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NOTES

H-NS Regulates OmpF Expression through *micF* Antisense RNA in *Escherichia coli*

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H-NS is a major constituent of the *Escherichia coli* nucleoid. Expression of the major outer membrane proteins, OmpC and OmpF, is influenced by *hns* mutations such that OmpC expression increases whereas OmpF expression decreases irrespective of the osmolarity of the medium (K. A. Graeme-Cook, G. May, E. Bremer, and C. F. Higgins, Mol. Microbiol. 3:1287–1294, 1989). In this study we show that the effect of an *hns*::*neo* mutation on OmpF expression is largely diminished in a deletion mutant carrying the *micF* gene that encodes the *ompF* mRNA-specific antisense RNA. In addition, the *micF* transcript levels in the *hns*::*neo* mutation are high compared with transcript levels in wild-type cells. On the basis of these results, we provide evidence for a link between OmpC/OmpF expression and the regulatory function of H-NS. We suggest that H-NS most likely affects OmpC expression directly at the level of transcription, but OmpF expression is indirectly regulated by *micF* antisense RNA.

We have a long-standing interest in the molecular mechanisms by which expression of the *Escherichia coli* outer membrane proteins OmpC and OmpF is regulated in response to various environmental stimuli such as the osmolarity of the medium (reference 14 and references therein). The regulatory factors EnvZ (an osmosensory kinase) and OmpR (a positive regulator) are crucially involved in the osmotic regulation of *ompC* and *ompF*. This EnvZ/OmpR regulatory system is one of the best characterized examples of phosphotransfer signal transduction through bacterial two-component regulatory factors (references 15 and 17 and references therein). Nevertheless, previous studies implicated other regulatory factors, in addition to EnvZ/OmpR, in the complex mechanisms underlying the expression of OmpC and OmpF. These factors include *micF* RNA (an antisense RNA for *ompF* mRNA) (12, 13), Lrp (a global regulator; leucine-responsive regulatory protein) (4), H-NS (a global regulator; nucleoid protein) (6), and SoxRS (positive regulators involved in the oxidative stress response) (3). For example, the 93-nucleotide *micF* antisense RNA was shown to regulate the level of OmpF in the outer membrane in response to temperature and other conditions by decreasing the level of *ompF* mRNA, presumably through a specific hybridization between them (1, 2). However, the complex mechanisms of the expression of OmpC and OmpF are not yet fully understood.

We have also studied the structure and function of H-NS (references 20 and 21, and references therein). This protein is a major constituent of the *E. coli* nucleoid (8). On the basis of recent genetic studies, it is clear that H-NS influences transcription of a number of apparently unlinked genes on the chromosome (reference 8 and references therein), although its underlying mechanism remains elusive. As mentioned above, the production profiles of OmpC and OmpF in the outer membrane are also markedly affected in a genetic background carrying an *hns* mutation (6). Here we wanted to elucidate this particular problem and provide evidence that H-NS affects the production of OmpF by regulating the level of *micF* RNA.

An *hns*::*neo* mutation results in altered expression of OmpC and OmpF. Figure 1A shows typical osmoregulatory profiles of OmpC and OmpF in the outer membrane. The wild-type cells (CSH26) were grown either in low- or high-osmolarity medium and then analyzed by urea-sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (lanes 1 and 2). When the profile was examined for an *hns*::*neo* derivative (CU211), however, expression of OmpC increased whereas that of OmpF decreased regardless of the medium osmolarity (lanes 3 and 4). Therefore, H-NS appears to affect the expression of both OmpC and OmpF somehow but in different directions. This is consistent with the previous observation by Graeme-Cook et al. (6). On the basis of their analyses of strains MH225 (*ompC-lacZ*) and MH513 (*ompF-lacZ*), these researchers suggested that the effect of H-NS on both OmpC and OmpF is at the level of transcription. If so, one can envisage that H-NS affects *ompC* transcription negatively but affects *ompF* transcription positively. However, it is well documented that H-NS influences transcription of a number of genes mainly in a negative fashion. Is the *ompF* gene a rare exception? In this respect, it may also be noted that the structures of the *ompC-lacZ* and *ompF-lacZ* fusion genes in MH225 and MH513 are not precisely known, since they were constructed by a classical method with λpl(209) (7). This prompted us to reexamine in more detail the intriguing effect of H-NS on OmpC and OmpF.

