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# Probing the Structure, Function, and Interactions of the *Escherichia coli* H-NS and StpA Proteins by Using Dominant Negative Derivatives

ROY M. WILLIAMS, SYLVIE RIMSKY,* AND HENRI BUC

*Unité de Physicochimie des Macromolécules Biologiques (URA 1149 du Centre National de la Recherche Scientifique), Institut Pasteur, 75724 Paris Cedex 15, France*

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**Twelve different dominant negative mutants of the *Escherichia coli* nucleoid-associated protein, H-NS, have been selected and characterized in vivo. The mutants are all severely defective in promoter repression activity in a strain lacking H-NS, and they all disrupt the repression normally exerted by H-NS at two of its target promoters. From the locations of the alterations in these mutants, which result in both large truncations and amino acid substitutions, we propose that H-NS contains at least two distinct domains. The in vitro protein-protein cross-linking data presented in this report indicate that the proposed N-terminal domain of H-NS has a role in H-NS multimerization. StpA is a protein with known structural and functional homologies to H-NS. We have analyzed the extent of these homologies by constructing and studying StpA mutants predicted to be dominant negative. Our data indicate that the substitutions and deletions found in dominant negative H-NS have similar effects in the context of StpA. We conclude that the domain organizations and functions in StpA and H-NS are closely related. Furthermore, dominant negative H-NS can disrupt the activity of native StpA, and reciprocally, dominant negative StpA can disrupt the activity of native H-NS. We demonstrate that the N-terminal domain of H-NS can be chemically cross-linked to both full-length H-NS and StpA. We account for these observations by proposing that H-NS and StpA have the ability to form hybrid species.**

Dominant negative mutations lead to the production of stable proteins capable of inhibiting the normal function of their wild-type counterpart in the cell (discussed in reference 16). The analysis of such mutants provides a powerful means for gaining insights into both the cellular function and the mechanism of action of the native protein. Early studies of such mutations in the *lacI* gene revealed the locations in the Lac repressor of the distinct domains involved in DNA binding and oligomerization and thus allowed the dissection of these two activities (summarized in reference 23). This study and others (for examples, see references 10, 17, 23, and 24) reveal that for oligomeric DNA-binding proteins, dominant negative inhibition commonly arises when partially functional or nonfunctional mixed oligomers containing both mutant and wild-type subunits are formed. The mixed oligomers have reduced activity, as the incorporation of the mutant subunit produces an overall reduction in the ability of the protein to carry out an essential function.

H-NS is an interesting target for investigation by the analysis of dominant negative derivatives: it is a major component of the bacterial nucleoid with roles in many disparate cellular processes (see references 36 and 41 and references therein). The many regulatory activities of H-NS apparently arise from two unexplained properties of the protein: its abilities to (i) discriminate between curved and linear DNA sequences (37, 38) and (ii) form higher-order oligomeric assemblies on DNA (11, 20, 21, 33). The study of dominant negative H-NS derivatives, and the mapping of alterations responsible for dominant negative effects, was anticipated to throw some light on the molecular details underlying the ability of H-NS to fulfill these two key functions.

Recent reports have revealed that *Escherichia coli* contains an H-NS analog, StpA, with 58% amino acid identity to H-NS (27, 39). StpA is normally very poorly expressed in standard laboratory *E. coli* strains; however, by using plasmid expression systems, StpA activity can be readily observed (41). In addition to the striking structural similarity between the two proteins, many functional and mechanistic parallels, such as the inhibition of transcription from promoters containing curved DNA and the ability to constrain DNA supercoils, have now been described. Even though StpA can apparently function as a molecular backup for H-NS (41), it is clear that StpA has some distinctive properties of its own, including an ability to act as a far superior RNA chaperone (40, 41). The available data have allowed the postulation of a global regulatory network involving both H-NS and StpA, with an opportunity for differential gene expression left open via the RNA specific effects of the StpA protein (41). To test and extend such a model, it is of interest to gain a molecular understanding of how significant the similarities and differences are between H-NS and StpA and to determine whether H-NS and StpA interact and form hybrid species.

Therefore, we have designed genetic tests to analyze dominant negative phenotypes at two different promoters. By applying the same genetic approach, we have used dominant negative effects to analyze the extent of the homologies between H-NS and StpA down to the level of protein structure and amino acid function. This was achieved by transplanting from H-NS to StpA changes originally found to cause dominant negative effects in H-NS. We also investigated whether the dominant negative properties of these H-NS and StpA derivatives were specific for only the respective parent protein. To do this, we examined the activities of the mutants in backgrounds in which either native H-NS only or native StpA only was responsible for promoter repression. From our genetic studies, we surmised that H-NS and StpA were capable of forming mixed hybrids. The results of in vitro protein-protein

* Corresponding author. Phone: (1) 45 68 86 43. Fax: (1) 40 61 30 60. Electronic mail address: srimsky@pasteur.fr.

