Microbiology (2005), **151**, 1199–1208 DOI 10.1099/mic.0.27734-0

The H-NS protein represses transcription of the *eltAB* operon, which encodes heat-labile enterotoxin in enterotoxigenic *Escherichia coli*, by binding to regions downstream of the promoter

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Received 29 October 2004
Revised 27 December 2004
Accepted 5 January 2005

Heat-labile enterotoxin, a major virulence determinant of enterotoxigenic *Escherichia coli*, is encoded by the *eltAB* operon. To elucidate the molecular mechanism by which the heat-stable nucleoid-structural (H-NS) protein controls transcription of *eltAB*, the authors constructed an *eltAB–lacZ* transcriptional fusion and performed β-galactosidase analysis. The results showed that H-NS protein exerts fivefold repression on transcription from the *eltAB* promoter at 37 °C and 10-fold repression at 22 °C. Two silencer regions that were required for H-NS-mediated repression of *eltAB* expression were identified, both of which were located downstream of the start site of transcription. One silencer was located between +31 and +110, the other between +460 and +556, relative to the start site of transcription, and they worked cooperatively in repression. DNA sequences containing the silencers were predicted to be curved by *in silico* analysis and bound H-NS protein directly *in vitro*. Repression of *eltAB* transcription by H-NS was independent of promoter strength, and the presence of H-NS protein did not affect promoter opening *in vitro*, indicating that repression was achieved by inhibiting promoter clearance or blocking transcription elongation, probably via DNA looping between the two silencers.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of watery, cholera-like diarrhoea in children in less-developed countries and in travellers to those regions (Black, 1993; Rowe *et al.*, 1970). It is also an important animal pathogen of considerable economic significance to the pig industry, amongst others. ETEC strains express one or both of two toxic proteins, known as heat-labile (LT) and heat-stable (ST) enterotoxins (Nataro & Kaper, 1998), which perturb the transport of fluid and electrolytes across the intestinal epithelium. LT is a heterohexameric protein of 84 kDa which contains one A subunit and five identical B subunits (Spangler, 1992; Nataro & Kaper, 1998). The B pentamer binds to the host membrane receptor ganglioside, GM1, after which the A subunit is cleaved within the epithelial cells, where it activates adenylate cyclase, resulting in accumulation of cAMP and the consequent disruption of ion channels across the intestinal lumen (Spangler, 1992; Nataro & Kaper, 1998).

Abbreviations: CT, cholera toxin; DRE, downstream regulatory element; EMSA, electrophoretic mobility shift assay; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; H-NS protein, heat-stable nucleoid-structural protein; LT, heat-labile enterotoxin.

LT is encoded on a 60 kb virulence plasmid termed Ent (Yamamoto *et al.*, 1984). The *eltA* and *eltB* genes encoding the A and B subunits, respectively, share extensive homology with those for cholera toxin (CT; Mekalanos *et al.*, 1983) and appear to form a single transcriptional unit (Yamamoto *et al.*, 1984). Transcriptional regulation of CT has been extensively studied. Expression of the *ctx* gene encoding CT is controlled by a regulatory cascade involving two cytoplasmic membrane pairs, ToxS/ToxR and TcpP/TcpH, and an AraC-like activator, ToxT (DiRita *et al.*, 1991; Higgins *et al.*, 1992; Skorupski & Taylor, 1997; Hase & Mekalanos, 1998; Krukonis *et al.*, 2000). In contrast, little is known about the regulation of the *eltAB* operon. It has been reported that LT transcription is negatively regulated by the heat-stable nucleoid-structural (H-NS) protein at low temperature and that a downstream regulatory element (DRE), which covers the coding region of *eltA*, is involved in H-NS mediated repression (Trachman & Maas, 1998; Trachman & Yasmin, 2004). However, the detailed molecular mechanism by which H-NS controls transcription of the *eltAB* operon is not known.

H-NS is a small (137 aa) but abundant protein (approximately 2×10^4 molecules per cell) (Ussery *et al.*, 1994; Rimsky, 2004; Dorman, 2004). It is a global regulator which

controls the expression of a large number of genes whose products are involved in a wide range of cellular processes (Ussery *et al.*, 1994; Rimsky, 2004; Dorman, 2004). In pathogenic enteric bacteria, such as enteropathogenic *E. coli* (EPEC) (Bustamante *et al.*, 2001; Haack *et al.*, 2003), ETEC (Murphree *et al.*, 1997), enteroinvasive *E. coli* (Falconi *et al.*, 2001), *Shigella flexneri* (Porter & Dorman, 1994) and *Vibrio cholerae* (Nye *et al.*, 2000; Yu & DiRita, 2002), H-NS participates in the regulation of virulence gene expression. In these bacteria, H-NS functions mainly as a negative regulator which represses transcription in response to environmental changes, such as temperature and osmolarity (Atlung & Ingmer, 1997). Unlike most other transcriptional regulatory proteins, H-NS binds to its DNA targets in a sequence-independent fashion, but shows a strong preference for regions of intrinsic curvature (Rimsky *et al.*, 2001). The minimum unit of H-NS protein in solution is a dimer but it exists in various oligomeric states (Badaut *et al.*, 2002; Rimsky, 2004). Structural and functional analyses have shown that H-NS contains two domains separated by a flexible linker. The N-terminal domain (aa 1–65) is responsible for dimerization and the C-terminal domain (aa 90–137) is involved in nucleic acid binding (Ueguchi *et al.*, 1996; Badaut *et al.*, 2002).