To address the issue, we first constructed CSH26 derivatives carrying either an *ompC-lacZ* or *ompF-lacZ* transcriptional fusion gene on the chromosome (strains TM2 and TM3, respectively), whose promoter structures are well-defined (Fig. 1B). A set of derivatives of TM2 and TM3, each carrying an *hns*::*neo* or Δ*envZ*/Δ*ompR* mutation, were also constructed (TM7 and TM8 from TM2; TM10 and TM11 from TM3). These strains were assayed for β-galactosidase activity after they were grown in either low- or high-osmolarity medium (Fig. 1C). Levels of the *ompC-lacZ* expression increased sig-

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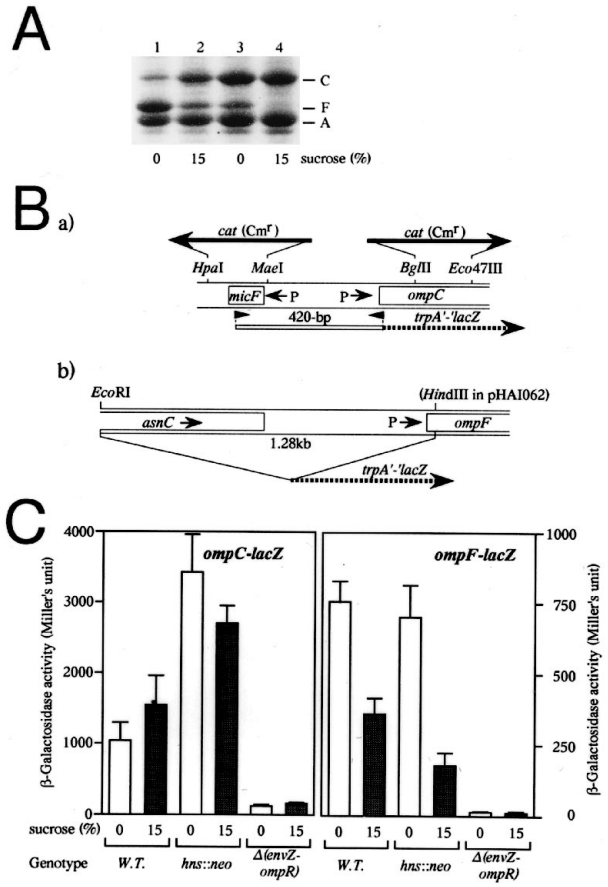

FIG. 1. Effect of an *hns* mutation on expression of OmpC and OmpF. (A) Urea-SDS polyacrylamide gel showing the profiles of outer membrane proteins. Strains CSH26 (wild type) (lanes 1 and 2) and CU211 (*hns*::*neo*) (lanes 3 and 4) were grown to the mid-logarithmic phase in medium A containing the indicated concentrations of sucrose (18). Outer membrane proteins were prepared and analyzed by urea-SDS polyacrylamide gel electrophoresis (12). C, OmpC; F, OmpF; A, OmpA. (B) Schematic representation of the promoter-*lacZ* operon fusions (*ompC-lacZ* and *ompF-lacZ*) and the deletion mutations (*ompC*::*cat* and *micF*::*cat*), which were constructed and used in this study. Strains carrying each promoter-*lacZ* fusion on the chromosome (TM2 carrying *ompC-lacZ*, TM3 carrying *ompF-lacZ*) were constructed by the conventional method described by Hirano et al. (9). Strains carrying each deletion mutation (TM27 carrying *ompC*::*cat*, CU270 carrying *micF*::*cat*) were constructed by the conventional method described by Russell et al. (16), respectively. Note that arrowheads represent PCR primers used for amplification of the 420-bp segment carrying both the *ompC* and *micF* promoters (see Fig. 4). (C) β-Galactosidase activity expressed by cells with the *ompC-lacZ* and *ompF-lacZ* operon fusions in appropriate genetic backgrounds. Cells carrying the respective fusion genes were grown in medium A (open bars) or medium A containing 15% sucrose (shaded bars). β-Galactosidase activities were measured, as described previously (18). Each value is the mean ± standard deviation of four independent assays. *W.T.*, wild type.

