canonical promoter for ehxCABD. Background activity (empty pRW50) is shown by a green line.

Widespread suppression of intragenic transcription
on genome-wide transcription initiation in *E. coli* K-12. We chose *E. coli* K-12 because [1] H-NS has been studied most extensively in this strain, [2] H-NS in *E. coli* K-12 is 100% identical to that in *E. coli* O157:H7, and [3] high-resolution genome-wide binding data for H-NS, generated using ChIP sequencing (ChIP-seq), are available. As a starting point, we determined association of σ70 with the *E. coli* K-12 genome in wild-type and Δ*hns* cells using ChIP-seq. Thus, we identified the location of promoter-bound RNAP and the level of occupancy (Supplemental Table S1). In parallel, we identified transcription start sites (TSSs) genome-wide in wild-type and Δ*hns* cells using a modified RNA sequencing (RNA-seq) approach (Singh and Wade 2014). This method also determines the relative abundance of RNAs associated with each TSS ("TSS strength"). Together, these data identify sites of transcription initiation with high confidence and nucleotide resolution. Visual inspection of the data revealed many promoters repressed by H-NS [Fig. 3A]. These promoters typically correspond to regions of high H-NS binding (Kahramanoglou et al. 2011). Repression was reflected by increases in both σ70 occupancy and TSS strength in the Δ*hns* mutant. Many of the H-NS-repressed promoters are located in genes.

We selected σ70 ChIP-seq peaks that collocated with TSSs in the Δ*hns* mutant. Thus, we identified 668 promoters with associated information for σ70 occupancy, TSS strength, and TSS orientation [Supplemental Table S1]. We divided the promoters into "low H-NS" (508 promoters) and "high H-NS" (160 promoters) categories based on the level of H-NS binding. For each promoter, σ70 binding or TSS strength was compared in Δ*hns* and wild-type cells. The result of the comparison is in Figure 3, B (for σ70 binding) and C (for TSS strength). In the scatter plots, each data point represents one of the 668 promoters. Consistent with H-NS-mediated repression, association of σ70 with promoters in high H-NS regions was significantly higher in Δ*hns* cells than in wild-type cells (Mann Whitney U test, normal approximation, *P* = 1.4 × 10^-39) [Fig. 3B]. Similarly, for high H-NS regions, TSS strength was significantly higher in Δ*hns* cells [Mann Whitney U test, normal approximation, *P* = 2.7 × 10^-10] [Fig. 3C].

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**Figure 2.** H-NS suppresses intragenic *ehxCABD* transcription. [A] H-NS suppresses intragenic *ehxCABD* transcription in vivo. β-Galactosidase activity stimulated by *ehxCABD* fragments fused to lacZ in plasmid pRW50. Intragenic promoters a–e are labeled. β-Galactosidase activity was measured in MG1655 and the Δ*hns* derivative. Background β-galactosidase activity, obtained with empty pRW50 vector, was subtracted from the values shown. Experiments were done in triplicate, and the standard deviation was always <10% of the mean. [B] H-NS binds promoters within the *ehxCABD* operon in vivo. ChIP-PCR was used to measure binding of H-NS at intragenic *ehxCABD* loci cloned in pRW50. PCR products were generated with primers to detect the five intragenic *ehxCABD* promoters, a–e. [C] H-NS binds promoters within the *ehxCABD* operon in vitro. Electrophoretic mobility shift assays with either small [62-bp] or large [5429-bp] fragments of *ehxCABD*. The different small DNA fragments are labeled a–e. H-NS was added at a concentration of 4 μM or 8 μM. [D] H-NS suppresses intragenic *ehxCABD* transcription in vitro. A run-off in vitro transcription assay using DNA templates consisting of the whole *ehxCABD* operon with [7435 bp] and without [6435 bp] the upstream regulatory region. The gel was calibrated with a sequencing reaction [lane 1] and the radiolabeled DNA template [lane 2]. RNAP was used at a concentration of 200 nM [lanes 3–16], and H-NS was added at concentrations of 150, 300, 600, 750, 1500, and 3000 nM [lanes 4–9,11–16].
contrast, for low H-NS regions, neither $\sigma^{70}$ binding nor TSS strength was significantly different in $\Delta hns$ and wild-type cells [Mann Whitney U test, normal approximation, $P = 0.29$ for $\sigma^{70}$ occupancy and $P = 0.23$ for TSS strength] [Fig. 3B,C]. Data for all 668 promoters are summarized in Figure 3, D (for $\sigma^{70}$ binding) and E (TSS strength). As expected, the mean wild-type/$\Delta hns$ ratio for both $\sigma^{70}$ binding and TSS strength is close to 1.0 in low H-NS regions [Fig. 3D,E, blue box]. Conversely, the equivalent ratio is 0.2 or 0.3 for high H-NS regions [Figs. 3D,E, first red box]. Taken together, our data indicate that high H-NS occupancy reduces the binding of initiating RNAP and represses transcription, consistent with previous studies [Dillon and Dorman 2010].

