Vol. 181, No. 3

Phenotypic Analysis of Random *hns* Mutations Differentiate DNA-Binding Activity from Properties of *fimA* Promoter Inversion Modulation and Bacterial Motility

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H-NS is a major *Escherichia coli* nucleoid-associated protein involved in bacterial DNA condensation and global modulation of gene expression. This protein exists in cells as at least two different isoforms separable by isoelectric focusing. Among other phenotypes, mutations in *hns* result in constitutive expression of the *proU* and *fimB* genes, increased *fimA* promoter inversion rates, and repression of the *flhCD* master operon required for flagellum biosynthesis. To understand the relationship between H-NS structure and function, we transformed a cloned *hns* gene into a mutator strain and collected a series of mutant alleles that failed to repress *proU* expression. Each of these isolated *hns* mutant alleles also failed to repress *fimB* expression, suggesting that H-NS-specific repression of *proU* and *fimB* occurs by similar mechanisms. Conversely, alleles encoding single amino acid substitutions in the C-terminal DNA-binding domain of H-NS resulted in significantly reduced affinity for DNA yet conferred a wild-type *fimA* promoter inversion frequency, indicating that the mechanism of H-NS activity in modulating promoter inversion is independent of DNA binding. Furthermore, two specific H-NS amino acid substitutions resulted in hypermotile bacteria, while C-terminal H-NS truncations exhibited reduced motility. We also analyzed H-NS isoform composition expressed by various *hns* mutations and found that the N-terminal 67 amino acids were sufficient to support posttranslational modification and that substitutions at positions 18 and 26 resulted in the expression of a single H-NS isoform. These results are discussed in terms of H-NS domain organization and implications for biological activity.

H-NS is a major component of the *Escherichia coli* nucleoid originally isolated by its ability to bind and compact chromosomal DNA (10, 34, 35, 44). This small (15.4 kDa), abundant (20,000 copies/cell), neutral (pI, ~7.5), heat-stable protein binds avidly to double-stranded (ds) DNA with higher affinity for curved DNA substrates (30, 37, 46). H-NS is a global modulator of gene expression affecting the synthesis, both positively and negatively, of more than 50 *E. coli* proteins (3, 22, 47). In addition, H-NS is also involved in regulating expression of virulence-associated genes in pathogenic *Shigella* spp. (25, 31), *Salmonella* spp. (15), and enteroinvasive *E. coli* (5, 32).

In the best-characterized H-NS-sensitive systems, such as *proU* (16, 26, 40), *fimB* (9), *bgl* (6), and *pap* (14), gene expression is derepressed in *hns* insertion mutant strains. Often H-NS acts as a transcriptional repressor binding to intrinsically curved DNA sequences located near prokaryotic promoters (37). In solution, H-NS acts as a homodimer and can form higher-order oligomers at increased protein concentrations (12, 34). Multimerization has recently been shown to be critical for H-NS repressor function at both the *proU* and *hns* promoters (36, 41).

In light of the fact that H-NS is found in a cross-section of bacterial species (43) and is part of global regulatory networks involved in virulence, metabolism, and environmental stress, yet contains no known structural peptide motifs (such as zinc finger or helix-turn-helix motifs), we randomly mutagenized *hns* to help delineate biologically active H-NS domains. To clarify protein structure and function, we examined H-NS isoform composition and the action of H-NS in the processes of piliation and motility, as well as the more frequently studied roles of DNA binding and gene repression.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, genetic techniques, and enzyme assays. The bacterial strains and plasmids used in this study are listed in Table 1. H-NS mutant derivatives are listed in Table 2. Media consisted of Luria-Bertani (LB) broth, LB agar, and MacConkey agar (Difco, Detroit, Mich.). When used, antibiotics were added to a final concentration of 100 μg (ampicillin) or 20 μg (tetracycline or chloramphenicol) per ml of medium. Restriction and other DNA-modifying enzymes were used as instructed by the manufacturers (Gibco-BRL, Gaithersburg, Md.; New England Biolabs, Beverly, Mass.; and Boehringer Mannheim, Indianapolis, Ind.). β-Galactosidase assays were performed as described elsewhere (27) with strains grown in LB broth.