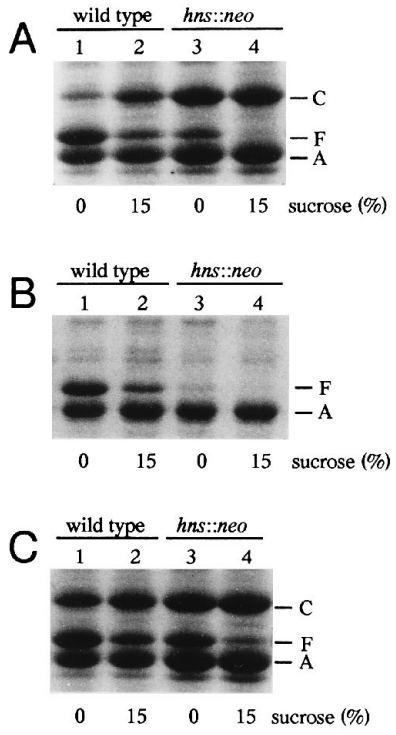

FIG. 2. Effects of the *ompC* and *micF* deletion mutations on OmpF expression. Osmoregulatory profiles of OmpC and OmpF expression in the outer membrane were examined by urea-SDS polyacrylamide gel electrophoresis in the various genetic backgrounds, as indicated (wild type with respect to both *ompC* and *micF* [A]; *ompC*::*cat* [B]; *micF*::*cat* [C]). The outer membranes were prepared and analyzed as described in the legend to Fig. 1A. The strains used are CSH26 and CU211 (*hns*::*neo*) (A), TM27 (*ompC*::*cat*) and TM29 (*ompC*::*cat* and *hns*::*neo*) (B), and CU270 (*micF*::*cat*) and CU271 (*micF*::*cat* and *hns*::*neo*) (C). C, OmpC; F, OmpF; A, OmpA.

nificantly in the *hns*::*neo* background, irrespective of the medium osmolarity. This is consistent with the observation by Graeme-Cook et al. (6). However, the effect of the *hns* lesion on the *ompF-lacZ* expression was not evident. The latter observation for the *ompF-lacZ* fusion gene is not fully consistent with that reported by Graeme-Cook et al. (6). These results suggest that OmpC expression is indeed regulated negatively by H-NS at the level of transcription but that the effect of H-NS on OmpF expression appears to be somewhat complicated.

H-NS influences OmpF expression through the function of *micF* RNA. We then supposed that H-NS may affect *ompC* transcription primarily, and this may in turn influence OmpF expression through a posttranscriptional or translational mechanism (e.g., competition for assembly into the outer membrane). We thus needed to examine the effect of H-NS in a Δ*ompC* background and so constructed such mutant derivatives of CSH26 and CU211 (Fig. 1B). The profiles of their outer membrane proteins are shown in Fig. 2B. The results show that OmpF expression is affected by the *hns*::*neo* mutation even in the Δ*ompC* background. The simple idea, described above, was thus dismissed. Here it should be recalled that the *micF* gene, which is located upstream of the *ompC* gene (Fig. 1B), is involved in the regulation of OmpF expression. Considering the fact that the *micF* RNA functions as a repressor for OmpF expression, we examined the effect of H-NS in a Δ*micF* background. Figure 2C shows that the effect of H-NS on OmpF expression is largely, if not completely, diminished in the Δ*micF* background compared with that in the wild-type background (Fig. 2A). In the Δ*micF* background, the level of OmpF revived to near the wild-type level (Fig. 2C). This observation is compatible with the idea that H-NS may affect the level of *micF* RNA primarily and thereby may influence OmpF expression. In other words, the *hns*::*neo* lesion may result in derepression of the expression of *micF* as well as of *ompC*.