We next examined the position of the 668 promoters with respect to genes. Promoters in high H-NS regions are far more likely to be inside genes (46% of promoters) than promoters in low H-NS regions (19% of promoters; Fisher’s exact test, $P = 2.0 \times 10^{-11}$). However, an intragenic or intergenic location for a promoter does not fully describe its genomic context. For example, an intergenic promoter may be oriented opposite to surrounding genes and unable to drive mRNA transcription. Conversely, an intragenic promoter may be close to a correctly oriented gene, as is the case for many known mRNA promoters [Keseler et al. 2013]. Hence, we next determined the distance between each of the 668 promoters and the nearest properly orientated gene. These distances represent the distance between each of the 668 promoters and the nearest orientated gene, as is the case for many known mRNA promoters (Fig. 3G). Interestingly, while both intragenic and intergenic promoters in high H-NS regions are repressed by H-NS, repression of intragenic promoters is significantly greater (Mann Whitney U test, normal approximation).

![Figure 3](image-url)
tion, $P = 0.03$ for $\sigma^{-70}$ occupancy and $P = 0.01$ for TSS strength) [Fig. 3D,E, cf. the first three red data points]. Thus, intragenic promoters are more likely to be repressed and are more strongly repressed by H-NS. Importantly, intragenic promoters in H-NS-bound regions are indistinguishable from all other promoters with respect to DNA sequence [Supplemental Fig. S4].

We next compared the position of H-NS-repressed promoters with horizontally acquired genes in the known H-NS regulon. Strikingly, intragenic promoters repressed by H-NS align with both horizontally acquired DNA and genes proposed to be repressed by H-NS [Fig. 3D,E, final two data points; Supplemental Table S1; Garcia-Vallve et al. 2003]. We note that previous studies used microarray-based approaches unable to distinguish canonical mRNAs and have many TpA steps common to both H-NS targets and promoter –10 elements [Bouffartigues et al. 2007; Singh et al. 2011]. Hence, these genes are ideal candidates for recognition by H-NS or its functional analogs and for spurious RNAP binding. In such regions, high-affinity canonical promoters and transcription factors likely play a key role, along with H-NS, in facilitating specific transcription.

### Materials and methods

**Strains and plasmids**

*E. coli* JCB887 $\Delta$arg $\Delta$lac and MG1655 have been described [Page et al. 1990; Keseler et al. 2013]. The $\Delta$hs MG1655 derivative (Fig. 2) was provided by Dong Jin. Plasmid PRW50 was described by Lodge et al. [1992]. The 745-base $\Delta$ehxBAD fragment was synthesized by DNA2.0 and contained silent mutations to remove restriction sites to facilitate cloning. The ~62-base $\Delta$ehxCARD fragments were generated with oligonucleotides (Supplemental Table S2). A small number of fragments contained more than one putative promoter and were thus larger (up to 80 bp). Mutations in –10 elements were introduced with oligonucleotide derivatives (Supplemental Table S2). Mutations were numbered on the assumption that –10 elements are 7 bp upstream of a TSS. The $\Delta$hs::kan$^R$ MG1655 derivative used for ChIP-seq was constructed by P1 transduction of $\Delta$hs::kan$^R$ from BW25113 $\Delta$hs::kan$^R$ [Baba et al. 2006] into MG1655. Derivatives of BW25113 $\Delta$(araD-araB)$^R$s677, $\Delta$(acz4787::$\Delta$rrnB-3), $\Delta$(rhaD::rhaB)$^R$s68, and $\Delta$(hsdR$^S$) were used for TSS mapping. Unmarked BW25113 $\Delta$rrpH was constructed by removal of kan$^R$ from BW25113 $\Delta$rrpH::kan$^R$ using FLP recombinase from plasmid pCP20 [Datsenko and Wanner 2000]. BW25113 $\Delta$rrpH::kan$^R$ was constructed by P1 transduction of $\Delta$hs::kan$^R$ from BW25113 $\Delta$hs::kan$^R$ into the unmarked BW25113 $\Delta$rrpH.**