In order to understand the molecular biology of H-NS-mediated transcriptional regulation of the *eltAB* operon of ETEC, we carried out a number of genetic and biochemical experiments to map and characterize the *eltAB* promoter and determine the H-NS binding sites which are required for repression. Using in-gel DNase I footprinting, we identified two regions, between +31 and +110 and +460 and +556 relative to the start site of transcription, as silencers that interact directly with the H-NS protein. *In silico* analysis indicated that both silencers correspond to regions that are intrinsically curved. Genetic analysis demonstrated that these silencers are critical for H-NS-mediated repression of *eltAB* expression, suggesting that repression occurs via DNA looping. Data from $KMnO_4$ footprinting indicated that generation of a repressing nucleoprotein complex at the *eltAB* operon inhibits a step(s) following promoter open complex formation.

METHODS

Strains, plasmids, oligonucleotides, reagents and media. The prototype ETEC strain H10407 (Evans *et al.*, 1973) was used as a source of DNA for PCR amplification of various *eltAB* fragments. The *E. coli* K-12 strains used in this study are as follows. JM101 [Δ(*lac–proAB*) *supE thi* F′(*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*ΔM15)] (Messing, 1983) was used for cloning and propagating M13 derivatives. *E. coli* XL-1 Blue MRF′ [Δ(*mcrA*)183 Δ(*mcrCB–hsdSMR–mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)] (Stratagene) was used for oligonucleotide-directed mutagenesis. *E. coli* MC4100 [Δ(*argF–lac*)*U169 rpsL150 relA araD139 flb-5301 deoC1 ptsF25*] (Casadaban, 1976) and *E. coli* PD145 (MC4100 *hns*::Tn*10*) (Dersch *et al.*, 1994) were used as hosts for analysis of the expression of various *eltAB–lacZ* fusions. Plasmids pMU2385 (Praszkier *et al.*, 1992), pUC19 (Vieira & Messing, 1982) and M13tg130 (Kieny *et al.*, 1983) have all been described previously. Oligonucleotides used in this study are listed in Table 1. Luria broth [1 % (w/v) tryptone, 0·5 % (w/v) yeast extract, 171 mM NaCl] was routinely used for growing *E. coli* strains. Trimethoprim was used at a final concentration of 40 μg ml^{-1} in Luria broth. Restriction enzymes and chemicals were purchased commercially. Highly purified RNA polymerase holoenzyme and native H-NS protein used in the *in vitro* experiments were provided by A. Ishihama (Nippon Inst. Biol. Sci., Tokyo, Japan).

Recombinant DNA techniques and oligonucleotide-directed mutagenesis. Standard recombinant DNA procedures were used as described by Sambrook *et al.* (1989). DNA was sequenced with a model 377 DNA sequencer and ABI Big Dye terminators (Perkin-Elmer). *In vitro* mutagenesis with synthetic oligonucleotides was performed on an M13tg130 derivative containing the $eltAB_{1–757}$ fragment using commercially available kits (Amersham and US Biochemical). Mutations were confirmed by DNA sequence analysis.

***β*-Galactosidase assay.** *β*-Galactosidase activity was assayed as described by Miller (1974). Specific activity was expressed in units described therein. The data are the results of at least three independent assays.

Table 1. Oligonucleotides used in this study

Oligo	Sequence	Used for
YJ001	5′TTTGGATCCTAATCAGGTTGCCATGATTC3′	PCR amplification of *eltAB* fragment
YJ003	5′TTTAAGCTTGTCAGCACGGTATAATCTG3′	PCR amplification of *eltAB* fragment and primer extension
YJ004	5′TTTAAGCTTCTGTCCTGCTAAGTGAGCAC3′	PCR amplification of *eltAB* fragment
YJ005	5′TTTAAGCTTGTAACCATCCTCTGCCGGAGC3′	PCR amplification of *eltAB* fragment
YJ017	5′TAAAAAACATGACCCTCATCATGTTG3′	Creating mutation −35 down
YJ018	5′GCATATAGGTCCAACAAAACAAG3′	Creating mutation −10 down
YJ020	5′CCGTGCTGACTCTAGACCCCC3′	PCR amplification of *eltAB* fragment
YJ025	5′CCTCGCATGGATGAACATGATTGACATC3′	Creating mutation ΔUP
YJ027	5′GCATATAGGTTATAATAAACAAGTGG3′	Creating mutation −10 up
YJ030	5′ACTACACATTGGATCCTCGCAT3′	Creating mutation Δ1–95
YJ031	5′GTGCTCACTTAGCAGGACA3′	PCR amplification of *eltAB* fragment
YJ041	5′GATTCTAGACAATCCGGAAAAAGATAACG3′	Creating mutation Δ192–297
YJ046	5′ACTACACATTTTATCCTCGCAT3′	PCR amplification of *eltAB* fragment and $KMnO_4$ footprinting

Primer extension. Primer YJ003 (Table 1) was labelled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega). The labelled primer was co-precipitated with 10 μg total RNA isolated from *E. coli* MC4100 carrying a pUC19 derivative containing either the $eltAB_{1–297}$ or $eltAB_{1–757}$ fragment. Hybridization was carried out at 45 °C for 15 min in 10 μl TE buffer containing 150 mM KCl. Primer extension reactions were started by the addition of 24 μl extension solution [20 mM Tris/HCl (pH 8·4), 10 mM $MgCl_2$, 10 mM DTT and 2 mM dNTPs and 1 U μl^{-1} AMVreverse transcriptase (Promega)] and were carried out at 42 °C for 60 min. Samples were then precipitated and analysed on a sequencing gel.

Construction of various *eltAB–lacZ* transcriptional fusions and deletion mutants. The three *eltAB–lacZ* fusions, $eltAB_{1–297}$–*lacZ*, $eltAB_{1–490}$–*lacZ*, $eltAB_{1–757}$–*lacZ*, were constructed as described below. The three *eltAB* fragments, which carry 297, 490 and 757 bp respectively, were generated by PCR using total cellular DNA isolated from ETEC strain H10407 as template and primers described in Table 1. Each of the PCR fragments, which were flanked by a *Bam*HI site and a *Hin*dIII site, was cloned into M13tg130 and sequenced. The three *eltAB* fragments were excised from the M13tg130 derivatives and cloned into the *Bam*HI and *Hin*dIII sites of the single-copy plasmid pMU2385, to create *eltAB–lacZ* transcriptional fusions.