The level of *micF* RNA increases in the *hns*::*neo* background. To examine if the production of *micF* transcript is influenced by the *hns*::*neo* lesion, we directly measured the level of *micF* RNA by primer extension analysis, which was designed to be

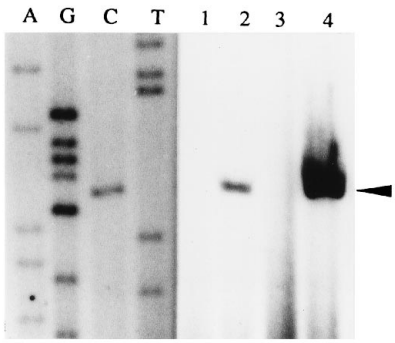

FIG. 3. Expression of *micF* RNA. Primer extension analysis of the *micF* gene product was carried out. The following strains were analyzed: CSH26 (lane 1), CU211 (*hns*::*neo*) (lane 2), CU270 (*micF*::*cat*) (lane 3), and CSH26 harboring pMAN055 (a high-copy-number plasmid carrying the *micF* gene) (lane 4). They were grown to the mid-logarithmic phase in medium A, and then total RNAs were isolated, as described previously (21). The level of *micF* RNA was examined by primer extension analysis with an oligonucleotide primer which specifically hybridize to *micF* RNA. The arrowhead indicates primer extension products. Products of sequencing reaction with the same primer were also electrophoresed together at the left-hand side of the gel.

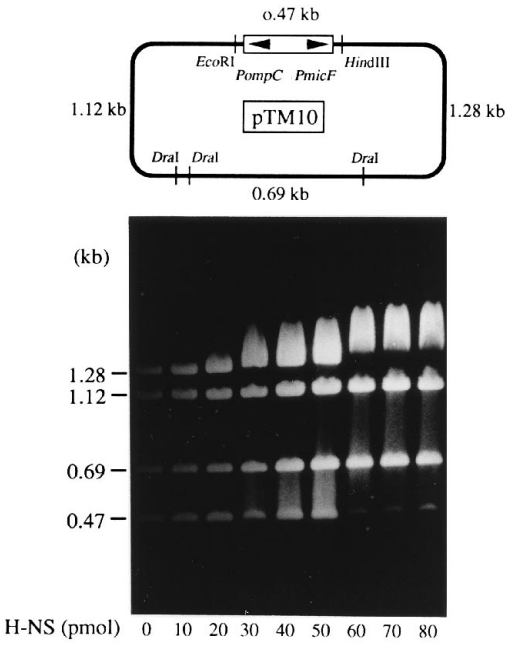

FIG. 4. Competitive gel shift analysis with H-NS and the DNA fragment encompassing the region of both the *ompC* and *micF* promoters. Plasmid pTM10 (a derivative of pUC118) carrying the 420-bp region encompassing both the *ompC* and *micF* promoters on the multicloning site was constructed (Fig. 1B). This plasmid was digested with *Eco*RI, *Hin*dIII, and *Dra*I to yield a mixture of DNA fragments which consists of four DNA fragments derived from the vector as well as the 420-bp *ompC/micF* promoter fragment. This mixture of DNA fragments (total, 1 μg) was directly used for a DNA-binding assay with the purified H-NS protein. The H-NS protein was purified as described previously (20). After incubation of the DNA fragments with the indicated amount of H-NS for 30 min at 37°C, they were applied to 1.2% agarose gels to detect the presumed protein-DNA complexes by electrophoresis, followed by ethidium bromide staining. In this particular experiment, note that the plasmid DNAs were used as competitive internal references.