Plasmid construction and *hns* mutagenesis. Plasmid pTHK116-7 was generated to serve as the *hns* template for the mutagenesis as follows. Plasmid pTHK116 was linearized by *Eco*RI digestion, blunt-ended with T4 DNA polymerase, and ligated to *Bam*HI linkers (Stratagene, La Jolla, Calif.). The 1.8-kb *hns* fragment was released by *Bam*HI-*Sal*I digestion, gel purified (Qiagen, Chatsworth, Calif.), and cloned into the *Bam*HI-*Sal*I sites of pACYC184. The ligation reaction was transformed into DH5α, and colonies were selected on chloramphenicol plates. DNA from Cm^r Tet^s transformants was subsequently verified by PCR screening and restriction digestion.

The *hns* gene was randomly mutated by transforming and propagating pTHK116-7 in the *mutD mutS mutT* strain XL1-Red as suggested by the manufacturer (Stratagene). Plasmid DNA was purified (Qiagen) from Cm^r transformants, retransformed into the indicator strain THK62, and plated on lactose MacConkey medium. Plasmid DNA from these strains was verified to contain *hns* by *Hpa*I digestion and sequenced to determine the mutations with forward 5′ CAG TCC TGC TCG CTT CGC 3′ and reverse 5′ GGT GTT ATC CAC GAA ACG GC 3′ primers.

Motility, gel shift assays, and protein purifications. Motility was assayed by measuring swarm diameters as previously described (8) except measurements were taken at only one time point. Gel shift assays and H-NS purifications were done as previously described (9) except the mutant protein lysate (T108I) was washed from the ds-DNA-cellulose column with a 125 mM NaCl solution.

Two-dimensional gel electrophoresis. Whole-cell lysates were prepared by using a French pressure cell as previously described (9). Samples were dissolved in O'Farrell's solution (28) containing 1.5% pH 5 to 7 and 0.5% pH 3 to 10

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TABLE 1. Bacteria and plasmids used

Strain or plasmid	Description	Reference or source
Bacterial strain		
THK38	*thr-1 leuB thi-1 Δ(argF-lac)U169 malA1 xyl-7 ara-13 mtl-2 gal-6 rpsL fhuA2 supE44 hns⁺*, linked to *tetR*	18
THK62	THK38 except Φ(*proU'-lacZYA-kan*) *hns2-tetR*	18
ORN116	THK38 except Φ(*fimA'-lacZYA-kan*) *hns-1*	29
AL90	MG1655 Δ*lacZYA hns2-tetR fimB-lacZYA*	9
AL29	ORN116 except *hns2-tetR*	29
XL1-Red	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn10(Tetʳ)	Stratagene
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI�q ZΔM15* Tn10(Tetʳ)]	Stratagene
Plasmids		
pACYC184	Low-copy-number cloning vector, Tetʳ, Cmʳ	New England Biolabs
pTHK116	pBR322 with wild-type *hns* gene under control of *hns-1* promoter mutation, Ampʳ	19
pTHK116-7	pACYC184 with 1.8-kb *Eco*RI-*Sal*I *hns* encompassing fragment from pTHK116	This study

ampholytes (Bio-Rad, Hercules, Calif.) and focused in the first dimension in tube gels according to the manufacturer's instructions (Hoefer Scientific Instruments, San Francisco, Calif.). After isoelectric focusing (IEF), proteins in the extracted tubes were separated in the second dimension by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with H-NS antiserum, and detected as described previously (9). Some filters were probed with H-NS antiserum preadsorbed against an *hns2 tetR* strain in order to eliminate cross-reactivity to non-H-NS proteins.

RESULTS

Isolation of *hns* mutations. H-NS is a global regulator of a variety of unlinked and unrelated genes in *E. coli* and *Salmonella typhimurium* (2, 43). Mutations in *hns* are pleiotropic, affecting the synthesis of a vast array of gene products involved in numerous biological processes (3, 22, 47). In an attempt to define and correlate separate H-NS domains with different biological activities, we subjected *hns* to a random mutagenesis scheme followed by an in vivo genetic screen to isolate a series of *hns* mutants.

We constructed plasmid pTHK116-7 (Table 1) to serve as the low-level *hns*-expressing template for the mutagenesis in order to avoid the potentially deleterious effects of overexpressing mutant *hns* protein products in the cell (35). This clone carried the wild-type *hns* gene driven by the down-regulated *hns-1* promoter mutation (19) in the low-copy-number vector pACYC184. Plasmid pTHK116-7 was transformed into a triple-repair-deficient *E. coli* mutator strain (XL1-Red) to generate spontaneous, random mutations. Approximately 100 antibiotic-resistant colonies were picked, and plasmid DNA was purified from each of these transformants and individually retransformed into strain THK62 carrying a chromosomal *hns2-tetR* insertion mutation and *proU'-lacZ* transcriptional fusion (Table 1). H-NS is a direct transcriptional repressor of *proU* (16, 23, 40), and expression is phenotypically detectable in strains with *proU-lacZ* fusions on lactose MacConkey indicator medium. Thus, our strategy was to select for mutations that were unable to complement the *hns2-tetR* mutation and therefore represented the derepression of *proU* expression (Lac⁺, red colony phenotype). Seventy-one red THK62-based

transformants from lactose MacConkey plates were chosen as putative *hns* mutant candidates.