Deletion mutant Δ1–95 was constructed as follows. A *Bam*HI site between positions 95 and 100 (Fig. 1a) was created by oligonucleotide-directed mutagenesis on M13tg130-$eltAB_{1–757}$ using primer YJ030 (Table 1). The *eltAB* fragment (Δ1–95) lacking the first 95 nt was cloned into pMU2385 to create the $eltAB_{(\Delta 1–95)}$–*lacZ* transcriptional fusion.

Deletion mutant Δ192–297 was constructed as follows. The *Xba*I–*Hin*dIII *eltAB* fragment containing the region between positions 297 and 757 (Fig. 1a) was excised from M13tg130-$eltAB_{1–757}$. A *Bam*HI–*Xba*I *eltAB* fragment containing the region between positions 1 and 192 was generated by PCR using primers YJ001 and YJ041 (Table 1). The two fragments were ligated at the *Xba*I site and the resulting fragment was cloned into the *Bam*HI and *Hin*dIII sites of pMU2385 to create the $eltAB_{(\Delta 192–297)}$–*lacZ* transcriptional fusion.

Electrophoretic mobility shift assay (EMSA) and DNase I footprinting. The ^{32}P-labelled *eltAB* fragments (−79 to +137, +129 to +330 and +313 to +597) used in the EMSA or in-gel

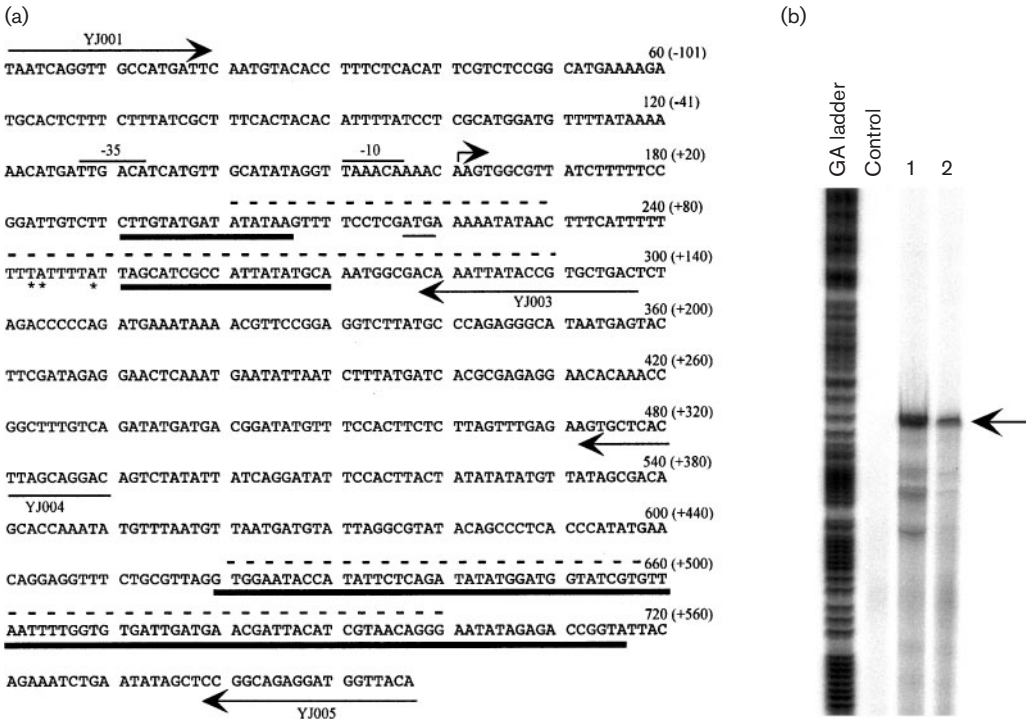


Fig. 1. Sequence of the *eltAB* regulatory region and determination of transcription start site. (a) The transcription start site of the *eltAB* promoter is marked with an angled arrow. The numbers in parentheses represent positions relative to the start site of transcription. The −35 and −10 regions of the *eltAB* promoter are indicated with lines above the sequence. The translation start codon (ATG) for the A subunit of heat-labile enterotoxin is underlined. The regions that are protected by H-NS protein in in-gel DNase I assays are indicated with bold lines beneath the sequence, and the hypersensitive positions revealed at high concentrations of H-NS are indicated with asterisks. The regions predicted to be curved are indicated with dashed lines above the sequence. The regions that are primed by oligonucleotides (YJ001, YJ003, YJ004 and YJ005) for PCR amplification of the various *eltAB* fragments are marked by arrows. (b) Determination of the start site of transcription of the *eltAB* operon by primer extension. *E. coli* MC4100 strains carrying pUC19, or a pUC19 derivative containing the $eltAB_{1–297}$ or $eltAB_{1–757}$ fragment, were grown in Luria broth at 37 °C to mid-exponential phase. Total cellular RNA isolated from each of the strains was hybridized with ^{32}P-labelled primer YJ003. Lanes: control, reaction with RNA from MC4100 carrying pUC19; 1, reaction with RNA from MC4100 carrying a pUC19 derivative containing the $eltAB_{1–297}$ fragment; 2, reaction with RNA from a pUC19 derivative containing the $eltAB_{1–757}$ fragment. The extension product is marked with an arrow.

