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Temperature Regulation of Heat-Labile Enterotoxin (LT) Synthesis in *Escherichia coli* Is Mediated by an Interaction of H-NS Protein with the LT A-Subunit DNA

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Protein and mRNA levels of heat-labile enterotoxin (LT) of *Escherichia coli* are highest at 37°C, and they decrease gradually as temperature is decreased. This temperature effect is eliminated in an Hns$^-$ mutant. Deletion of portions of DNA coding for the LT A subunit also results in an increase in LT expression at low temperatures, suggesting that the H-NS protein causes inhibition of transcription at low temperatures by interacting with the LT A-subunit DNA. The region that interacts with H-NS is referred to as the downstream regulatory element (DRE). Plasmids in an *hns* strain from which the DRE has been deleted still produce elevated levels of LT at 18°C, suggesting that intact DRE is not required for transcription from the LT promoter.

A major proportion of infectious diarrhea in humans in third-world countries and in domestic animals worldwide is caused by enterotoxigenic strains of *Escherichia coli* (ETEC strains). ETEC strains carry transmissible plasmids, called Ent plasmids, that encode heat-stable enterotoxin (ST) or heat-labile enterotoxin (LT) or both.

LT is very similar antigenically and pharmacologically to cholera toxin (CT) produced by *Vibrio cholerae*. However, based on the considerable amount of information known about the regulation of CT (23) and from what has been learned about LT regulation, there is no evidence that LT, like CT, is regulated by a two-component regulatory system (26).

LT mRNA and protein levels are significantly affected by temperature but are only slightly affected by some of the other environmental conditions known to strongly influence CT expression (23). When LT is in a native plasmid (8) or when LT is subcloned into vectors, it is optimally expressed at 37°C and its expression decreases as temperature is decreased to 18°C (26).

Recently, much has been reported about the global regulator H-NS with respect to its ability to mediate the response of many operons to environmental changes (2, 28). HN-S mediates the temperature regulation of several virulence factor operons, including the CFA/I fimbriae, CS1 pili, and *pap* pili of *E. coli* and the *virF* and *virB* genes of enteroinvasive *E. coli* and *Shigella* species (10, 12, 18–20, 29). H-NS also mediates osmoregulation of the *proU* operon in *E. coli* and *Salmonella* species (9). Interestingly, it has been shown through extensive genetic analysis that H-NS influences *proU* expression by binding to a downstream regulatory element (the DRE) in the *proU* structural gene (11, 14, 15, 27).

We shall present results obtained with an H-NS mutant that produces a truncated H-NS protein with altered DNA binding capacities (5). Hns$^+$ (GM37) and Hns$^-$ mutant (GM230) strains (9) carrying LT plasmids with various deletions located in the structural gene coding for the LT A subunit or upstream of the −35 and −10 promoter elements were grown at either 37 or 18°C to analyze the role that H-NS plays in mediating temperature control of LT expression.

Plasmid construction. Standard DNA manipulations were carried out as described by Sambrook et al. (21). The plasmid pLT was derived from plasmid pJT2, which contains the entire LT operon on an *Hpa*I-*Bam*HI fragment from pEWD030 (24) subcloned into the *Eco*RV and *Bam*HI sites of pBR322. Previously, we sequenced approximately 725 bp upstream of the LT promoter subcloned from pEWD030 (GenBank accession no. M61015). *Bal*31 mutagenesis and primer extension were performed to characterize the region upstream of the promoter elements and to confirm the precise locations of the promoter elements (26).

The 2,800 bp upstream of the LT mRNA start site from pJT2 was substituted with a fragment containing only 723 bp upstream of the LT mRNA start site. This fragment was isolated from one of the plasmids obtained from *Bal*31 deletion analysis of an LT–β-galactosidase translational fusion construct. Likewise, a second fragment containing only 34 bp upstream of the LT mRNA start site was used to construct pLTΔUCR (26).

pLTΔNC was constructed by excision of a 686-bp *Xba*I-*Eco*RI fragment from pLT, pLTΔN was constructed by excision of a 422-bp *Xba*I-*Age*I fragment from pLT, and pLTΔC was constructed by excision of a 264-bp *Age*I-*Eco*RI fragment from pLT. pLTΔUCRΔNC was constructed by excision of a 686-bp *Xba*I-*Eco*RI fragment from pLTΔUCR. The *Xba*I, *Age*I, and *Eco*RI sites are located 184, 550, and 814 bp downstream of the LT mRNA start site, respectively.