specific for natural *micF* RNA (Fig. 3). The result showed that the level of *micF* RNA increases significantly in the *hns*::*neo* background (lane 2). To confirm that this primer extension product is indeed *micF* RNA, the Δ*micF* strain and the cells carrying the *micF* gene on a multicopy plasmid were also analyzed for the production of *micF* transcript (lanes 3 and 4). These results indicated that *micF* transcript is accumulated in the *hns*::*neo* mutant to high levels. The stability of *micF* RNA in the *hns*::*neo* cells was then needed to compare with that in the wild-type cells (RNAs were isolated at intervals from rifampin-treated cells and then subjected to the primer extension analysis). However, the stability of *micF* RNA in the *hns*::*neo* cells was more or less the same as that in the wild-type cells (data not shown). Taking all these results together (Fig. 2 and 3), we would like to propose that H-NS influences indirectly OmpF expression by regulating the expression of *micF* antisense RNA at the level of transcription.

H-NS binds preferentially to a DNA region encompassing the *ompC* and *micF* promoters. Finally, we asked how does H-NS influence both *ompC* and *micF* expression. One of the current views of the mode of the regulatory function of H-NS is that this relatively nonspecific DNA-binding protein preferentially recognizes a certain set of genes and functions as a global transcriptional repressor (20). To examine this possibility in the case of *ompC* and *micF*, a 420-bp sequence encompassing both the *ompC* and *micF* promoters was cloned in a vector (Fig. 4). This plasmid was digested with *Eco*RI, *Hin*dIII, and *Dra*I into five discrete pieces (drawing at top of Fig. 4). These DNA segments were used as probes for an in vitro DNA-binding gel shift assay with H-NS (Fig. 4). The result showed a highly preferential binding of H-NS to the *Hin*dIII-*Eco*RI 0.47-kb fragment encompassing the 420-bp *ompC* and *micF* sequences. Note also that H-NS binds preferentially to the largest fragment encompassing the *bla* region of pBR322, as reported previously (11). The result supported the view that H-NS may directly bind to a region around the *ompC* and *micF* promoters, thereby influencing expression of these genes negatively. This view is consistent with our previous observation that there is a *cis*-acting sequence upstream of the *micF* promoter, which affects *micF* expression not only in a negative fashion but also in an OmpR-independent manner (18).

Concluding remarks. Besides the main regulators (EnvZ/OmpR), other regulatory factors have been implicated in the complex OmpC/OmpF regulatory circuit. For example, *micF* antisense RNA was proposed to play an important role for OmpC/OmpF expression in response to medium osmolarity (12), temperature (2), and oxidative stress (3). On the other hand, H-NS has been demonstrated to act as a transcriptional regulator for a variety of unlinked genes (8). Most of the genes are known to be regulated by various environmental stimuli such as osmolarity (e.g., *proU*) (10), temperature (e.g., *pap*) (5), pH (e.g., *gad*) (22), and host infection (e.g., *virB*) (19). Here we provide evidence for a link between OmpC/OmpF expression and H-NS function, by suggesting that H-NS most likely affects OmpC expression at the level of transcription and influences OmpF expression indirectly by affecting the production of *micF* antisense RNA. In this mechanism, H-NS appears to bind to the region encompassing both the *ompC* and *micF* promoters. It is also worth mentioning that a scenario similar to that proposed here for H-NS was recently proposed for another global regulator, Lrp (4). Lrp also appears to influence the OmpF expression through its negative effect on the expression of *micF*. In any event, in considering the current view of the regulatory function of H-NS (8, 20), one can envisage the following physiological relevance of our finding. That is, although normal osmoregulation is governed mainly by the EnvZ/OmpR system, H-NS may be capable of integrating the effects of other environmental stimuli (e.g., temperature and

host infection) into this complex circuitry of the regulation of OmpC/OmpF expression in order to adjust the levels of these outer membrane proteins very sensitively in response to other aspects of *E. coli* physiology. In short, H-NS (perhaps together with Lrp) and *micF* RNA can be implicated as important auxiliary regulators for OmpC/OmpF expression under the more stressful natural habitat of this bacterium.

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