

Interaction of Ler at the *LEE5* (*tir*) Operon of Enteropathogenic *Escherichia coli*

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The genome of enteropathogenic *Escherichia coli* (EPEC) encodes a global regulator, Ler (locus of enterocyte effacement [LEE]-encoded regulator), which activates expression of several polycistronic operons within the 35.6-kb LEE pathogenicity island, including the *LEE2-LEE3* divergent operon pair containing overlapping –10 regions and the *LEE5* (*tir*) operon. Ler is a predicted 15-kDa protein that exhibits amino acid similarity with the nucleoid protein H-NS. In order to study Ler-mediated activation of virulence operons in EPEC, we used a molecular approach to characterize the interactions of purified Ler protein with the upstream regulatory sequences of the *LEE5* operon. We determined the *cis*-acting DNA sequences necessary for Ler binding at *LEE5* by mobility shift and DNase I protection assays, demonstrating that Ler acts directly at *LEE5* by binding sequences between positions –190 and –73 in relation to the transcriptional start site. Based on the molecular weight of Ler, the similarity to H-NS, and the extended region of protection observed in a DNase I footprint at *LEE5*, we hypothesized that multiple Ler proteins bind upstream of the *LEE5* promoter to increase transcriptional activity from a distance. Using an *hns* deletion strain, we demonstrated that like the *LEE2-LEE3* operon pair, H-NS represses *LEE5* transcription. We describe a model in which Ler activates transcription at both divergent overlapping paired and single promoters by displacing H-NS, which results in the disruption of a repressing nucleoprotein complex.

Enteropathogenic *E. coli* (EPEC) is a leading cause of infant diarrhea in developing countries (35), and there is some indication that diarrhea caused by EPEC in the United States is underreported (5). EPEC is the prototype organism of a group of pathogenic bacteria that cause attaching and effacing (AE) intestinal lesions (25, 33, 35). A variety of gram-negative pathogens are capable of forming AE lesions, including enterohemorrhagic *E. coli* serotype O157:H7, which causes hemorrhagic colitis and hemolytic-uremic syndrome (52), *Hafnia alvei*, which causes diarrhea in children (2), the mouse pathogen *Citrobacter rodentium*, (43), and rabbit enteropathogenic *E. coli*, which causes diarrhea in rabbits (40).

All genes necessary for the AE phenotype in EPEC are encoded in a 35.6-kb pathogenicity island that contains 41 predicted open reading frames termed the locus of enterocyte effacement (LEE) (15, 19, 28, 35). The genetic organization of the EPEC LEE was determined previously by defining transcriptional units and mapping transcriptional start points (30). Components of the type III secretion system are transcribed from three polycistronic operons designated *LEE1*, *LEE2*, and *LEE3*, and the secreted Esp molecules are part of a fourth polycistronic operon designated *LEE4*. Genes involved in intimate attachment to the host cell, *tir*, *cesT*, and *ea*e, were found to be transcribed from a fifth polycistronic operon termed *LEE5* (*tir*) (30, 42).

Ler (LEE-encoded regulator) increases expression of at

least four polycistronic operons found within the LEE (14, 30). In a cascade fashion, the EAF plasmid-encoded regulator Per modulates the expression of the Ler protein, which goes on to increase expression of most, if not all, of the genes necessary for the AE phenotype (30). Ler increases the transcription of divergent operons of the LEE possessing overlapping promoter regions (e.g., the *LEE2-LEE3* promoter pair), as well as single nondivergent operons. Interestingly, Ler activates transcription from both *LEE2* and *LEE3* by binding over an extended region (positions –221 to –100) on only one side of the overlapping promoters, upstream of *LEE2* (47). In addition, Ler activates the expression of *espC*, which encodes an enterotoxin (14) and is contained within a second pathogenicity island in EPEC (31), and thus Ler is considered to be a global regulator of virulence genes.

The predicted 15-kDa Ler protein exhibits significant amino acid similarity to the H-NS family of DNA-binding proteins, including 24% identity and 44% similarity to H-NS of *Salmonella enterica* serovar Typhimurium (47). The C-terminal domain of H-NS contains a conserved DNA-binding domain (4), whereas the N terminus contains a coiled-coil domain involved in protein oligomerization (27). Greater amino acid sequence similarity to H-NS was observed in the C terminus than in the N terminus. Base substitution in the putative oligomerization domain in the N terminus of Ler eliminated the ability of the protein to bind to DNA and the ability to activate expression of a *LEE2-lacZ* fusion, strongly suggesting that Ler must form oligomers in order to activate transcription (47).

H-NS is a 15-kDa, histone-like, nucleoid-associated protein that was originally described as a protein that compacts and alters the topology of DNA (20, 37). This protein is also a pleiotropic transcription factor, affecting the expression of ap-