To ensure that these potential *hns* mutations encoded stable proteins and to study point mutations rather than large gene deletions, we probed mutant strains with anti-H-NS antiserum in Western blots (data not shown). H-NS from 29 of 71 strains reacted with anti-H-NS antibody with 25 isolates producing apparently full-length protein on SDS-PAGE. We transformed plasmids representing 17 *hns* isolates into a clean background strain (XL1-Blue) and sequenced the DNA. We found seven independent missense and five nonsense mutations, which all maintained the original pTHK116-7 promoter sequence. The remaining five isolates expressed duplicate *hns* mutations. Each missense mutation encoded inferred single amino acid substitutions while the nonsense mutations resulted in C-terminally truncated forms of H-NS (Table 2).

Effects of *hns* mutations on gene expression. We measured β-galactosidase activity (27) from THK62 carrying each *hns* mutant allele to quantitate the effects of the mutations on *proU* expression (Fig. 1A). As expected, *proU-lacZ* expression increased significantly in all *hns* mutant strains tested, albeit to various degrees. We also examined the effects of these mutations on *fimB* expression by using a *fimB-lacZ* fusion strain (AL90). We have recently shown that H-NS acts as a repressor of *fimB* expression by directly binding to the promoter region and inhibiting transcription (9). As with *proU*, *fimB-lacZ* expression was 6- to 30-fold higher in the *hns* mutant strains relative to that of the wild-type *hns*-expressing strain (Fig. 1B). Similar trends develop if one compares the level of derepression of both promoters over the course of each *hns* mutation. That is, each *hns* mutation affected *proU* and *fimB* expression in a similar fashion. For example, *hnsL26P* greatly increased expression whereas *hnsT108I* and *hnsA18E* resulted in the least severe mutant phenotypes at both promoter fusions. We concluded that (i) all of the *hns* mutations isolated induced *proU* and *fimB* expression, (ii) these mutants were defective in their repression ability, and (iii) H-NS probably employs similar repression mechanisms at each promoter.

Effects of *hns* mutations on *fimA* promoter inversion. In order to sort our series of *hns* mutants into separate phenotypic classes we tested their effects on *fimA* promoter inversion. Type 1 pilus expression is controlled transcriptionally by the inversion of a DNA element encompassing the *fimA* promoter

TABLE 2. Sequence analysis of *hns* mutations

H-NS derivative[a]	Codon change or alteration	No. of isolates
M1I	ATG→ATA	1
A18E	GCA→GAA	2
L26P	CTG→CCG	2
L65P	CTG→CCG	1
R93H	CGT→CAT	1
T108I	ACC→ATC	1
G111S	GGC→AGC	2
N67	1-bp insertion causing frameshift and new sequence encoding 64MLIA67→64NADR67 before early stop codon	1
N82	AAA→TAA stop codon	1
N93	CCG→TGA stop codon	1
N123	2-bp deletion causing frameshift and new sequence encoding 122AM→122DG before early stop codon	1
N124	1-bp insertion causing frameshift and new sequence encoding 122AMD→122SNG before early stop codon	3