DNase I footprinting experiments were generated as follows. The oligonucleotide primers YJ046, YJ020 and YJ005 (Table 1) were each labelled with ^{32}P at the 5′ end by using [γ-^{32}P]ATP and T4 polynucleotide kinase. The *eltAB* fragments (−79 to +137, +129 to +330 and +313 to +597) were each amplified by PCR using the ^{32}P-labelled primers, unlabelled primers (^{32}P-YJ046-YJ003, ^{32}P-YJ020-YJ004 and ^{32}P-YJ005-YJ031) (Table 1) and M13tg130-$eltAB_{1–757}$. The amplified *eltAB* fragments were then purified on a native polyacrylamide gel. The reactions for EMSA (25 µl) were carried out in transcription buffer [50 mM Tris/HCl (pH 7·8), 50 mM NaCl, 3 mM magnesium acetate, 0·1 mM EDTA, 0·1 mM DTT and 25 µg BSA ml^{-1}] containing approximately 3 nM end-labelled *eltAB* fragment and 250 or 500 nM purified H-NS protein. The samples were incubated at 22 °C for 20 min. Six microlitres glycerol (40 %) was added to each sample before electrophoresis on 5 % native polyacrylamide gels (37·5 : 1). The polyacrylamide gels and running buffer contained the following: 50 mM Tris base, 50 mM boric acid, 1 mM $MgCl_2$ and 1 % glycerol. Following a pre-run at 4 °C for 1 h at 20 V cm^{-1}, samples were loaded onto the gel and electrophoresis was carried out at 4 °C for approximately 12 h at 10 V cm^{-1}.

The in-gel DNase I assay was performed essentially as described by Yang *et al.* (2004). Gel slices containing various protein–DNA complexes, as well as free DNA fragments from EMSA, were excised from the polyacrylamide gel. The gel slices were each incubated in 10 µl of covering buffer (10 mM Tris/HCl, pH 8·0, 2 mM DTT, 5 % glycerol, 0·5 mg BSA ml^{-1} and 0·4 U DNase I ml^{-1}; Amersham Biosciences) at room temperature for 15 min. Five microlitres starting solution (50 mM $MgCl_2$ and 50 mM NaCl) was added to each sample and the reactions continued for a further 2 min before being terminated by addition of 30 µl stop solution (100 mM EDTA and 2 % SDS). DNA was extracted from the gel slices and analysed on a sequencing gel.

$KMnO_4$ footprinting. A 0·5 µg sample of the linear $eltAB_{1–757}$ fragment carrying the *eltAB* promoter and regulatory elements was incubated in 20 µl *in vitro* transcription buffer [50 mM Tris/HCl (pH 7·8), 50 mM NaCl, 3 mM magnesium acetate, 0·1 mM EDTA, 0·1 mM DTT and 25 µg BSA ml^{-1}] in the presence or absence of H-NS protein at 22 °C for 10 min. RNA polymerase (100 nM) was then added to the reaction mixtures. Following a further incubation at 30 °C for 30 min, to allow open complex formation, the samples were treated with 2·5 µl $KMnO_4$ (80 mM) for 3 min at room temperature. The reaction was quenched with 2 µl β-mercaptoethanol (14·7 M). The DNA was cleaved with piperidine at 90 °C for 20 min. A primer extension experiment using α-^{32}P-labelled oligonucleotide (YJ046) was carried out to probe for the modified bases in the template strand of the open complex. Primer extension reactions were carried out in a Thermal Cycler for 25 cycles, using PCR Mastermix (Promega) and ^{32}P-labelled primer (YJ046). The extension products were analysed on a 6 % denaturing polyacrylamide gel next to the GA track generated by Maxam and Gilbert sequencing.

RESULTS

Identification of a region involved in H-NS-mediated repression of *eltAB* transcription

Analysis of the DNA sequences flanking the *eltAB* operon of a number of clinical ETEC isolates revealed the presence of a conserved region extending from the translation start codon to a position 236 bp further upstream (Schlör *et al.*, 2000). The sequences upstream of this conserved region contain partial insertion sequence elements and vary greatly between different ETEC strains. Therefore any *cis*-acting element which controls *eltAB* transcription should be located within or downstream of this conserved region. To determine the degree of H-NS-mediated repression of *eltAB* transcription, we amplified three DNA fragments by PCR using total cellular DNA isolated from the prototype ETEC strain H10407 as template, and primers based on the previously published sequence of the *etlAB* genes (Yamamoto *et al.*, 1984) (Fig. 1a). All three fragments started at the same point, 217 bp upstream of the start site of translation of the A subunit (Fig. 1a), but varied at their downstream ends. The three fragments, which comprised 297, 490 and 757 bp, respectively, were cloned into pMU2385 to create *eltAB–lacZ* transcriptional fusions. pMU2385 is a single-copy vector which carries a promoterless *lacZ* structural gene with its own translational signals (Yang *et al.*, 2004). Each of the three pMU2385 derivatives, $eltAB_{1–297}$–*lacZ*, $eltAB_{1–490}$–*lacZ* and $eltAB_{1–757}$–*lacZ*, and the control plasmid, pMU2385, were transformed into isogenic Hns^+ or Hns^- *E. coli* K-12 strains, MC4100 (Hns^+, $LacZ^-$) and PD145 (Hns^-, $LacZ^-$) (Casadaban, 1976; Dersch *et al.*, 1994). β-Galactosidase levels were assessed for each of the transformants grown in Luria broth at 37 or 22 °C.