Effect of temperature and H-NS on LT mRNA levels. The promoter activity of the LT gene was measured in primer extension experiments. The segment of LT mRNA chosen for the primer extension experiments is shown in Fig. 1A. It is located between the promoter and the 5′ end of the LT A gene. This location avoids complications that we have previously found to arise in assessing promoter activity by using reporter genes in translational fusion plasmids that encode β-galactosidase or alkaline phosphatase (26).

A variation of the hot phenol procedure (1) was used to isolate RNA from cells grown to mid-log phase (optical density

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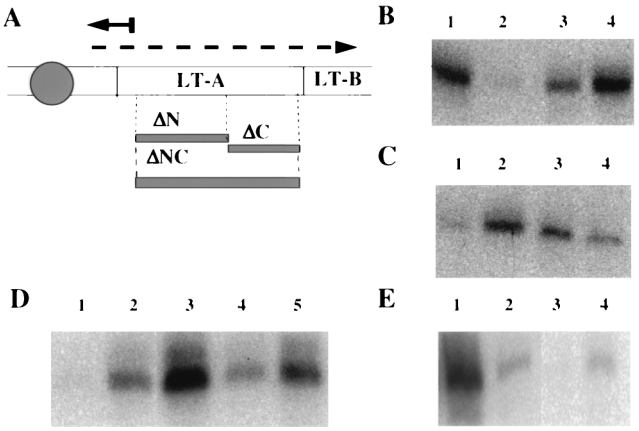

FIG. 1. (A) Schematic representation of the LT operon and location of the DRE deletions with respect to the location of the primer extension product. LT A and LT B code for subunits of LT. Vertical bars, locations of the LT A and B structural genes; gray circle, the promoter region; dashed arrow, the LT mRNA; solid arrow, the LT primer extension product; solid bar, the LT primer; filled-in gray bars, approximate locations of the DRE deletions described in the text. (B) Effect of temperature and H-NS on LT mRNA production. Lanes: 1, *hns*$^+$ pLT at 37°C; 2, *hns*$^+$ pLT at 18°C; 3, *hns* pLT at 37°C; and 4, *hns* pLT at 18°C. (C) Effect of DRE deletions on LT mRNA production in an *hns*$^+$ strain at 18°C. Lanes: 1, *hns*$^+$ pLT; 2, *hns*$^+$ pLTΔNC; 3, *hns*$^+$ pLTΔN; and 4, *hns*$^+$ pLTΔC. (D) Effect of DRE deletions on LT mRNA production in *hns* strains at 18°C. Lanes: 1, *hns*$^+$ pLT; 2, *hns* pLT; 3, *hns* pLTΔNC; 4, *hns* pLTΔN; and 5, *hns* pLTΔC. (E) Effect of UCR deletion or UCR and DRE deletions on LT mRNA production in *hns*$^+$ strains at 37 and at 18°C. Lanes: 1, pLT at 37°C; 2, pLTΔUCR at 37°C; 3, pLTΔUCR at 18°C; and 4, pLTΔUCRΔNC at 18°C. Exposure of the autoradiograph for panel E was five times longer than that for panels B to D.

at 580 nm [OD_{580}] of 0.3 as determined by a model 401A Lumetron colorimeter) in LB broth (17) containing ampicillin (30 μg/ml) at either 37 or 18°C. RNA purity, quantity, and quality were assessed by measuring the OD_{260}/OD_{280} in a Beckman DU-40 spectrophotometer and electrophoresis on a 1.5% agarose gel before use for primer extension.

Primer extension on total RNA (10 μg per sample) was performed under the conditions described by Curtis (3), except that the oligonucleotide 5′-AGTCAGCACGGTATAATCTG-3′ (the binding site on RNA is at positions +119 through +138 relative to the mRNA start site) was end labeled with [γ-^{32}P] dATP by using T4 polynucleotide kinase, and none of the added nucleotides was radioactive. Reaction products were analyzed on a 6% acrylamide-urea gel. The same primer was used for dideoxy chain termination sequencing reactions (with an AmpliCycle Sequencing kit from Perkin-Elmer Corp. and [α-^{33}P]dATP) to serve as a size marker for the extended fragments (data not shown).