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TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid or phage	Genotype or description	Source or reference
Strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Lab stocks
MC4100	<i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	7
HN4104	MC4100 Δ (<i>hns idk adhE oppABCD</i>)118 <i>zch-506::Tn10</i>	8
KMR551	MC4100 ϕ (promoterless) <i>lacZ</i>	This study
KMTIR2	MC4100 ϕ <i>LEE5-lacZ</i> (-405 to +172)	This study
KMTIR3	MC4100 ϕ <i>LEE5-lacZ</i> (-303 to +172)	This study
KMTIR4	MC4100 ϕ <i>LEE5-lacZ</i> (-198 to +172)	This study
CRTIR5	MC4100 ϕ <i>LEE5-lacZ</i> (-75 to +172)	This study
JFHNTIR3	HN4104 ϕ <i>LEE5-lacZ</i> (-303 to +172)	This study
JFHNTIR4	HN4104 ϕ <i>LEE5-lacZ</i> (-198 to +172)	This study
JFHNTIR5	HN4104 ϕ <i>LEE5-lacZ</i> (-75 to +172)	This study
KHTIR3M	MC4100 ϕ Δ (-51 to -47) <i>LEE5-lacZ</i> (-303 to 172)	This study
Plasmids		
pBR322	Cloning vector, <i>bla</i>	49
pBluescript-II KS	Cloning vector, <i>bla</i>	Stratagene
pVS45	Minimal <i>ler</i> in pBADMyHis	47
pSE1100	Minimal <i>ler</i> in pBR322, <i>bla</i>	30
pTHK113	Minimal <i>hns</i> in pBR322, <i>bla</i>	23
pRS551	(Promoterless) <i>lacZ</i> reporter fusion vector	45
pKMTIR2	<i>LEE5</i> (-405 to +172) in pRS551	This study
pKMTIR3	<i>LEE5</i> (-303 to +172) in pRS551	This study
pKMTIR4	<i>LEE5</i> (-198 to +172) in pRS551	This study
pCRTIR5	<i>LEE5</i> (-75 to +172) in pRS551	This study
pKHTIR3M	Δ (-51 to -47) <i>LEE5</i> (-303 to 172) in pRS551	This study
pBlueTIR3	<i>LEE5</i> (-303 to +172) in pBluescript	This study
pBlueTIR4	<i>LEE5</i> (-198 to +172) in pBluescript	This study
pBlueTIR5	<i>LEE5</i> (-75 to +172) in pBluescript	This study
pBlueTIR3M	Δ (-51 to -47) <i>LEE5</i> (-303 to 172) in pBluescript	This study
pKH273	<i>LEE5</i> (-272 to -31) in pBluescript	This study
Phage λ RS45	Specialized transducing phage	45

proximately 5% of the genes in *E. coli* (3, 21). One commonality of the seemingly unrelated genes regulated by H-NS is that many respond to environmental stress conditions, such as osmotic shock and cold shock (13, 16, 26, 29, 50). H-NS was shown to influence the thermo-osmotic regulation of virulence genes in *Shigella* (38). H-NS is now known to regulate virulence gene expression in several gram-negative pathogens (16, 18, 41, 54).

H-NS forms homodimers or tetramers (48) and is known to form higher-order oligomeric structures in solution (46). It is now apparent that the H-NS protein binds to DNA possessing a particular conformation rather than to a specific consensus binding sequence (10, 12, 50). There is a large body of data that demonstrates that there is transcriptional repression by H-NS binding to promoter regions (1, 17, 26, 39, 53). Recent discoveries indicate that H-NS can hold RNA polymerase in an open complex while it forms a nucleoprotein structure that represses transcription at the *rnnB* P1 promoter (11, 44). Ler has been shown to relieve the transcriptional repression caused by H-NS at the *LEE2* and *LEE3* operons (6) and the transcriptional repression of the *LEE5* operon caused by an unknown negative regulator (42).

In order to learn more about the molecular pathogenesis of EPEC, we used a biochemical approach to study the mechanism of Ler binding at the *LEE5* operon. Ler is a key virulence gene regulator in EPEC, and a better understanding of the details of its DNA binding activity and the DNA sequences

required for binding should provide insight into to how Ler activates transcription. The ability of the Ler protein to increase transcriptional activity from a distance at both divergent overlapping and nondivergent promoter sequences and the similarity to the H-NS family of proteins that, in most cases, repress transcription prompted us to study the mechanism of Ler binding at the molecular level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. The plasmids, strains, and phage used in this study are listed in Table 1. Strains were grown at 37°C with aeration in Luria-Bertani medium supplemented with ampicillin (100 μ g/ml) or kanamycin (50 μ g/ml). Single-copy chromosomal *lacZ* fusions were constructed by homologous recombination between λ RS45 and plasmids containing *LEE5* regulatory fragments cloned upstream of the promoterless *lacZYA* operon contained in pRS551 and were subsequently transduced into MC4100, selecting for kanamycin resistance as previously described (30, 45).

Generation of *LEE5* regulatory fragments. Specific *LEE5* regulatory fragments were generated by PCR by using *Pwo* DNA polymerase, which contains proofreading activity (Boehringer Mannheim). The oligonucleotides used in the study are listed in Table 2 and were purchased from Invitrogen. PCRs were performed by using standard protocols. Restriction endonucleases and DNA ligase were obtained from New England Biolabs and used according to the manufacturer's instructions. The PCR-generated *LEE5* regulatory fragments were gel isolated with a Qiaquick gel extraction kit obtained from Qiagen. DNA fragments were then cloned into pRS551 by using a Bio-Rad Micropulser electroporation apparatus to transform *E. coli* strains DH5 α and MC4100. Clones containing plasmids with *LEE5* regulatory sequences fused to *lacZ* were verified by restriction mapping, PCR, DNA sequence analysis, and β -galactosidase assays.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5' to 3') ^a
TIR1.....	CGCGGATCCGCGCCGTCTGTTTGTGAA
TIR2.....	CCGGAATTCGGTAAAGGAGTGGATCCCA
TIR3.....	CCGGAATTCAGTGATATCAAGGCTCTAA
TIR4.....	CCGGAATTCAGGTCTCTATAGACGTTTAAA
TIR5.....	CCGGAATTCGTTGGAAATACAGACATGCA
JFTIR1	AATACAGACATGCATTTCTGTTATTTGCTTG CATCAAAA
JFTIR3	TTTTGATGCAAGCAAATAACAGAAATGCAT GTCTGTATT
TF1.....	CCGGAATTCCTTGAGACAATGTGCAGGAT
TF5.....	CGCGGATCCATGCAAGCAAATAACGCAC
BlueNot	GGCCGCTCTAGAAGTAGTG

^a Restriction sites used in cloning are underlined.