As shown in Fig. 2, at 37 °C, in the Hns^+ background (MC4100), the fusions $eltAB_{1–297}$–*lacZ* and $eltAB_{1–490}$–*lacZ* produced similar levels of β-galactosidase activity of 675 and 791 units, respectively. In contrast, the level of expression from $eltAB_{1–757}$–*lacZ* was only 238 units, about one-third of that of the other two constructs. In the Hns^- background (PD145), the β-galactosidase activities for the fusions $eltAB_{1–297}$–*lacZ* and $eltAB_{1–490}$–*lacZ* were increased 1·3- or 1·6-fold, respectively, relative to the levels in the Hns^+ host. A far more pronounced increase was observed

	Specific activity of β-galactosidase (Miller units)					
	37 °C			22 °C		
	Hns^+	Hns^-	Fold rep.	Hns^+	Hns^-	Fold rep.
Control *lacZ*	0·2	0·2		0·2	0·2	
$eltA_{1-297}$ *lacZ*	675	881	1·3	582	732	1·3
$eltA_{1-490}$ *lacZ*	791	1244	1·6	987	1238	1·3
$eltA_{1-757}$ *lacZ*	238	1162	4·9	101	978	9·7

Fig. 2. Promoter activities of various *eltAB* transcriptional fusions and effects of H-NS and temperature on *eltAB* expression. β-Galactosidase activities are the mean values from three independent assays, with variation <15 %. See Methods for details. Fold repression (Fold rep.) is the specific activity of β-galactosidase of the Hns^- strain (*E. coli* PD145) divided by that of the Hns^+ strain (*E. coli* MC4100).

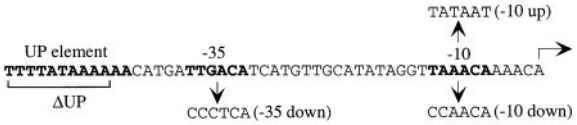
with the construct $eltAB_{1–757}$–*lacZ*, where the β-galactosidase expression was approximately fivefold higher in the Hns^- host than in the Hns^+ host.

At 22 °C, there were no major changes in expression with constructs $eltAB_{1–297}$–*lacZ* and $eltAB_{1–490}$–*lacZ* in either the Hns^+ or the Hns^- background, but the expression of the construct $eltAB_{1–757}$–*lacZ* was only 101 units in the Hns^+ strain compared to 978 units in the Hns^- strain, which represented a 10-fold repression of *eltAB* transcription by H-NS overall. The difference in the levels of expression in the Hns^+ background at 22 °C (101 units) and 37 °C (238 units) indicates a twofold induction at the permissive temperature of 37 °C. The results from this analysis indicated that all three DNA fragments contain the *eltAB* promoter region and that the $eltAB_{1–757}$ fragment carries a region responsible for temperature-mediated repression by H-NS. It was also apparent that an H-NS binding site is located in the structural gene between positions 491 and 757.

Characterization of the *eltAB* promoter

To determine the start site(s) of transcription for the *eltAB* operon, we carried out a primer extension experiment. A ^{32}P-labelled primer was hybridized with 10 μg total RNA isolated from strain MC4100 carrying a pUC19 derivative containing either the $eltAB_{1–297}$ or the $eltAB_{1–757}$ fragment. Following extension using reverse transcriptase in the presence of dNTPs, the samples were analysed on a sequencing gel. Only one major extension product was seen in each sample, indicating the presence of a single promoter for the *eltAB* operon (Fig. 1b). Judged by the intensity of the extended product, more *eltA* transcripts were produced by pUC19-$eltAB_{1–297}$ than by pUC19-$eltAB_{1–757}$, consistent with the observation that $eltAB_{1–757}$ is more repressible by H-NS than $eltAB_{1–297}$. The start site of transcription was mapped to an adenine residue located 56 nt upstream of the start codon for the A-subunit of LT (Fig. 1a). Based on this start site, a putative *eltAB* promoter was identified (Fig. 1a). This contained a perfect −35 region (TTGACA) but had a less conserved −10 region (TAAACA), which were separated by an ideal spacing of 17 bp. In addition, a cluster of 12 AT pairs was found 5 bp upstream of the −35 sequence, which represents a putative UP element of the *eltAB* promoter (Gourse *et al.*, 2000).

In order to confirm the identity of the *eltAB* promoter, mutations were introduced into the putative −10 region, −35 region and UP element of the $eltAB_{1–757}$ fragment by site-directed mutagenesis. The promoter activities of the down mutations (−10 down and −35 down) were reduced to about 5 % of the wild-type level in both the Hns^+ and Hns^- hosts at both 37 and 22 °C (Fig. 3). On the other hand, improving the −10 region by making it into a perfect hexamer (−10 up) resulted in a twofold increase in promoter activity at 37 °C. At 22 °C there was only an approximately 1·5-fold increase in promoter strength with this mutant in the Hns^+ host. Although the genetic changes in these three mutant promoters affected *eltAB* expression, the levels of H-NS-mediated repression remained similar to that of the wild-type.



Specific activity of β-galactosidase (Miller units)

	37 °C			22 °C		
	Hns⁺	Hns⁻	Fold rep.	Hns⁺	Hns⁻	Fold rep.
WT	238	1162	4·9	101	978	9·7
−35 down	11	45	4	6	48	8
−10 down	10	41	4	6	54	9
−10 up	548	2051	3·7	147	930	6·3
ΔUP	226	922	4·1	77	715	9·3

Fig. 3. Effect of mutations in the *eltAB* promoter on transcription and repression by H-NS. The sequence of core promoter elements is shown and the various genetic changes of mutants are marked. β-Galactosidase activities were determined as described in Methods.

To assess the role of the putative UP element in *eltAB* expression, the AT-rich cluster was removed from the $eltAB_{1–757}$ fragment (ΔUP). Functional analysis showed that this mutation had no significant effect either on transcriptional expression or on repression by H-NS (Fig. 3).