TABLE 1. *E. coli* strains used in this study

| Strain | Genotype | Source | Reference(s) |
|---|---|---|---|
| M182*hns*$^+$ | Δ*lacX74 galU galK recA strA srl*::Tn*10* | Laboratory stocks | 5 |
| M182*hns* | Δ*lacX74 galU galK recA strA srl*::Tn*10 hns2600* | Laboratory stocks | 5, 43 |
| TP2600 | Δ*lacX74 xyl-7 arg H1 hns2600* | P. Bertin | 18 |
| GJ74 | Chromosomal *proU-lacZ* fusion derivative of MC4100 | J. Gowrishankar | 12 |
| BL21 | *ompT* (*lon*) λDE3 | Novagen | 1 |

cross-linking experiments support the conclusion that the proteins can assemble into heteromeric species.

## MATERIALS AND METHODS

Tables 1 and 2 list the strains and plasmids used throughout this work. Unless otherwise indicated, all standard DNA manipulations were carried out as described elsewhere (26).

**Construction of plasmids.** Three series of plasmids containing the *proU* promoter upstream of a reporter gene were constructed. An *Eco*RI-*Hin*dIII-tagged DNA fragment carrying a *proU* promoter region homologous to that present in plasmid pHYD 270 (8) was generated by PCR amplification using M182 genomic DNA as the template. This promoter fragment was chosen as it has been shown to contain both the downstream response element and the upstream H-NS target sites (8, 20). The *proU Eco*RI-*Hin*dIII promoter DNA fragment was then inserted between the corresponding sites in the pAA *lac* expression vector (6). A pAA*proU* clone having the published promoter sequence (13) was retained. This *proU* promoter fragment was then transferred into both the RK2-based *lac* expression vector pRW2 (19), producing pRW*proU*, and the pBR322-based *cat* expression vector pKK232.8, producing pKK*proU*.

Plasmid pLG H-NS was constructed by inserting a DNA fragment carrying the *hns* open reading frame (ORF) and promoter region between the *Eco*RI and *Sal*I sites in the low-copy-number, Kan$^r$, pSC101-based vector pLG339 (31). The *Eco*RI-*Sal*I-tagged DNA fragment was generated by PCR amplification using M182 (*hns*$^+$) genomic DNA as the template. Clones which complemented the *hns* (*bglY*) phenotype of strain TP2600, as indicated by white colony color on MacConkey salicin plates (18), were picked, and the DNA sequence of the insert carrying *hns* was determined. Sequence determination was performed by using the Pharmacia T7 sequencing kit in combination with 5′ biotinylated DNA templates immobilized on streptavidin-coated magnetic Dynabeads (32). One pLG H-NS clone which both complemented TP2600 and contained an insert with the correct inferred amino acid sequence for native H-NS was retained for further manipulation.

**Random mutagenesis of *hns*.** Random mutations in the *hns* gene, cloned in pLG H-NS, were created by exploiting *Taq* DNA polymerase as previously described, with slight modification (42). For genes cloned in low-copy-number plasmids, such as *hns* in pLG H-NS, effective library creation is simplified when the whole plasmid is amplified in vitro. The amplification of the 6.6-kbp pLG H-NS plasmid was performed with *Taq* (Exo$^-$) DNA polymerase (Bethesda Research Laboratories) plus *Pfu* (Exo$^+$) DNA polymerase (Stratagene). The method used to perform the amplification with this mix of polymerases was essentially as described previously (2). The divergent oligonucleotide primers used for plasmid amplification were complementary to the region around and just overlapping the unique *Hin*dIII site in the Tn*903* Kan$^r$ gene carried by pLG H-NS. After purification of the PCR product by using Qiaspin columns (Qiagen), the termini of the fragment were cleaved with *Hin*dIII and the plasmid was recircularized with T4 DNA ligase. Each 50-μl reaction yielded approximately 5 μg of amplified pLG H-NS DNA. Mutant *hns* alleles isolated from this library were transferred to a nonmutagenized plasmid background before further study.

**StpA plasmids and site-directed mutagenesis of *stpA*.** Plasmid pD StpA was constructed by inserting a DNA fragment carrying the *stpA* ORF and promoter region between the *Bam*HI and *Sal*I sites in the pBR322-based, Amp$^r$ cloning vector pDU9 (4). A *Bam*HI-*Sal*I-tagged DNA fragment containing the *stpA* gene was generated by PCR amplification as described previously (27), using M182 genomic DNA as the template. A pD StpA clone that complemented the *hns* (*bglY*) phenotype of strain TP2600 was selected on MacConkey salicin plates as described above for pLG H-NS. Double-stranded DNA sequencing revealed that this clone encoded wild-type StpA protein.