Enzymatic assays. Lysogens of *E. coli* strain MC4100 containing single-copy chromosomal *lacZ* fusions were transformed with pSE1100 (minimal *ler* clone [30]), pTHK113 (minimal *hms* clone [23]), or pBR322 (control) and grown at 37°C in Luria-Bertani medium supplemented with the appropriate antibiotics with aeration to an absorbance at 600 nm of approximately 0.3 to 0.5 and then subjected to β -galactosidase assays performed by the method described by Miller (32). Assays were performed in triplicate, and values (in Miller units) were expressed as the mean \pm one standard deviation. Statistical analysis was performed with raw data generated from β -galactosidase assays by using StatView (SAS Institute Inc.).

Ler protein purification. His-tagged Ler protein was isolated from DH5 α (pVS45) as described previously (47). The purified Ler protein migrated as a single species when it was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and stained by Coomassie brilliant blue. The concentration of purified Ler protein, 50 μ g/ml, was estimated by comparison with a known concentration of a standard protein having a similar molecular weight (lysozyme from Sigma).

DNA mobility assays. The *LEE5* regulatory fragments were subcloned into pBluescript by using *E. coli* strain DH5 α . Plasmid DNA was isolated with a Qiagen plasmid midi kit (Qiagen). The purified plasmid DNA was cut with *NotI* restriction endonuclease, which left a 5' overhang that was subsequently filled in by using [α -³²P]dCTP (Amersham), dGTP, and Klenow DNA polymerase (Promega). The plasmid DNA was then cut with *EcoRI*, releasing the *LEE5* regulatory fragments. Radiolabeled and nonlabeled DNA fragments were separated in a 6% polyacrylamide-1 \times Tris-borate-EDTA (TBE) gel and were isolated by soaking the gel slice in elution buffer (0.5 M ammonium acetate, 0.1% SDS, 1 mM EDTA) overnight and precipitating the DNA with ethanol. Ler binding reactions were performed by incubating 10 μ g of poly(dI-dC) (U.S. Biochemicals) per ml (to prevent nonspecific binding), 8 μ g of purified Ler protein per ml, and 20,000 cpm of probe DNA in binding buffer (10 mM Tris-HCl [pH 7.4], 1

mM EDTA, 50 μ g of bovine serum albumin per ml, 5 mM NaCl, 50 mM KCl) for 30 min at room temperature. A 50-fold excess of an identical unlabeled fragment was used as a specific competitor for each fragment as a control. The binding reaction mixtures were electrophoresed in a 6% polyacrylamide-1 \times TBE gel at 4°C, dried at 80°C for 1 h, and exposed to Kodak X-OMAT film.

PCR-generated deletions. Overlapping PCR was performed by using oligonucleotide primers which contained deletions of the *LEE5* sequence corresponding to positions -51 to -47. Primers JFTIR1 and JFTIR3 contain corresponding five-base deletions and were used with primers TIR1 and TIR3, respectively, to independently amplify *LEE5* sequences. Individual PCR products were amplified, gel isolated, mixed, and used as template DNA for a secondary overlapping PCR (22), which resulted in a product that contained the 5-bp deletion. The PCR product was cloned into pRS551 and sequenced to verify the deletion.

DNase I footprint analysis. Ler binding reactions were performed as described above, except that various amounts of Ler protein, 70,000 cpm of radiolabeled probe DNA (both coding and noncoding strands individually from positions -272 to -31 of the *LEE5* regulatory region), and 100 μ g of poly(dI-dC) (U.S. Biochemicals) per ml were used. The coding and noncoding strands were labeled individually by cutting the plasmid, pKH273, with either *EcoRI* or *NotI* first, followed by filling in of the *EcoRI* site with [α -³²P]dATP or of the *NotI* site with [α -³²P]dCTP and the Klenow fragment of DNA polymerase. The polymerase was then heat inactivated by heating the preparation at 70°C for 15 min. Subsequently, the fragments were released from the plasmid by a final restriction with the alternate enzyme *NotI* or *EcoRI* and isolated as described above. After Ler binding, 5 mU of DNase I (Sigma), MgCl₂ (final concentration, 5 mM), and CaCl₂ (final concentration, 1 mM) were added and incubated at room temperature for 2 min. The reaction was terminated by addition of stop solution (200 mM NaCl, 2 mM EDTA, 1% SDS), and this was followed by phenol-chloroform extraction and ethanol precipitation with 7.5 M ammonium acetate (pH 7.5) and 1 μ l of glycogen (Invitrogen). The footprint reaction mixtures were denatured at 75°C for 2 min prior to loading and then were electrophoresed in a denaturing 6% polyacrylamide-1 \times TBE gel, dried at 80°C for 1 h, and exposed to Kodak X-OMAT film. Sequencing reaction mixtures (Sequenase DNA sequencing kit, version 2; U.S. Biochemicals) with the TF1 and BlueNot primers were loaded adjacent to the footprint reaction mixtures to determine the positions of protected sequences.