[a] In order to maintain consistency with previously published mutants (41, 42, 45), the initial methionine is represented by amino acid position no. 1. Derivatives N67, N82, N93, N123, and N124 are N-terminal truncations.
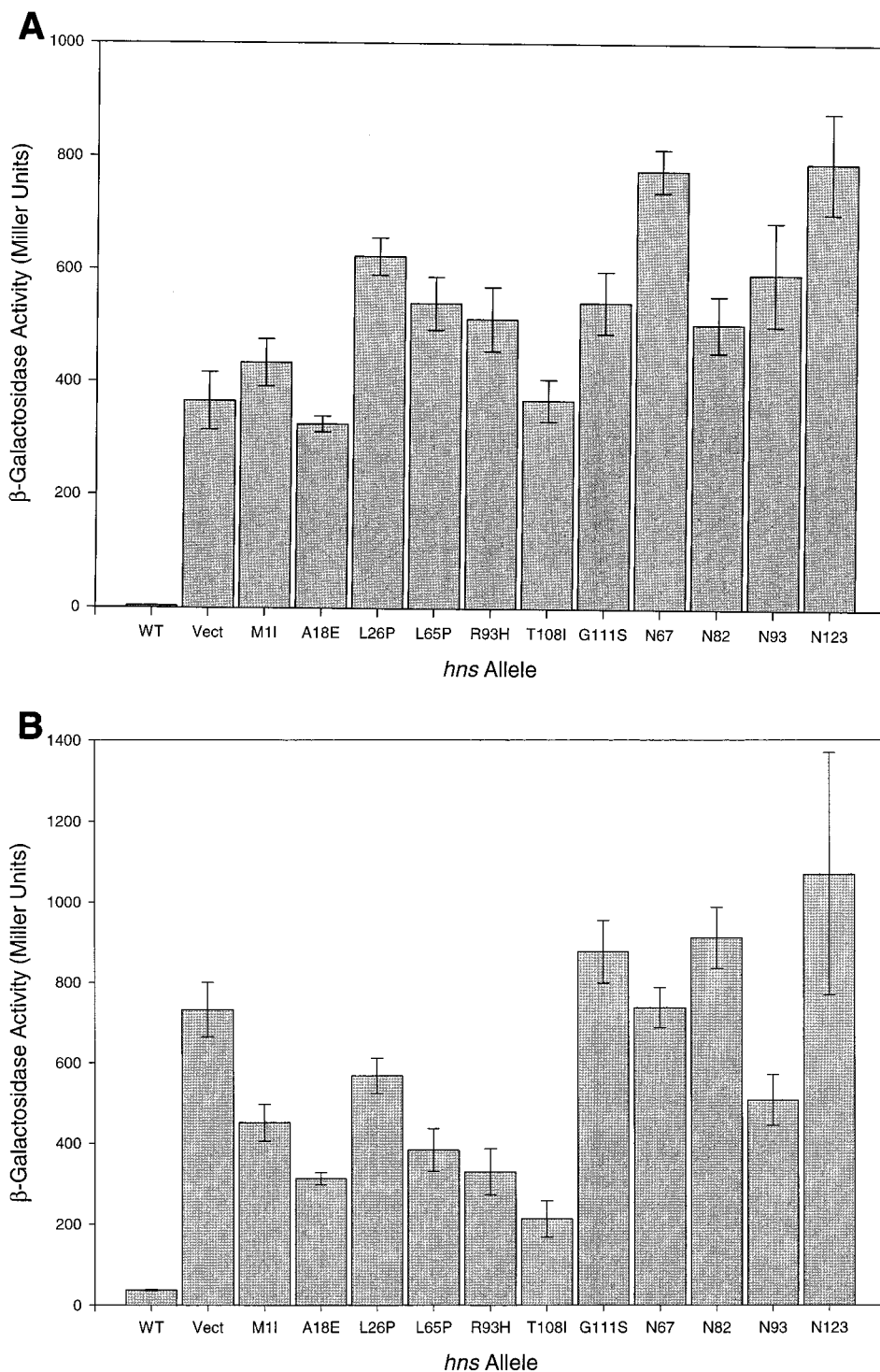
FIG. 1. Effects of *hns* mutant alleles on gene expression. All strains containing either *proU-lacZ* (THK62) (A) *fimB-lacZ* (AL90) (B) fusions and the indicated *hns* plasmids were grown to mid-log phase at 30°C and assayed for β-galactosidase activity (27). Data represent the averages of three to four independent experiments in duplicate. Error bars, standard errors of the means.

(1, 11). Piliation is phase variable (on ⇔ off), and changes in expression may be monitored phenotypically on lactose Mac-Conkey agar (29). Individual colonies from wild-type strains carrying a *fimA'-lacZ* fusion are either red (Lac⁺, *fimA* promoter on) or white (Lac⁻, *fimA* promoter off). Our laboratory has previously shown that *hns* mutations cause a 100-fold in-

crease in the *fimA* promoter inversion rate, yielding pink colonies consisting of approximately equal numbers of on and off promoter-oriented cells (18, 19).