Prediction of DNA curvature for the *eltA* gene

The DNA sequence containing the *eltAB* promoter region and the entire *eltA* structural gene was examined *in silico* for the presence of any intrinsic DNA curvature by using the program BEND-IT (http://www.icgeb.org/dna/bend_it.html). As shown in Fig. 4, the sequence spanning the region between positions 1 and 300 or −160 and +140, relative to

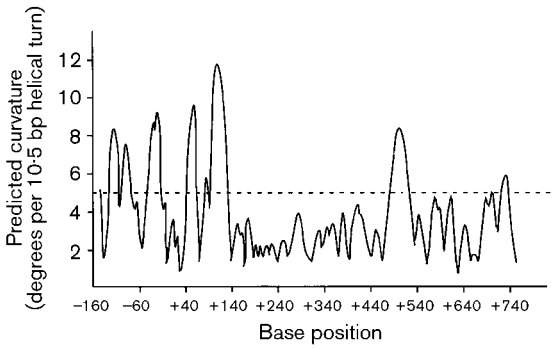


Fig. 4. *In silico* analysis of intrinsic curvature of the *eltA* gene and regulatory region, using the BEND-IT program. The regions with >5 degrees per helical turn of DNA (dashed line) represent curved sequences.

the start site of transcription, which encompasses the *eltAB* promoter and the initially transcribed region, is predicted to contain several curved DNA motifs (with predicted curvature >5 degrees). In addition, a region in the structural gene between positions 600 and 700 on the DNA sequence, or between +440 and +540, relative to the start site of transcription, is also predicted to have intrinsic DNA curvature. This is consistent with the observation that an H-NS binding site, which is critical for temperature-mediated repression, is located at the downstream end of the 757 bp fragment (see above).

Deletion of the region between positions 192 and 297 affects H-NS-mediated repression

The prediction of highly curved sections upstream of the *eltA* fragment, between positions 1 and 300, led us to investigate the possible involvement of this region in H-NS-mediated repression. Two deletion mutants, Δ1–95 and Δ192–297, were constructed. Δ1–95 lacks a region of 95 bp between positions 1 and 95 and Δ192–297 lacks 106 bp between positions 192 and 297 (Fig. 1a). Functional analysis of the mutants showed that removing the region upstream of the *eltAB* promoter (Δ1–95) had no significant effect on H-NS-mediated repression, whereas deleting the region downstream of the start site of transcription (Δ192–297) reduced H-NS-mediated repression from around fivefold at 37 °C and 10-fold at 22 °C to less than twofold at both temperatures (Fig. 5).

EMSA and in-gel DNase I footprinting analysis

To test if H-NS can bind directly to the regions that are essential for *eltAB* repression, we carried out an EMSA. Three ^{32}P end-labelled fragments covering the regions between positions −79 and +137, +129 and +330, and +313 and +597, respectively (Fig. 1a), were generated by PCR. Each of the DNA fragments was incubated with varying amounts of purified H-NS at 22 °C for 30 min and the samples were analysed on native polyacrylamide gels. The H-NS protein formed discrete protein–DNA complexes with fragments −79 to +137 and +313 to +597 but not with fragment +129 to +330 (Fig. 6a). In the EMSA with fragments −79 to +137 and +313 to +597, a second retarded protein–DNA complex (II) was seen at the higher concentration of H-NS (500 nM) (Fig. 6a).

To characterize the H-NS–DNA complexes, each of the retarded bands (I and II), as well as the band containing free DNA (F) (Fig. 6a), was excised from the native polyacrylamide gels, and the gel slices were subjected to limited digestion by DNase I. The samples were then analysed on a sequencing gel. In the case of fragment −79 to +137 (Fig. 6b), two patches of sequence, between positions +31 and +46 and +91 and +110, were protected by H-NS in both complexes I and II. Several hypersensitive sites were detected in complex II, indicating distortion of DNA at higher H-NS concentrations. The in-gel DNase I footprinting with fragment +313 to +597 showed that both complexes I and II consisted of H-NS protein bound to a region between positions +460 and +556 (Fig. 6b). Complex II was formed at a higher H-NS concentration (500 nM) but it had the same protection profile as complex I which was detected at a lower H-NS concentration (Fig. 6b). This suggests that H-NS molecules may be able to pack together during oligomerization on DNA.

H-NS does not inhibit open complex formation at the *eltAB* promoter

To assess the effect of H-NS on promoter opening during transcription initiation of the *eltAB* promoter, we carried out $KMnO_4$ footprinting. The 757 bp linear DNA fragment that contains the *eltAB* promoter and H-NS binding sites was incubated with purified H-NS protein (200 nM, 400 nM and 1000 nM) at 22 °C for 10 min, after which RNA polymerase (100 nM) was added to the reaction. The samples were incubated for a further 25 min at 30 °C to allow the formation of open complexes. Following treatment with $KMnO_4$, primer extension analysis was used to measure the extent of promoter opening. In the presence of RNA polymerase, cleavage of the thymine residues at positions −7, −8 and −9 in the bottom strand was detected (Fig. 7). Addition of H-NS had no effect on promoter opening.

DISCUSSION

In this study, we carried out a series of *in vivo* and *in vitro* experiments to elucidate the molecular mechanism by which H-NS protein controls transcription of the *eltAB* operon encoding LT, a major virulence factor of ETEC. The results from β-galactosidase analysis using a single-copy plasmid carrying an $eltAB_{1-757}$–*lacZ* fusion show that H-NS exerts fivefold repression of transcription from the *eltAB* promoter at 37 °C, and that this repression is increased to 10-fold in response to growth at 22 °C. Deletion analysis demonstrates that efficient repression requires binding of H-NS protein to two regions that are separated by 350 bp.

	Specific activity of β-galactosidase (Miller units)					
	37 °C			22 °C		
	Hns$^+$	Hns$^-$	Fold rep.	Hns$^+$	Hns$^-$	Fold rep.
WT (1 → 757)	238	1162	4·9	101	978	9·7
Δ1-95	170	1065	6·3	72	782	10·7
Δ192-297	486	851	1·8	386	667	1·7

Fig. 5. Effect of deletion mutations on H-NS-mediated repression of *eltAB* transcription. β-Galactosidase activity was determined as described in Methods.