RESULTS

Deletion analysis of *LEE5* regulatory sequences. In order to define *LEE5* regulatory fragments that were appropriate for Ler binding studies, sequences upstream of the *LEE5* promoter were deleted (Fig. 1A). PCR amplification of *LEE5* sequences from positions -405, -303, -198, and -75 to position +172 relative to the transcriptional start site of the *tir* gene, which is the first gene of the *LEE5* operon, were fused to a promoterless *lacZ* reporter gene in pRS551 (45). Individual

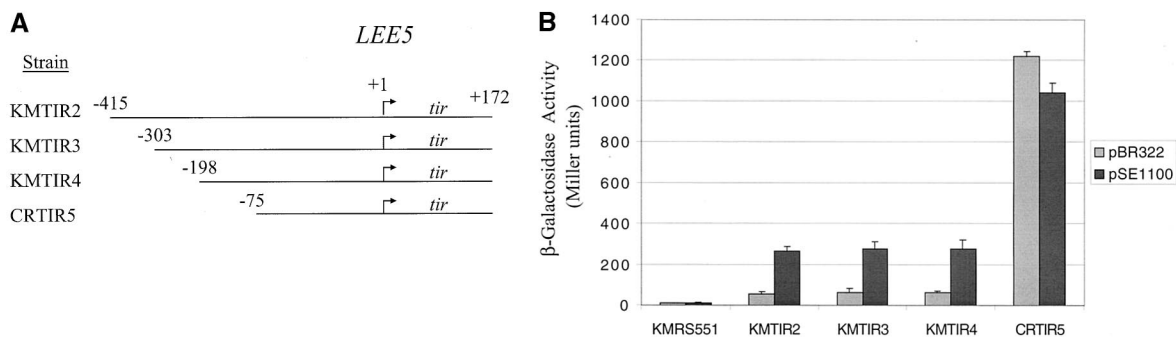


FIG. 1. Deletion analysis of the *LEE5* regulatory region. (A) Schematic representation of *LEE5* fragments generated by PCR for Ler binding experiments. The strains used were K-12 derived and contained single-copy, chromosomal *LEE5-lacZ* fusions. The first gene of the *LEE5* operon is *tir*. The termini of each fragment in relation to the *LEE5* transcriptional start site (+1) are indicated (30, 42). (B) β -Galactosidase activities derived from the single-copy, chromosomal *LEE5-lacZ* fusion strains shown in panel A. Plasmid pSE1100 contains a minimal *ler* fragment cloned into pBR322 and was described previously (30). The error bars indicate one standard deviation. The values are the means for multiple independent assays performed in triplicate. Strain KMRS551 contained the single-copy, chromosomal, promoterless *lacZ* fusion in MC4100 as a negative control.

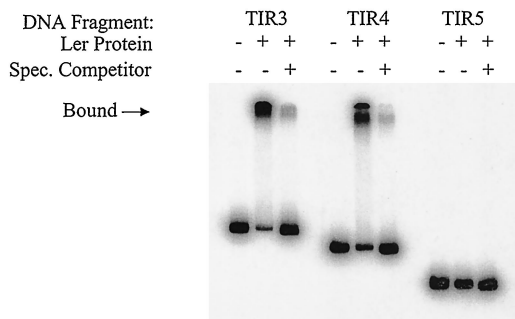


FIG. 2. Mobility shift assay of *LEE5* regulatory fragments. Purified Ler protein (8 μ g/ml) was incubated with the radiolabeled *LEE5* regulatory fragments shown in Fig. 1A and 10 μ g of nonspecific competitor DNA [poly(dI-dC)] per ml. The specific competitor DNA (Spec. Competitor) consisted of a 50-fold excess of the identical fragment that was not labeled and added to the binding reaction mixture. Unbound species and bound species (indicated by the arrow) were separated in a 6% nondenaturing polyacrylamide gel and visualized by autoradiography.

fusions were allowed to recombine with λ RS45 and inserted, as single copies, into the chromosome of MC4100 (see Materials and Methods). The resulting isogenic, single-copy fusion strains were transformed with pBR322 as a negative control or pSE1100, which encodes a minimal *ler* fragment in pBR322 (30). The β -galactosidase activities of these strains were assayed in the presence and absence of the Ler protein expressed in *trans* from plasmid pSE1100. Fusion strains KMTIR2 (positions -405 to +172), KMTIR3 (positions -303 to +172), and KMTIR4 (positions -198 to 172) were all induced approximately four- to fivefold in the presence of pSE1100 (Fig. 1B). Fusion strain CRTIR5 (positions -75 to +172) exhibited high levels of β -galactosidase activity independent of *ler* expression. These results indicated that Ler-mediated activation of the *LEE5* operon required sequences between positions -198 and -75. The observation that high levels of β -galactosidase activity were detected independent of Ler in the CRTIR5 strain was consistent with the activity of a negative regulator that represses *LEE5* expression and with the hypothesis that sequences necessary for repression also reside within the same region required for activation by Ler.

DNA mobility assays with purified Ler protein. To identify sequences necessary for Ler binding, we performed mobility shift experiments with purified Ler protein (47) and radiolabeled DNA fragments corresponding to the *LEE5* regulatory sequences used in the deletion analysis (Fig. 1A). As expected, DNA fragments corresponding to TIR3 and TIR4 exhibited pronounced shifts in mobility when purified Ler protein was added to the binding reaction mixture compared to the results obtained with an identical reaction mixture without the Ler protein (Fig. 2). DNA fragment TIR5, which showed no Ler-dependent activation (Fig. 1B), also exhibited no shift in mobility in the presence of purified Ler protein (Fig. 2). This observation clearly demonstrated that Ler did not bind to sequences from positions -75 to +172. These results were consistent with the fusion data in that Ler-mediated transcriptional activation was observed with the same regulatory fragments that also exhibited shifts in mobility due to Ler binding.