The *hns* mutations fell into two classes in regards to *fimA* promoter inversion (Table 3). The point mutations in the C-terminal third of the protein did not affect inversion, whereas

TABLE 3. Effects of *hns* mutations on *fimA* promoter inversion[a]

H-NS derivative	Inversion phenotype
pTHK116-7 (wild-type *hns*)	Red and white (normal)
pACYC184 (vector)	Pink (rapid)
M1I	Pink
A18E	Pink
L26P	Pink
L65P	Pink
R93H	Red and white
T108I	Red and white
G111S	Red and white
N67	Pink
N93	Pink
N123	Pink

[a] All plasmids were transformed into AL29 (*fimA'lacZYA-kan, hns2-tetR*) and grown at 37°C on lactose MacConkey indicator medium. Individual colonies were picked and restruck to verify phenotypes.

the remaining four point mutations and three C-terminal truncations all resulted in the mutant, rapid promoter-inversion phenotype. It has been well documented that the DNA-binding domain is located in the C terminus of H-NS (33, 42). Thus, our results suggest that H-NS regulated type 1 pilus promoter inversion by some non-DNA-binding mechanism. We concluded that the ability of H-NS to control promoter flipping resides in the N-terminal half of the protein and that the C terminus may be needed only for some undefined role.

Effects of *hns* mutations on motility. H-NS is a positive regulator of *flhCD*, the master operon which controls the expression of all flagellar genes required for motility (24). Mutations in *hns* render bacteria nonmotile due to the reduced expression of *flhCD* and subsequent lack of flagellar biogenesis (4, 17, 20). Strains harboring each of the *hns* plasmids were inoculated onto semisolid agar plates and incubated at 30°C for 13 to 17 h. Motility was determined by measuring the diameter of the bacterial swarms and calculating a relative rate (Table 4). Most of the mutations had no major effect on motility with swarm rates close to 1. However the two *hns* point mutations *A18E* and *T108I* exhibited a unique hypermotile phenotype. We independently analyzed the basis for this hypermotile phenotype and determined that *A18E* and *T108I* affected flagellar rotational speed (8). Three other mutants were severely defective in the ability to move through the soft agar. The initial M1I mutant was completely nonmotile and two truncations, N93 and N124, displayed two- to threefold decreases in motility rates, respectively. These results suggested that the carboxy-terminal portion of H-NS may be involved in some aspect of bacterial motility that does not necessarily include DNA binding.

DNA-binding activity of altered H-NS protein. One of the most extensively studied properties of H-NS is its ability to bind DNA, since this interaction is often coupled to gene regulation (9, 38, 40). Combined genetic and biochemical efforts have isolated the DNA-binding region of H-NS to the C-terminal third of the protein (33, 42). We performed gel shift assays to compare the binding affinities of wild-type H-NS to protein encoded by *hns* with a mutation in the proposed DNA-binding fragment (T108I). Equal and increasing amounts of each purified protein were added to a known H-NS target, the *fimB* promoter (9) (Fig. 2).

In agreement with our previous study on H-NS–*fimB* interactions (9), we determined that (i) wild-type H-NS avidly bound the *fimB* promoter fragment, (ii) binding occurred at a low protein-to-DNA molar ratio, indicating a high-affinity

binding capacity, and (iii) there were multiple H-NS binding sites within the *fimB* promoter region. In contrast, H-NS encoded by *hnsT108I* was unable to bind and retard *fimB* DNA. Even at the maximum protein concentrations added, complexes were undetectable even though the intensity of free DNA slightly decreased. These results demonstrated that H-NST108I is defective in its ability to bind relevant *fimB* DNA and corroborates evidence that the H-NS C terminus is essential for this function.

H-NS isoforms. In the cell, H-NS exists as two or three isoforms differing only in their pI values (21, 34, 43). Presumably, H-NS undergoes a posttranslational modification, yet the type and site of modification and biological function of each form is still unknown. To begin uncovering these details we subjected whole-cell lysates of the *hns* mutant strains to two-dimensional electrophoresis followed by H-NS identification via Western blot analysis (Fig. 3). We consistently observed two isoforms present at 15.4 kDa that reacted with anti-H-NS antiserum in the wild-type *hns* strain (Fig. 3A) but absent in the vector-only control (Fig. 3B). The same two polypeptides were also synthesized from the *hnsT108I* mutant allele (Fig. 3C). Two low-intensity H-NS specific spots were also visible with the N67 derivative (Fig. 3D). This mutation encodes a C-terminally truncated form of H-NS that is approximately 7 kDa. However, each of the N-terminal point mutations, *A18E* and *L26P*, yielded only one H-NS isoform (Fig. 3E and F, respectively). Thus, these results suggested that the H-NS modification site is located within the amino half of the protein, potentially near residues 18 to 26.