Promoter activities of the LT gene were determined at 37 and 18°C in an H-NS mutant and the corresponding wild-type strain. As shown in Fig. 1B, the wild type produces much less mRNA at the lower temperature (lane 2), whereas in the H-NS mutant, mRNA production is not inhibited at the lower temperature (lane 4). We also showed that the mRNA start sites were the same in the wild type and in the Hns$^-$ mutant (Fig. 1B).

To test if the difference seen between the wild type and the mutant was due to differences in plasmid levels, we measured the respective plasmid yields. Plasmid DNA was isolated from 4.5 ml of cultures grown to an OD_{580} of 0.3, linearized, and electrophoresed on a 1% agarose gel which was stained with ethidium bromide. We found plasmid levels to be essentially the same regardless of the growth conditions and host strains (data not shown). We have found this method to be sensitive enough to detect a twofold difference.

Effect of DRE deletions on mRNA levels. To test for the presence of a site that interacts with H-NS in the structural part of the LT gene, we generated the three deletions shown in Fig. 1A. The effect of these deletions on promoter activity at 18°C was tested. As shown in Fig. 1C, the deletions alleviate the inhibition seen in the undeleted plasmid. Two of the plasmids, pLTΔN and pLTΔNC, eliminate the inhibition almost completely. pLTΔC has a weaker effect. We conclude from these experiments that H-NS protein interacts with the region of LT A DNA encoding the N terminus to cause the inhibition, presumably by binding to this region.

We also tested the effect of the three deletions at 18°C in the H-NS mutant. It can be seen (Fig. 1D) that all promoters in the mutant are active (lanes 2, 3, 4, and 5), in contrast to the undeleted plasmid in the H-NS$^+$ strain (lane 1). There is some variability among the plasmids in the mutant. LTΔN has about the same activity as the undeleted plasmid (lanes 2 and 4), whereas activities are stronger in LTΔNC and LTΔC. So far, we have not found the cause for these differences. One possible explanation is that they are due to the H-NS homolog StpA. It has been shown that StpA production is increased in H-NS$^-$ strains (25, 30). Our results indicate that StpA binds to the C-terminal part of LT A and that this leads to inhibition of LT synthesis. However, in contrast to the inhibition by H-NS, this inhibition is not dependent on temperature. If anything, it is stronger at 37 than at 18°C (Fig. 1B).

A similar deletion analysis of the region upstream of the −35 element of the promoter (the upstream control region [UCR]) was performed. Deletion of 300 bp or more immediately upstream of the −35 element of the promoter and not including the promoter elements results in a considerable decrease in LT production at the protein and mRNA levels (only mRNA data shown [Fig. 1E, lane 2]). This effect is also H-NS dependent (data not shown) but is not affected by temperature. Hns$^+$ *E. coli* cells carrying an LT plasmid with deletion of the UCR still manifest an observable decrease in LT protein and mRNA production at low temperatures (only mRNA data shown [Fig. 1E, lane 3]). Furthermore, Hns$^+$ *E. coli* cells carrying a plasmid containing deletions in both the DRE and the UCR do not result in LT levels fully restored to wild-type levels at high temperatures (Fig. 1E, lane 4), suggesting that there is an additional H-NS-sensitive but temperature-independent control region in the UCR. Since the present paper is concerned with temperature control and since the UCR effect is independent of temperature and the interaction of H-NS with the DRE, it will not be considered further.

Effect of temperature and H-NS on LT protein synthesis. In parallel with the above-described studies of mRNA synthesis, we measured LT protein synthesis. Cells were grown in modified K medium containing 171 mM NaCl, Casamino Acids (7), and ampicillin (30 μg/ml) or LB (17) and ampicillin (30 μg/ml) at 18°C and subcultured at either 37 or 18°C. After cultures had reached desired cell densities, polymyxin B (90 μg per ml of culture) (6) and $MgSO_4$ (1.725 mg per ml of culture) were added directly to cell aliquots and were incubated at their respective temperatures until the cells appeared to be completely lysed. Determinations of amounts of protein in the extracts were made with the Bio-Rad system. Cell extracts were assayed by a G_{M1}-enzyme-linked immunosorbent assay under conditions similar to those described by Scotland et al. (22). Microtiter plates were analyzed with a Dynatech MR5000 Microplate Reader at OD_{410}.