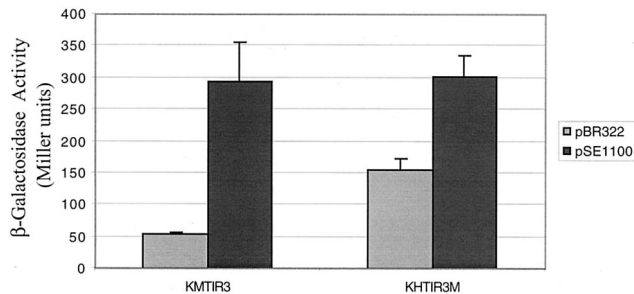


FIG. 3. β -Galactosidase activity derived from a *LEE5-lacZ* fusion containing a 5-bp deletion centered at position -49. Wild-type TIR3 and TIR3M (5-bp deletion) regulatory fragments were fused to *lacZ*, and β -galactosidase activities were assayed. The error bars indicate one standard deviation. Construct pSE1100 described in the legend to Fig. 1B encodes a minimal *ler* clone that constitutively expresses Ler protein (30). The values are means for two experiments performed in triplicate.

To examine the specificity of Ler binding, we added nonspecific competitor DNA [unlabeled poly(dI-dC)] at a concentration of 10 μ g/ml to each binding reaction mixture to minimize nonspecific interactions. In addition, a 50-fold excess of unlabeled specific competitor DNA (identical to the labeled probe DNA) was added to the reaction mixtures (Fig. 2), which resulted in specific competition. Under these conditions, the binding of Ler protein to radiolabeled TIR3 and TIR4 fragments was severely reduced (Fig. 2). We therefore concluded that the Ler protein bound specifically to sequences upstream of position -75 and acted directly to increase transcription at *LEE5*.

Deletion of sequences between the regulatory region and the promoter affected repression but not activation of the *LEE5* operon. Since Ler bound at a distance from the *LEE5* promoter and also the *LEE2* promoter (47), we hypothesized that Ler may influence transcription by altering DNA topology. By deleting 5 bp at positions -51 to -47, we altered the spacing of the *LEE5* regulatory region (positions -198 to -75) in relation to the promoter by one half-helical turn of DNA without altering the sequences required for repression or Ler-mediated activation. Strains with single-copy *lacZ* fusions, encoding the wild-type TIR3 regulatory fragment or the TIR3M (five-base deletion) regulatory fragment, were assayed for Ler-dependent β -galactosidase activity as described above. Strains KMTIR3 and KHTIR3M transformed with pSE1100 exhibited Ler-mediated activation of the same magnitude, ~300 Miller units (Fig. 3). Strain KHTIR3M transformed with pBR322, as a control, exhibited threefold-higher β -galactosidase activity (155 Miller units) than strain KMTIR3 (53 Miller units) also transformed with pBR322 ($P < 0.0001$). These results indicated that repression of the *LEE5* operon was compromised by the 5-bp deletion.

Mobility shift experiments were performed with the TIR3M fragment and purified Ler protein. The deletion fragment TIR3M exhibited shifts in mobility in the presence of Ler protein that were identical to those exhibited by the wild-type TIR3 regulatory fragment, indicating that the five-base deletion had no effect on Ler binding (data not shown).

H-NS-dependent repression of *LEE5*. As indicated previously, H-NS is involved in repression of the *LEE2* and *LEE3*

TABLE 3. Effect of *hns* deletion on the expression of *LEE5-lacZ* fusions^a

Relevant genotype ^b	Plasmid	β -Galactosidase activity ^c
MC4100 Φ TIR3- <i>lacZ</i>		61 (3)
MC4100 Φ TIR3- <i>lacZ</i>	pBR322	64 (3)
MC4100 Φ TIR3- <i>lacZ</i>	pSE1100 (<i>ler</i> ⁺)	307 (36)
MC4100 Φ TIR3- <i>lacZ</i>	pTHK113 (<i>hns</i> ⁺)	48 (6)
HN4104 Φ TIR3- <i>lacZ</i> (Δ <i>hns</i>)		225 (15)
HN4104 Φ TIR3- <i>lacZ</i> (Δ <i>hns</i>)	pBR322	252 (23)
HN4104 Φ TIR3- <i>lacZ</i> (Δ <i>hns</i>)	pSE1100 (<i>ler</i> ⁺)	2,074 (79)
HN4104 Φ TIR3- <i>lacZ</i> (Δ <i>hns</i>)	pTHK113 (<i>hns</i> ⁺)	67 (12)
MC4100 Φ TIR4- <i>lacZ</i>		77 (4)
MC4100 Φ TIR4- <i>lacZ</i>	pBR322	60 (5)
MC4100 Φ TIR4- <i>lacZ</i>	pSE1100 (<i>ler</i> ⁺)	581 (52)
MC4100 Φ TIR4- <i>lacZ</i>	pTHK113 (<i>hns</i> ⁺)	39 (6)
HN4104 Φ TIR4- <i>lacZ</i> (Δ <i>hns</i>)		243 (14)
HN4104 Φ TIR4- <i>lacZ</i> (Δ <i>hns</i>)	pBR322	318 (20)
HN4104 Φ TIR4- <i>lacZ</i> (Δ <i>hns</i>)	pSE1100 (<i>ler</i> ⁺)	1,566 (41)
HN4104 Φ TIR4- <i>lacZ</i> (Δ <i>hns</i>)	pTHK113 (<i>hns</i> ⁺)	96 (21)
MC4100 Φ TIR5- <i>lacZ</i>		1,405 (307)
MC4100 Φ TIR5- <i>lacZ</i>	pBR322	2,319 (91)
MC4100 Φ TIR5- <i>lacZ</i>	pSE1100 (<i>ler</i> ⁺)	1,432 (111)
MC4100 Φ TIR5- <i>lacZ</i>	pTHK113 (<i>hns</i> ⁺)	1,188 (37)
HN4104 Φ TIR5- <i>lacZ</i> (Δ <i>hns</i>)		1,850 (498)
HN4104 Φ TIR5- <i>lacZ</i> (Δ <i>hns</i>)	pBR322	2,176 (124)
HN4104 Φ TIR5- <i>lacZ</i> (Δ <i>hns</i>)	pSE1100 (<i>ler</i> ⁺)	2,040 (69)
HN4104 Φ TIR5- <i>lacZ</i> (Δ <i>hns</i>)	pTHK113 (<i>hns</i> ⁺)	1,520 (151)