DISCUSSION

In this study, we combined genetic and biochemical approaches to identify key H-NS features necessary for a number of different functions. Transforming an *hns*-bearing plasmid into an *E. coli* mutator strain provided a simple method to obtain a set of random *hns* mutations. This technique, as opposed to chemical mutagenesis, had the advantage of being truly random and unbiased. We isolated *hns* mutant alleles with base pair insertions, deletions, transitions, and transversions which resulted in the alteration of different individual amino acid residues (Table 2). The locations of these amino acid substitutions and their effects on various H-NS activities allowed us to construct a model of H-NS domain organization (Fig. 4).

As anticipated, all of the *hns* mutations isolated were defec-

TABLE 4. Effects of *hns* mutations on motility

H-NS derivative	Relative motility rate[a]
pACYC184 (vector)	0.20 ± .05
M1I	0.17 ± .03
A18E	2.50 ± .17
L26P	0.79 ± .17
L65P	0.80 ± .11
R93H	0.99 ± .15
T108I	2.10 ± .21
G111S	0.94 ± .20
N67	0.60 ± .13
N93	0.43 ± .06
N124	0.32 ± .08

[a] Motility rates were determined by comparing the swarm diameters of the *hns2-tetR* strains expressing the mutant *hns* plasmids relative to those of the same strains, on the same plates, expressing the wild-type *hns* plasmid. A swarm rate of 1 indicates identical swarm sizes between the two strains. Data represent the means ± standard errors of the means for five to seven individual experiments.
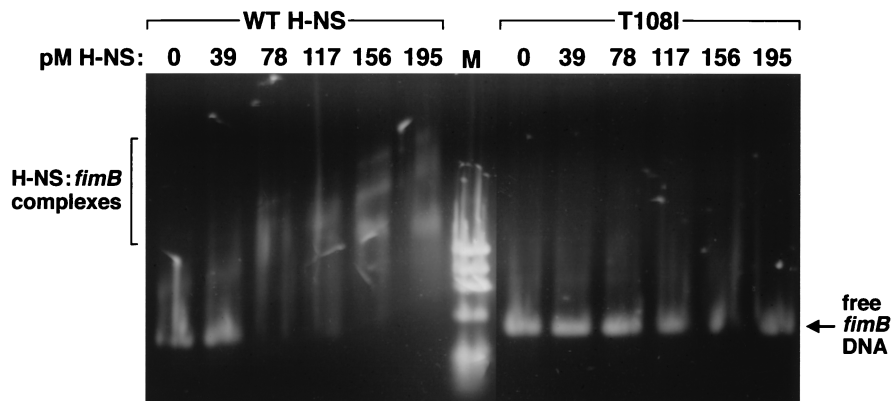
FIG. 2. Gel shift assay comparing the DNA-binding abilities of wild-type H-NS and altered T108I H-NS. Each lane contains 1.6 pM *fimB* promoter DNA and the indicated amount of either wild-type or T108I H-NS. Reaction mixtures were incubated, electrophoresed on a 1% agarose gel, and stained with ethidium bromide (9). M, ΦX174 marker.

tive in their ability to repress the expression of both *proU*- and *fimB-lacZ* promoter fusions (Fig. 1). The magnitude of induction varied greatly between mutations with the major difference between test promoters being that *proU* expression was ~10 times more repressed by wild-type H-NS than *fimB*. Moreover, individual *hns* mutations had equivalent effects at both promoters. This observation led us to postulate that the

mode of operation of repression is probably the same for genes at which H-NS has been shown to be a direct transcriptional inhibitor such as *fimB* (9), *proU* (40), and *rrnB* (38). Other investigators have suggested alternate mechanisms for H-NS repression of the semi-synthetic *gal* promoter (45) and the *bgl* operon (42).