The results of experiments with intact LT genes are shown in Table 1. At 37°C, both the H-NS mutant and its wild-type

TABLE 1. Effects of temperature and H-NS mutation on LT expression

Genotype	Strain (plasmid)	Temp (°C)	OD_{410}[a]	
			Mid-log phase[b]	Late-log phase[b]
hns$^+$	GM37(pLT)	37	0.32	0.45
hns$^+$	GM37(pLT)	18	0.13	0.001
hns	GM230(pLT)	37	0.50	0.60
hns	GM230(pLT)	18	0.60	0.40

[a] OD_{410}s measure the amounts of LT protein determined by G_{M1}-ELISA. See text for details.

[b] Sample extracts were derived from the same cultures at different points in the growth curve. Replicate OD_{410}s for each sample extract were obtained. The reported values are the averages of these determinations.

parent produce approximately the same amounts of LT (rows 1 and 3). At 18°C, LT formation is considerably less in the wild type but not in the mutant. These results confirm our findings on mRNA synthesis. It should be noted that the inhibition is greater at the end of growth than during the exponential phase (Table 1, row 2).

In these experiments, the bacteria were grown in a minimal medium (modified K medium) rather than in the rich medium used for the mRNA experiments. This medium was used because it permits LT extraction directly from cells suspended in the culture medium with polymyxin B, thereby allowing us to measure all of the fully and partially assembled LT that had accumulated in the periplasmic space and in the supernatant (6). We also carried out these experiments with the bacteria grown in rich medium and obtained similar results (data not shown).

Effect of DRE deletions on the inhibition of LT protein synthesis. We measured LT protein production in strains carrying plasmids with deletions at 18°C and compared them with the same strain carrying a complete plasmid. We found a restoration of LT formation in the strains with deleted plasmids similar to restoration of mRNA synthesis (Fig. 1C). However, the restoration was only approximately twofold, which is not as great as the restoration of mRNA levels. We believe that this may be due to technical factors, because it is likely that with the deleted LT A subunits, the incomplete toxin molecules gave values in the ELISA, which measures the amount of LT B subunit, that were lower than the values obtained with complete LT molecules (4, 16).

Mechanism of H-NS temperature regulation. It has been shown that H-NS is a member of the cold shock regulon and that its expression increases by three- to fourfold when the bacteria are shifted from 37 to 10°C (13). Here, we have shown that H-NS inhibits LT synthesis at the transcriptional level at low temperatures by interacting with a DNA sequence located at the part of the LT A gene encoding the N terminus, which is referred to as the DRE. The inhibition is exerted at the LT promoter upstream of the LT A gene.

Our results permit us to discriminate between two possible explanations for the inhibitory action of H-NS, with inhibition starting at the promoter or within the DRE. Since the inhibition of mRNA formation extends to the start site of transcription, the inhibition occurs at the promoter rather than in the DRE. Presumably, it prevents the action of RNA polymerase in initiating transcription.

We can also distinguish between an action of H-NS as an antagonist in preventing stimulation of promoter activity by the DRE and a cooperative action with DRE in inhibiting promoter activity. For the first explanation to be correct, DRE must by itself activate the promoter. We have shown that in the H-NS mutant, deletion of the DRE does not diminish mRNA synthesis (Fig. 1D). Therefore, the DRE is not required for activation of transcription but is required for inhibition by H-NS. Just how the interaction between DRE and H-NS brings about inhibition of promoter activity is not known at present. Presumably, H-NS has to bind to DRE in a specific manner to exert its effect. In the similar case of the *proU* operon (11, 14, 15, 27), it has been shown that H-NS also has to bind specifically to a DRE in the first structural gene in order to inhibit promoter activity and that other H-NS binding DNA segments could not replace DRE.

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