^a Overnight cultures of each strain were diluted 1:500 into fresh Luria-Bertani medium containing the appropriate antibiotics. Strains were grown to an optical density at 600 nm of 0.3 to 0.5 and assayed as described in Materials and Methods.

^b Strain genotypes are shown in Table 1.

^c β -Galactosidase activity is expressed in Miller units, and the standard error is indicated in parentheses. Assays were performed in triplicate with at least two independent cultures.

operons (6). However, because the identity of the negative-acting factor for the *LEE5* operon remained unclear, we wished to determine whether H-NS affected the expression of *LEE5* as well. Therefore, we assayed β -galactosidase activities derived from *LEE5-lacZ* single-copy fusions in HN4104 strains having a deletion of *hns* and the isogenic parent strain, MC4100. The TIR3 and TIR4 fusion strains which contained all known regulatory sequences and from which *hns* was deleted exhibited β -galactosidase activities that were approximately fourfold greater than the β -galactosidase activities of isogenic strains with wild-type *hns* (60 versus 250 Miller units) (Table 3). Providing H-NS expressed from the minimal *hns* gene on a plasmid restored transcriptional activity to low basal levels (67 to 96 Miller units) in the TIR3 and TIR4 fusion strains from which *hns* was deleted. Under the conditions tested, maximal *LEE5* expression was observed in the absence of H-NS and in the presence of Ler; under these conditions the β -galactosidase activities reached 1,400 to 2,000 Miller units. β -Galactosidase activities were similarly high for all of the strains containing the TIR5 fragment fused to *lacZ*, from which all currently known positive and negative regulatory sequences were deleted (Table 3).

To ensure that H-NS activity was indeed absent from the

HN4104 *hns* deletion strain used for our assays, phenotypes associated with this strain were confirmed prior to and after transduction of the *LEE5-lacZ* fusions into the strains. As expected, HN4104 and its derivatives were able to ferment salicin, presumably by expression of the cryptic *bgl* operon, which is known to be expressed in the absence of H-NS (34). Thus, we concluded that like the *LEE2-LEE3* operon pair, H-NS was involved in the negative regulation of *LEE5*.

DNase I footprint analysis of purified Ler bound to *LEE5* regulatory sequences. A 241-bp fragment (positions -272 to -31) containing the *LEE5* regulatory region and flanking sequences was radiolabeled in separate reactions on both the coding and noncoding strands and subjected to DNase I digestion in the presence and absence of purified Ler protein. Ler protein protected approximately 117 bp of DNA (positions -190 to -73) from digestion by DNase I (Fig. 4). Because of the low predicted molecular mass of Ler protein (15 kDa), multiple Ler proteins would be required to protect such an extended region of DNA (>100 bp). Consistently, Ler appeared to exhibit cooperative binding. At low concentrations of Ler protein (0 to 0.15 μ g of Ler protein per ml), little or no protection was observed, yet when the protein level reached 0.5 μ g/ml Ler protein protected an extended region of DNA. One striking feature of these footprints was that Ler protein protected a region of DNA rich in AT (A+T content, $>80\%$).

At high concentrations of Ler protein protection extended slightly further downstream on the coding strand (position -60) and slightly further upstream on the noncoding strand (position -221). This most likely represented weak binding and was observed only at high concentrations of Ler protein. Thus, similar to binding upstream of the *LEE2* promoter (from positions -221 to -100 [47]), Ler bound between positions -190 and -73 in relation to the *LEE5* transcriptional start site. A Ler-dependent DNase I-hypersensitive site was observed at position -111 on the coding strand. We concluded from this finding that the binding of Ler protein to the *LEE5* regulatory region induced a structural change in the DNA that resulted in increased cleavage, specifically at position -111 , by DNase I.

DISCUSSION

We demonstrated that Ler acts directly to increase transcription of the *LEE5* operon. As determined by DNase I footprint analyses, purified Ler protein bound to an approximately 117-bp region of DNA (positions -190 to -73) upstream of the *LEE5* transcriptional start site. As determined by a mobility shift assay, Ler protein specifically bound to sequences between positions -198 and -75 , which is consistent with the *lacZ* fusion data (Fig. 1), with which we independently identified and confirmed a regulatory region (42) required for both H-NS-dependent repression and Ler-mediated derepression. One apparent conflict in our data was that as determined by a mobility shift assay, the TIR5 regulatory fragment (positions -75 to $+172$) did not bind Ler, whereas a DNase I protection assay showed that there was protection to the -60 position on the coding strand (Fig. 4A). This might be explained by the observation that Ler did not protect sequences downstream of the -79 position on the noncoding strand, and thus the binary