The pYC StpA plasmids were constructed by inserting the desired *stpA* gene from pD StpA between the *Bam*HI and *Sal*I sites in the medium-copy-number cloning vector pACYC184 (7). Hence, the pYC StpA plasmids carry Cm$^r$ and have the P15A origin of replication.

Site-directed mutations were introduced into *stpA* by a two-step PCR process as described previously (25). This process exploited the two primers originally used to amplify *stpA*, a mutagenic primer for creating a deletion between codon 65 and the stop codon, and primers for the substitutions L26P, Y97C, and P116S. The presence of only the desired alterations was confirmed by double-stranded DNA sequencing of the entire *stpA* gene in pD StpA.

Further details of plasmid construction and PCR conditions are available on request.

**Growth conditions and β-galactosidase assays.** The *hns* phenotype of cells was judged by using the reporter plasmids described above in combination with MacConkey lactose indicator plates, MacConkey salicin indicator plates, and 15% LB-chloramphenicol plates. Where necessary, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; tetracycline, 35 μg/ml; and chloramphenicol, 35 to 55 μg/ml. For β-galactosidase assays, cells were grown aerobically in LB and after permeabilization assayed as described previously (22). The data for β-galactosidase activities are expressed in Miller units: 1 U = 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg (dry weight) of cells. The values presented represent data from at least three independent determinations that differed by less than 10%.

**RNA analysis.** Cells were grown to an optical density at 600 nm of 0.7, and total RNA was isolated by hot phenol extraction (3). RNA was quantified by $A_{260}$, and its integrity was checked by electrophoresis in agarose gels followed by ethidium bromide staining. Primer extension analysis was performed with 50 μg of RNA, using either the PEHW (for *hns* mRNA detection; 5′CTAATTTTTC CAGCATTTCTTCCA3′) or PESW (for *stpA* mRNA detection; 5′CTGAATT TTCGAGCATTTCTTCAA3′) antisense oligonucleotide 5′ end labeled with $^{32}$P as described previously (1). The transcript-specific probes correspond to the regions in the mRNAs coding for amino acids 26 to 33. The cDNA products of the primer extension reactions were separated on 8% sequencing gels and visualized by autoradiography. Dideoxy sequencing reactions were run in parallel to verify the positions of the transcription start points. Quantification of the transcripts was performed with a PhosphorImager scanner, and relative quantities of transcript are given in arbitrary units as calculated by using ImageQuant software (Molecular Dynamics).

**Purification of StpA, H-NS, and H-NSΔ64 protein.** Recombinant genes encoding C-terminally $His_6$-tagged StpA, H-NS, and H-NSΔ64 (StpA $His_6$, H-NS $His_6$, and H-NSΔ64 $His_6$, respectively) were constructed by PCR using primers that (i) included the additional sequence encoding the hexahistidine tail and (ii) allowed the retention of the natural ribosome binding sites. The recombinant

TABLE 2. Plasmids used in this study[a]

| Plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| pLG339 | Kan$^r$; ori-pSC101; low-copy-number cloning vector | 31 |
| pLG H-NS | Kan$^r$; ori-pSC101; *hns*$^+$ | This work |
| pDU9 | Amp$^r$; ori-ColE1; pBR322-based cloning vector | 4 |
| pD StpA | Amp$^r$; ori-ColE1; *stpA*$^+$ | This work |
| pACYC184 | Cm$^r$; Tet$^r$; ori-P15A; medium-copy-number cloning vector | 7 |
| pYC StpA | Cm$^r$; ori-P15A; *stpA*$^+$ | This work |
| pKK232.8 | Amp$^r$; ori-ColE1; promoterless *cat* expression vector | Pharmacia |
| pKK*proU* | Amp$^r$; ori-ColE1; $P_{proU}$-*cat* | This work |
| pAA187 | Amp$^r$; ori-ColE1; promoterless *lac* expression vector | 6 |
| pAA*5A6AgalP1* | Amp$^r$; ori-ColE1; $P_{5A6AgalP1}$-*lacZYA* | 5 |
| pAA*proU* | Amp$^r$; ori-ColE1; $P_{proU}$-*lacZYA* | This work |
| pRW2A | Tc$^r$; ori-RK2; promoterless *lac* expression vector | 19 |
| pRW*proU* | Tc$^r$; ori-RK2; $P_{proU}$-*lacZYA* | This work |
| pBluescript SK | Amp$^r$; ori-pUC; T7 promoter upstream of polylinker | Stratagene |
| pBSK H-NS His6 | Amp$^r$; ori-pUC; H-NS $His_6$ overexpression vector | This work |
| pBSK H-NSΔ64C His6 | Amp$^r$; ori-pUC; H-NSΔ64C $His_6$ overexpression vector | This work |
| pBSK StpA His6 | Amp$^r$; ori-pUC; StpA $His_6$ overexpression vector | This work |
| placI$^q$ | Kan$^r$; ori-p15A; high-level expression of Lac repressor | Laboratory stocks |