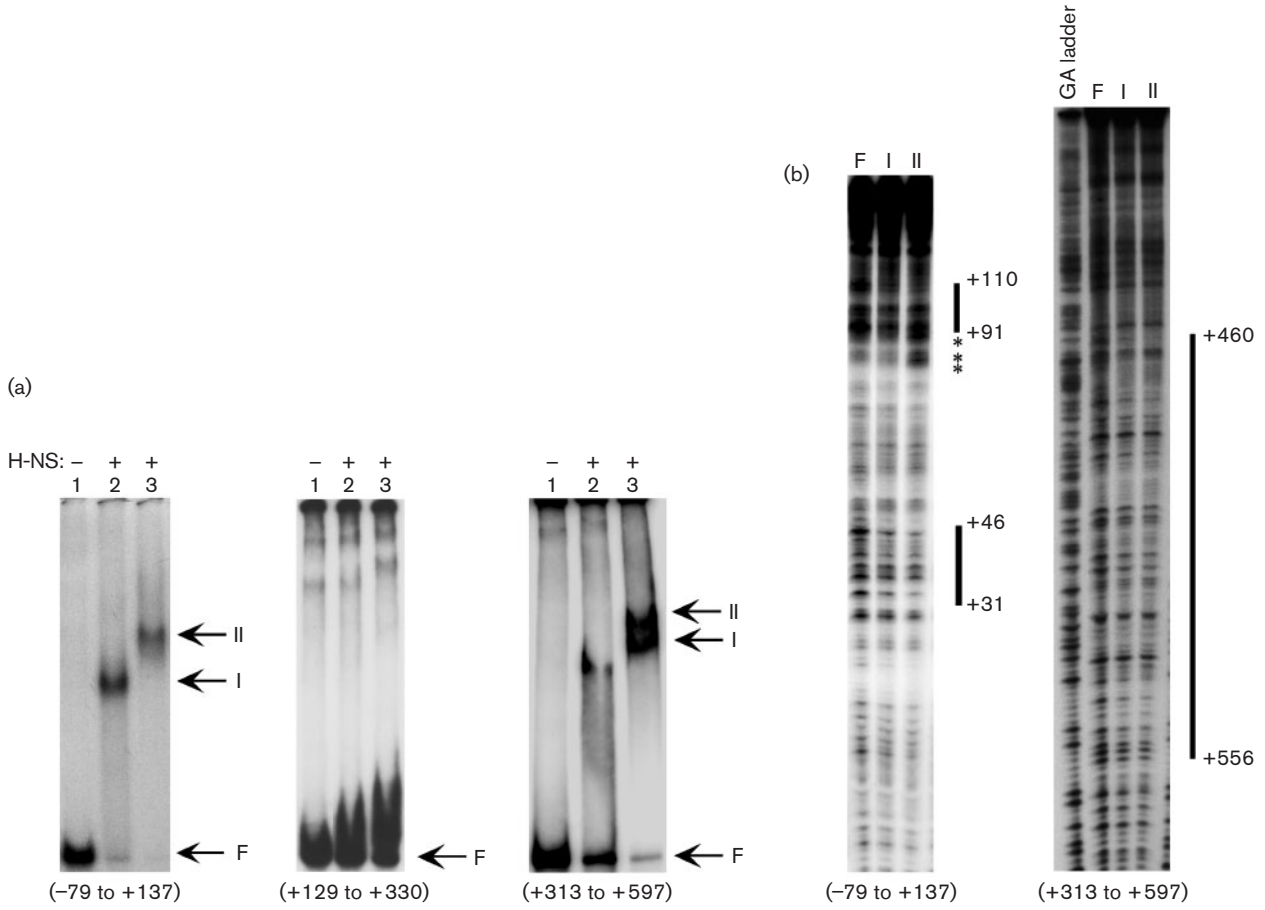


Fig. 6. EMSA and in-gel DNase I footprinting. (a) EMSA analysis of H-NS binding to *eltAB* fragments −79 to +137, +129 to +330 and +313 to +597. EMSA was carried out as described in the Methods, using ^{32}P end-labelled *eltAB* fragments. Lane 1, sample without H-NS; lane 2, sample with 250 nM H-NS; lane 3, sample with 500 nM H-NS. (b) Gel slices containing free DNA (F), H-NS–DNA complex I (I) and H-NS–DNA complex II (II) from the EMSA (a) were treated with DNase I, then analysed on an 8 % sequencing gel. Regions protected by H-NS are indicated by bars. Hypersensitive sites are marked with asterisks.

The results of this analysis correlated well with data obtained from *in silico* analysis, EMSA and in-gel DNase I footprinting of the *eltAB* fragment. The regions that are essential for repression *in vivo* were predicted to contain intrinsic curvature and can interact directly with the H-NS protein.

Both of the H-NS-binding regions were located downstream of the *eltAB* promoter, one between +31 and +110 and the other between +460 and +556, relative to the start site of transcription. These results are in agreement with Trachman & Maas (1998), who showed that deleting the *eltA* structural gene increased LT mRNA synthesis in an Hns$^+$ strain at low temperature. The upstream site contained two patches of approximately 20 bp that were protected by H-NS, whereas the downstream H-NS-binding motif encompassed a single sequence of >90 bp. The presence of multiple H-NS-binding sites over a long distance of DNA occurs in most genes whose expression is repressed by H-NS. The observation that the presence of only one binding region did not bring about significant repression by H-NS, whereas the presence of both regions led to a maximal 10-fold repression, indicates cooperative interactions between the H-NS molecules bound at the two sites. Such interactions could induce DNA loop formation and compact the sequence of the *eltA* structural gene into a nucleoprotein complex, inhibiting transcription by RNA polymerase. The stronger repression observed at 22 °C was probably due to an enhanced DNA curvature, which resulted in increased affinity of H-NS for its binding sites.