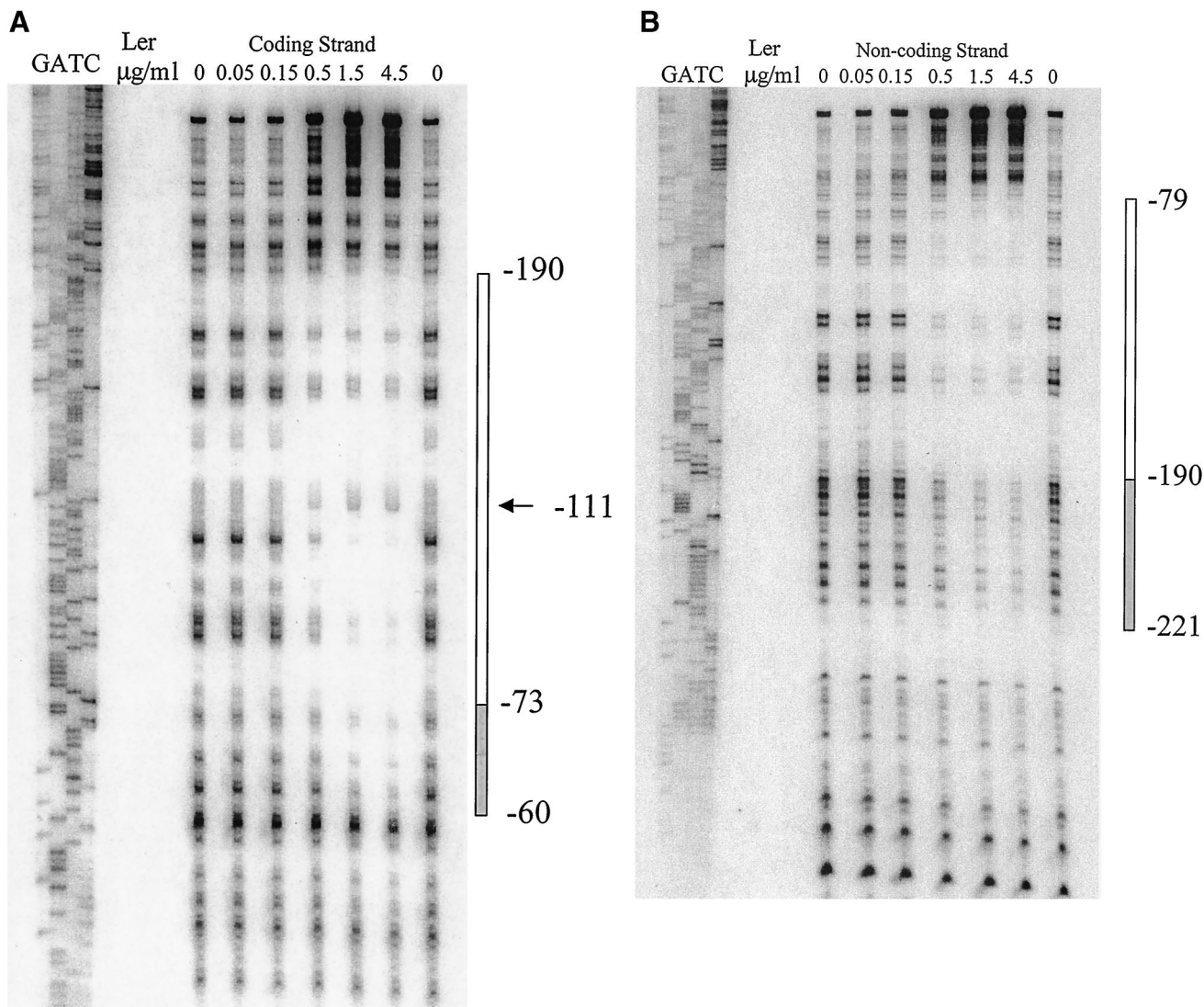


FIG. 4. DNase I footprint of Ler protein on the upstream *LEE5* regulatory region. Concentrations of Ler protein (in micrograms per milliliter) are indicated at the top. (A) DNase I protection of the *LEE5* coding strand. The Ler protein protected a region of DNA from DNase I digestion from positions -190 to -73, which is represented by an open bar. A DNase I-hypersensitive site was observed at position -111 on the coding strand and is indicated by an arrow. (B) DNase I protection of the *LEE5* noncoding strand. Ler protein protected a region of DNA from positions -190 to -79, which is represented by an open bar. At higher concentrations of Ler protein, the regions of protection extended to positions -60 and -221 on the coding and noncoding strands, respectively, as indicated by shaded bars.

complex (TIR5 DNA and Ler protein) was most likely too unstable to remain intact in the mobility shift assay.

Our data clearly demonstrated that H-NS is involved in the negative regulation of the *LEE5* operon (Table 3). The observation that under the conditions tested, maximal activity was achieved only in the presence of Ler and in the absence of H-NS may suggest that an additional protein(s) acts negatively at *LEE5*. An alternate explanation for the high transcriptional activity observed in the presence of Ler and in the absence of H-NS is that greater Ler activation of *LEE5* requires environmental conditions specific to conditions inside the human gut. Whether H-NS acts directly or indirectly through control of expression of an alternate negative regulator to repress *LEE5* transcription remains to be determined.

There is an extensive body of work which suggests that the

global regulator H-NS is a non-sequence-specific DNA-binding protein yet specifically recognizes structural features of DNA (10, 12, 50). When we aligned the upstream *LEE5* and *LEE2* (47) regulatory sequences to which Ler bound, we found no significant sequence similarity that allowed us to identify a Ler consensus binding sequence (data not shown). These sequences were, however, similar lengths, approximately 120 bp, and the A+T contents were high, approximately 80%. AT-rich DNA, particularly poly(A) tracts, are known to form curves (24). Based on these observations, the molecular mass of Ler (15 kDa), the amino acid sequence similarity to H-NS, the extended protection from DNase I digestion, and the likelihood that multiple Ler proteins bind upstream of *LEE5* and *LEE2* (47), we propose that like H-NS, Ler recognizes DNA structural motifs instead of specific nucleotide sequences.