Since its discovery (34, 44), the ability to bind DNA has been
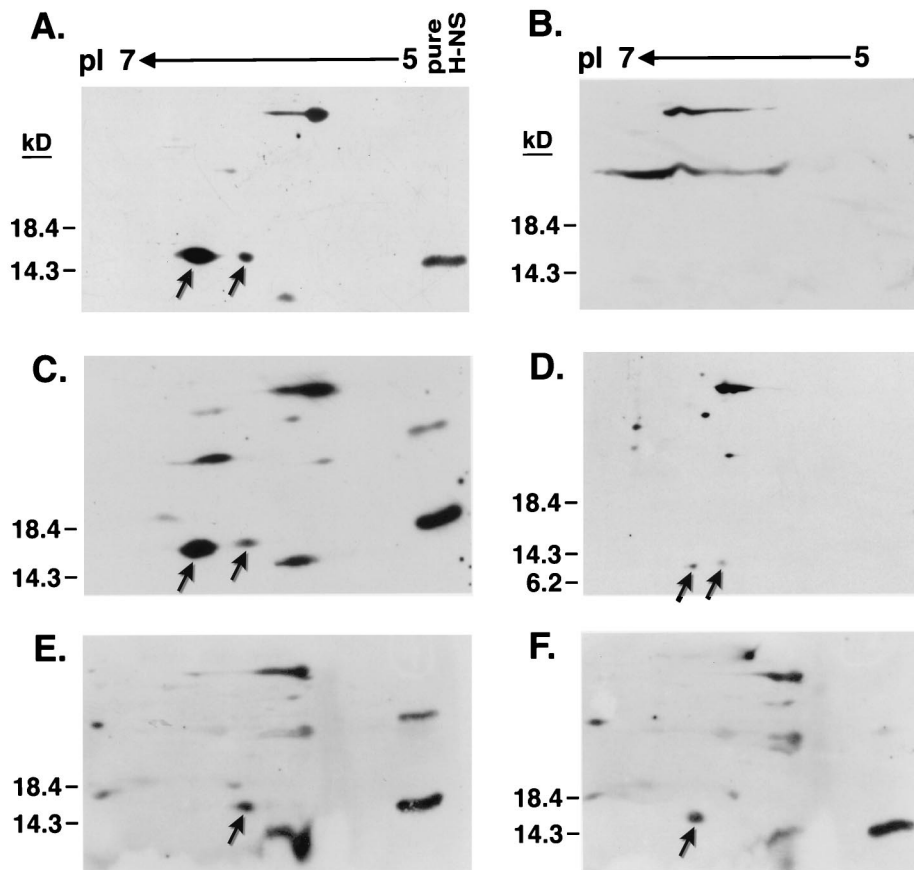

FIG. 3. Identification of H-NS isoforms by two-dimensional gel electrophoresis. Shown are Western blots of H-NS lysates separated by pI in the first dimension and by size in the second dimension and probed with anti-H-NS antiserum. All blots are in the same orientation with the same pI scale and protein standards indicated. Arrows indicate H-NS isoforms. For some panels e.g., panel A, purified H-NS (pure H-NS) was included as an internal marker. The H-NS protein and derivatives used were wild-type (A), vector (B), T108I (C), N67 (D), A18E (E), and L26P (F).
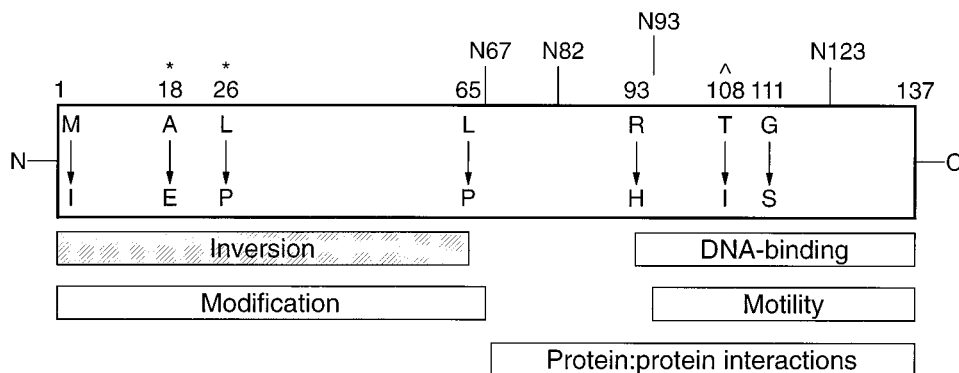
FIG. 4. Schematic representation of H-NS. Altered residues are indicated by position number and amino acid substitution. Truncations are denoted by vertical lines. Rectangles represent domains involved in the indicated H-NS functions based on this study. The DNA-binding domain is as defined by Ueguchi et al. (42). Caret denotes residue implicated in DNA binding in this study; asterisks represent potential sites of H-NS modification.