In Gram-negative bacteria, H-NS is one of the few repressor proteins that can act by binding to a region located far downstream of the start site of transcription. The *proU* operons from *E. coli* and *Salmonella typhimurium* were the first genes identified with a DRE (Dattananda *et al.*, 1991; Overdier & Csonka, 1992; Lucht *et al.*, 1994). In *S. typhimurium*, the DRE of *proU* is positioned between +73 and +274, relative to the start site of transcription, and is essential for repression by H-NS. Detailed analysis of the mechanism of repression of the *proU* promoter has shown

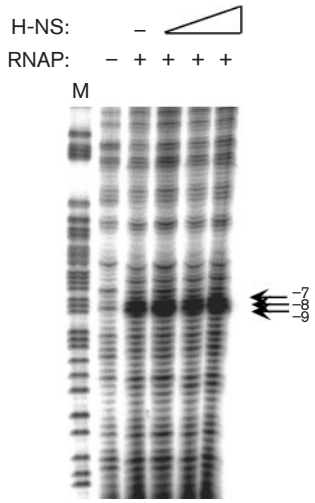


Fig. 7. $KMnO_4$ footprinting analysis of the effect of H-NS on open complex formation of the *eltAB* promoter. Linear template ($eltAB_{1-757}$) was incubated with RNA polymerase (100 nM) in the absence or presence of H-NS (200 nM, 400 nM and 1000 nM). The positions of the DNA template strand which are reactive to $KMnO_4$ are marked with arrows. M, size standards.

that binding of H-NS to DREs inhibits open complex formation by RNA polymerase (Jordi & Higgins, 2000). The *bgl* operon from *E. coli* carries two silencers to which H-NS binds preferentially (Schnetz, 1995; Dole *et al.*, 2004). One silencer is located upstream of the promoter and the other is located 600–700 bp downstream of the start site of transcription (Dole *et al.*, 2004). The two silencers function independently in the repression of *bgl* expression. While binding of H-NS to the upstream silencer represses the *bgl* promoter, presumably by inhibiting transcription initiation, binding of H-NS to the downstream silencer blocks chain elongation during mRNA synthesis by RNA polymerase. In the case of the *eltAB* operon, however, the two H-NS-binding regions appear to work cooperatively rather than additively. In addition, as demonstrated by $KMnO_4$ footprinting, occupation of the two binding sites by H-NS molecules did not affect formation of open complexes by RNA polymerase at the *eltAB* promoter. Mutational studies showed that H-NS-mediated repression is independent of promoter strength, as the repression ratio was essentially the same after altering the sequence of the promoter core elements to reduce or strengthen promoter activity. Together, these observations support the idea that H-NS represses expression of the *eltAB* promoter either by inhibiting later stages of transcription initiation (e.g. promoter clearance) or by aborting elongation of RNA polymerase immediately after transcription initiation.

The *eltAB* operon is encoded by a mobile genetic element on a virulence plasmid and the structural genes for the A- and B-subunits share approximately 80 % DNA and protein sequence homology with those for CT (*ctx*). However, the promoter regions of the two operons have undergone considerable genetic changes during evolution with regard to regulation of transcriptional expression in their respective hosts. Although transcription of *ctx* is also negatively regulated by H-NS, the region responsible for H-NS-mediated repression is situated upstream of the promoter core elements, between −69 and −400 relative to the start site of transcription (Nye *et al.*, 2000; Yu & DiRita, 2002). In the case of the *eltAB* operon, deleting the sequence between −65 and −160 had no effect on H-NS-mediated repression. The different binding locations of the H-NS protein may have resulted from co-evolution or incorporation with other regulatory systems specific for *V. cholerae* and ETEC, and could play a role in fine-tuning the levels of the enterotoxin production under different conditions.

The *eltAB* operon contains a moderately strong promoter, as judged by the levels of β-galactosidase expression from *eltAB–lacZ* fusions in the Hns^- background. The core promoter elements include a perfect −35 region and an optimal spacer of 17 bp, which are ideal for binding of the σ subunit of RNA polymerase to the promoter. In addition, the −10 region and the sequence between the −10 region and the start site of transcription are highly AT-rich, which facilitates promoter opening. All these factors contribute to the strength of the *eltAB* promoter and may explain why the putative AT-rich UP element is dispensable for promoter activity. Given the high intracellular levels of H-NS that vary little during bacterial growth, it is likely that the *eltAB* operon is normally repressed by H-NS when ETEC strains are not in their host environment. This effect would be overcome by ETEC-specific regulatory proteins which, under appropriate environmental conditions, could act as an anti-repressor to alleviate the effect of H-NS on *eltAB* expression. Indeed, several bacterial regulatory proteins are known to relieve H-NS repression by displacing the protein from DNA, e.g. the Fis protein at the *rrnB* P1 promoter in *E. coli* K-12 (Schneider *et al.*, 2003), the ToxT protein at the *ctx* promoter in *Vibrio cholerae* (Yu & DiRita, 2002), the Ler protein at the *LEE2*, *LEE3* and *LEE5* promoters in EPEC (Bustamante *et al.*, 2001; Haack *et al.*, 2003) and the VirF protein at the *virB* promoter in *Shigella flexneri* (Beloin & Dorman, 2003). The discovery of analogous proteins in ETEC would provide further insight into the regulation of enterotoxin production by these bacteria.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian National Health and Medical Research Council. We thank J. Gowrishankar, S. Rimsky, A. Ishihama and J. Pittard for strains, plasmids and proteins used in this study.

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