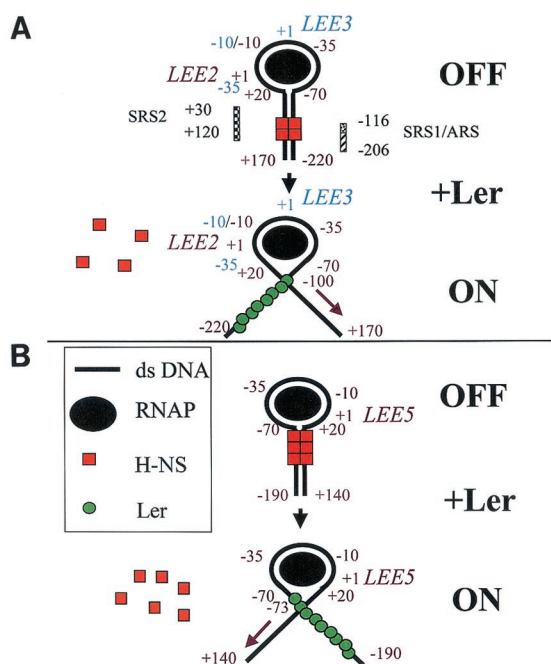


FIG. 5. Model for Ler action at LEE virulence operons. See the text for details. (A) *LEE2-LEE3* divergent operon pair. (B) *LEE5* operon. Assignment of the region of DNA contact with RNA polymerase (positions -70 to $+20$) was adapted from the three-step model of initiation by *E. coli* RNA polymerase previously described (9). Silencer regulatory sequences (SRS1 and SRS2), which were proposed to bind H-NS, and the putative attenuator regulatory sequence (ARS) (6) are shown. *LEE2* and *LEE5* operon regulatory positions, in relation to the starts of transcription ($+1$), are indicated by purple type, while *LEE3* positions are indicated by blue type. Operon labels are adjacent to their cognate transcriptional start sites. The purple diagonal arrows indicate the directions of transcription for *LEE2* and *LEE5*. ds DNA, double-stranded DNA; RNAP, RNA polymerase.

Our current model of Ler function is that multiple Ler proteins bind to the upstream regulatory regions of both *LEE5* and *LEE2*, disrupting a nucleoprotein complex responsible for the repression of the *LEE5*, as well as both the *LEE2* and *LEE3* operons (Fig. 5). It has been proposed that in the presence of H-NS, *LEE2-LEE3* transcription is repressed by the binding of H-NS to the silencing regulatory sequences, identified previously by genetic analysis (6). Thus, repression requires both upstream and downstream silencing regions (Fig. 5A). By using scanning force microscopy, it was recently demonstrated that H-NS represses the *rmB* P1 promoter by forming a collar-like structure wrapping RNA polymerase in an open complex (11), and some evidence suggests that this type of repression may be a general phenomenon (9). Thus, our model includes RNA polymerase in the nucleoprotein complex, which is disrupted by the binding of Ler to the *LEE2* upstream region, allowing transcription of *LEE2*. It is also possible, however, that RNA polymerase is not part of the repressing nucleoprotein complex and that H-NS simply occludes RNA polymerase binding. We propose that transcription of the *LEE3* operon is also increased due to the absence of the repressing nucleoprotein complex.

As shown in Fig. 5B, *LEE5* is also negatively regulated by H-NS. It is reasonable to propose that *LEE5* transcription is

inhibited by the formation of a nucleoprotein complex similar to that proposed for the *LEE2-LEE3* operon pair. Ler binding to the upstream region of *LEE5* disrupts the H-NS-dependent nucleoprotein complex, allowing an increase in *LEE5* transcription. Thus, our model begins to explain how Ler can bind on only one side of divergent operon pairs to increase transcriptional activity at both promoters, as well as single nondivergent operons which are repressed by the same protein, H-NS.

At *LEE5*, the upstream regulatory region (between positions -198 and -75) is required for both H-NS-dependent repression and Ler-mediated activation. KHTIR3M, the strain containing the 5-bp deletion fragment fused to *lacZ*, which showed significant derepression with no alteration of Ler-mediated activation or Ler binding, provides a unique opportunity to study potential topology constraints required for the nucleoprotein complex. If the five-base deletion exhibits derepression due to the one half-helical turn of DNA difference between the regulatory region and the putative downstream regulatory region of *LEE5*, one could then predict the transcriptional activity of multiple deletion derivatives.

H-NS is a modulator of expression of environmentally regulated genes, particularly those that respond to temperature, osmolarity, and pH (for a review see reference 3), and has been demonstrated to control the expression of virulence-associated genes in several genera, including *Shigella* (8, 51) and *Vibrio* (36, 54). The *proU* operon of *E. coli*, which is activated in response to osmotic stress, is negatively regulated by H-NS binding to sites located both upstream and downstream of the promoter (26), similar to what has been proposed for the *LEE2-LEE3* operon pair (6) and *LEE5*. Experiments performed with a *proU'-lacZ* transcriptional fusion showed that Ler can neither substitute for nor exert a dominant negative effect on H-NS, demonstrating that Ler is distinct from H-NS and suggesting that Ler does not interact directly with H-NS (14). These data, combined with our observation that Ler does not derepress the expression of the cryptic *bgl* operon in the presence of H-NS, demonstrate that Ler is a specific regulator of virulence genes rather than a general antagonist of H-NS. Thus, we concluded that the specific regulator Ler is part of a complex regulatory network controlling the expression of EPEC virulence genes in response to environmental signals inside the human gut.

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