a major focus of H-NS research. Based on the binding properties of an H-NS truncation containing only the C-terminal 47 amino acids of the protein (33) and analysis of H-NS mutants (42), it has been established that the C-terminal third of H-NS encompasses the DNA-binding domain. Two lines of evidence generated here with our *hnsT108I* mutation confirmed these data. First, during purification H-NS T108I did not bind as tightly as wild-type H-NS to a ds-DNA-cellulose column (7), dissociating at lower salt concentrations (data not shown). Secondly, in gel shift assays T108I did not bind a *fimB* promoter fragment at a protein-to-DNA molar ratio upwards of 120:1 (Fig. 2). This result is in contrast to the strong binding exhibited by a low 45:1 molar ratio of wild-type H-NS to the same piece of DNA. Thus, our work on T108I complemented previous intrinsic fluorescence H-NS studies (13, 39) that suggested that the proximal tryptophan-109 closely interacted with DNA. The caveat of in vitro binding assays is that other factors that may be present in the cell and necessary for efficient protein binding may not be included. However, this problem is minimized when comparing the binding affinities of two proteins, such as wild-type and T108I H-NS, under the exact same conditions. Also, as previously suggested (42), a defect in DNA binding is only partially responsible for maximal gene derepression. This observation is exemplified by the fact that H-NS encoded by *hnsT108I* was unable to bind *fimB* DNA (Fig. 2) yet this mutation caused the smallest increase in *fimB-lacZ* expression (Fig. 1B).

The molecular mechanism by which H-NS influences promoter inversion and type 1 piliation is unknown. By inversion assays (Table 3) we showed that the residues within the N-terminal half of H-NS were important for controlling inversion since changing them resulted in a mutant inversion phenotype. In contrast, inversion regulation was not dependent on DNA binding since strains with altered proposed H-NS–DNA contact sites (R93H, T108I, and G111S) retained the wild-type inversion frequency. In particular, substitutions at Thr-108 (Fig. 2) and Gly-111 (42) have specifically been shown to cause drastic reductions in the ability of H-NS to bind DNA yet neither alteration adversely affected *fimA* promoter inversion frequency. Interestingly, even though residues within the DNA-binding domain of H-NS were not required for proper *fimA* promoter flipping, the entire C-terminal half of the protein was needed in some capacity, as evidenced by the mutant inversion phenotype exhibited by the H-NS truncations. We postulate that H-NS regulates inversion without binding DNA, possibly through protein-protein interactions with other mol-

ecules present at the *fimA* invertible element, and that the C-terminal domain may be important for overall structural integrity and protein stabilization. Data presented here also corroborated our previous conclusion that the regulation of FimB recombinase levels in the cell by H-NS is not the sole cause of the rapid inversion witnessed in *hns* mutant backgrounds (9). Consistent with this hypothesis, H-NS derivatives R93H, T108I, and G111S caused 6- to 25-fold increases in *fimB* expression (Fig. 1B) while maintaining a wild-type inversion phenotype (Table 3).

As with inversion, the motility results (Table 4) implicated the nonregulatory role of the H-NS carboxy terminus. The negative effects of *hns* mutations on bacterial motility were mainly clustered to the H-NS truncations, arguing that the conformation of this domain, rather than individual residues, is relevant to this motility phenotype. Rather than acting solely as a transcriptional activator of flagellar gene expression (4) the effect of H-NS on motility may be determined by protein interactions between H-NS and flagellar rotor components, as we have recently demonstrated with FliG (8).

Determining the type and location of the modified group(s) of H-NS is valuable for devising a mechanistic view of multiple H-NS activities. It is possible that separate H-NS isoforms possess differential functions. Using two-dimensional gel analysis (Fig. 3), we showed for the first time that multiple isoforms were present when an *hns* clone encoding only residues 1 through 67 was the sole source of H-NS expression. Further H-NS analysis revealed that a potential modification site may lie within or near a nine-residue span from A18 to L26. Thus, alterations in these specific amino acids may directly interfere with the addition of a modified group or cause local structural rearrangements in proximal residues.

Utilizing a random mutagenesis scheme followed by multiple phenotypic analyses, we have been able to aid in defining the relationship between H-NS structure and function. Several general themes have arisen from this study. The gene expression experiments in conjunction with the H-NS–DNA binding assays have confirmed that the ability to bind DNA is only one of the duties H-NS has in modulating gene expression. Taken together, the effects of *hns* mutations and deletions on *fimA* promoter inversion and bacterial motility suggested that the H-NS C-terminal domain may have multiple functions including involvement in protein-protein interactions as well as interactions with DNA. In agreement with this concept, we have recently shown that alterations in H-NS at residue 108 affect binding to FliG (8) and Spurio et al. (36) have shown that

changes and deletions at Pro-116 impair H-NS oligomerization. We also speculate that the N and C termini may interact with each other, and we await determination of the three-dimensional crystal structure of H-NS to test this hypothesis. Although some H-NS functions may be isolated to particular domains, it seems likely that all portions of the protein work coordinately and in concert with each other to exert full H-NS activity.

ACKNOWLEDGMENTS

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