### Promoter DNA fragments and in vivo gene expression assays

In this study, we used a set of 745-base, horizontally acquired DNA fragments and their homologs in *E. coli* to test the ability of H-NS to repress transcription. We also used a panel of 28 micrococcal endonuclease-sensitive TSSs to map transcription initiation sites. These TSSs were selected from the GenoList database (http://genolist.pasteur.fr) and were used to interrogate bacterial genomes and determine the distribution of potential –10 hexamers between genes and noncoding DNA. We used the list of MvaT-bound intragenic promoters to identify potential targets for H-NS regulation. We have previously described the presence of H-NS and RNAP at the TSS of the hns and rppH promoters and have shown that H-NS is required for efficient transcription at these loci.**

### Binding of H-NS analogs coincides with predicted intragenic promoters in diverse bacterial species

MvaT and Lsr2 are functional analogs of H-NS in *Pseudomonas* spp. and *Mycobacterium* spp., respectively. For both proteins, ChIP-chip has been used to measure binding across the cognate genome [Castang et al. 2008; Gordon et al. 2010]. Fortuitously, promoters for primary proteins do not vary greatly between these species and *E. coli* [Potvin et al. 2008; Sachdeva et al. 2010]. Hence, we were able to use the same approach used for the *E. coli* genomes to search for –10 hexamers. We then compared the position of these putative –10 hexamers with binding of MvaT/Lsr2. Putative promoters were enriched approximately fivefold in MvaT-bound ($\chi^2$ test, $P = 8.9 \times 10^{-9}$) and Lsr2-bound ($\chi^2$ test, $P = 5.5 \times 10^{-4}$) regions. Furthermore, most putative promoters in MvaT- and Lsr2-bound regions were located in genes [58% for *Pseudomonas aeruginosa* and 78% for *Mycobacterium tuberculosis*]. Thus, MvaT and Lsr2 are likely to be suppressors of intragenic transcription.

### Conclusions

H-NS has been proposed to facilitate genome evolution by binding horizontally acquired DNA. Previous studies have concluded that H-NS silences such genes solely by repressing canonical transcription [Fig. 4A]. In contrast, we show that the primary function of H-NS is to silence spurious transcription of ncRNAs, often inside the genes previously described as being H-NS-repressed [Fig. 4B]. Hence, the fitness defect of deleting hns is likely due in part to increased spurious transcription. We suggest that, by suppressing intragenic transcription, H-NS prevents misappropriation of cellular resources, the formation of dangerous nucleic acid structures, and transcription interference. We note that the two models for H-NS function shown in Figure 4 are indistinguishable using DNA microarray-based approaches that cannot resolve canonical mRNAs and spurious ncRNAs. Thus, while we have identified a largely new set of H-NS regulated promoters, these promoters coincide with the existing H-NS regulon. Previous bioinformatic screens predicted that H-NS-bound genes contain promoters [Tutukina et al. 2007]. Such genes are often horizontally acquired, are A/T-rich, and have many TpA steps common to both H-NS targets and promoter –10 elements [Bouffartigues et al. 2007; Singh et al. 2011]. Hence, these genes are ideal candidates for recognition by H-NS or its functional analogs and for spurious RNAP binding. In such regions, high-affinity canonical promoters and transcription factors likely play a key role, along with H-NS, in facilitating specific transcription.

### Bioinformatic analysis of promoters

We used our alignment of 554 *E. coli* promoters to determine selection criteria [Singh et al. 2011]. Thus, putative –10 elements were chosen if they matched the sequence 5'-TAnAT-3', 5'-TATnAT-3' or 5'-TATAnt-3'. The GenoList database (http://genolist.pasteur.fr) was used to interrogate bacterial genomes and determine the distribution of potential –10 hexamers between genes and noncoding DNA. We used the list of MvaT-bound
regions described by Castang et al. (2008). Lsr2-bound regions in *M. tuberculosis* H37Rv were determined by applying a cutoff of 1.5 to the ChIP-chip data set of Gordon et al. (2010).

**ChIP, Chip-seq, and TSS mapping**

Targeted ChIP experiments (Fig. 2) were done as described (Singh and Grainger 2013) using exchCARD fragments cloned in pRW50. For ChIP-seq experiments, cultures were incubated 1:100 in LB with fresh overnight cultures of MG1655 or MG1655 Δhns:kan^r, and cells were grown at 225 rpm at 30 °C to an OD600 of 0.5–0.6. Protocols for ChIP-seq, TSS mapping, and analysis of these data are described in Supplemental Appendix S1.

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