[a] The ColE1, SC101, p15A, and RK2 origins of replication (ori) used in these plasmids are compatible and can be stably maintained together in the same host cell.

genes were then inserted into the polylinker in pBluescript SK (Stratagene) such that their expression was dependent on the Bluescript T7 promoter. Clones containing the desired insert were sequenced in their entirety, using the Pharmacia T7 sequencing kit with the T7 and T3 promoter primers. Genes with the correct predicted amino acid sequence were then transformed into the *E. coli* BL21[placI$^q$] overexpression strain. Growth of this strain, induction of T7 RNA polymerase, and protein overexpression were carried out essentially as described previously (9).

One-liter cultures of cells containing overexpressed protein were pelleted by centrifugation and washed in 50 ml of buffer A (750 mM NaCl, 10 mM Tris [pH 7.5]). After recentrifugation, the pellets were resuspended in 8 ml of buffer A containing phenylmethylsulfonyl fluoride and lysed by sonication. Cell debris was separated from the soluble fraction by centrifugation in a minicentrifuge at 13,000 rpm for 20 min at 4°C. The supernatant was loaded directly onto a 1-ml Hitrap metal chelation column (Pharmacia) pretreated with a solution of $NiSO_4$ and equilibrated in buffer A containing 70 mM imidazole. After loading, the column was washed with the same buffer until no further material was observed eluting. Recombinant His-tagged protein was then eluted in buffer A containing 50 mM EDTA (peak fraction concentration of ca. 5 mg/ml). The protein was separated from contaminating $NiSO_4$ and EDTA by exchanging buffer A for buffer B (350 mM NaCl, 7 mM morpholineethanesulfonic acid [MES; pH 6.0], 3% glycerol), using a PD10 desalting column (Pharmacia). Analysis of the purified proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining failed to reveal the presence of any contaminating protein. Protein concentrations were measured colorimetrically with the Bio-Rad reagent, using the Bradford procedure and commercial bovine serum albumin as a standard. Protein aliquots in buffer B were stored at −80°C.

**Protein-protein cross-linking.** The H-NSΔ64C $His_6$ polypeptide, H-NS $His_6$, and StpA $His_6$ were mixed as required in buffer B. Protein concentrations in a volume of 8 μl were 75 μM for H-NSΔ64C $His_6$ and 25 μM for H-NS $His_6$ or StpA $His_6$ as appropriate. After a 10-min incubation at room temperature, 2 μl of a solution containing a mixture of the zero-length chemical cross-linker 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC) and the catalyst *N*-hydroxysuccinimide (NHS) was added to the protein (final concentrations of 50 mM EDC and 200 mM NHS [15]). The cross-linking reaction mixture was then incubated at room temperature for 45 min, and the reaction was stopped by the addition of 10 μl of SDS–Blue–β-mercaptoethanol (β-mercaptoethanol final concentration, 150 mM) and heating at 95°C for 4 min. The sample was then loaded onto an SDS–10 to 20% polyacrylamide gradient gel (Readygel; Bio-Rad). Electrophoresis was carried out as directed by the supplier. The gel was stained with Coomassie brilliant blue and destained with a 30% methanol–10% acetic acid solution before being photographed.

TABLE 3. Sequences and in vivo activities of the 12 dominant negative H-NS mutants isolated

| pLG H-NS derivative | Codon change | Expression of test promoter[a] (U of β-galactosidase) | | | |
|---|---|---|---|---|---|
| | | *proU* | | *5A6AgalP1* | |
| | | *hns* | *hns*$^+$ | *hns* | *hns*$^+$ |
| Wild type | None | 23 | 20 | 130 | 75 |
| L26P | CTG to CCG | 300 | 70 | 1,040 | 180 |
| E53G T55P | GAG to GGG, ACT to CCT | 250 | 70 | 700 | 200 |
| Δ64C[b] | *A*TG to TGC | 190 | 55 | 685 | 210 |
| Y97C | TAT to TGT | 135 | 80 | 975 | 200 |
| Y97H | TAT to CAT | 120 | 80 | 980 | 240 |
| Y97S | TAT to TCT | 148 | 80 | 965 | 245 |
| T110A | ACT to GCT | 150 | 75 | 980 | 230 |
| G113S | GCA to TCA | 130 | 90 | 1,060 | 245 |
| P116S | CCA to TCA | 165 | 105 | 1,295 | 205 |
| I119T | ATC to ACC | 130 | 75 | 970 | 200 |
| Δ121[c] | AAA to AAG | 135 | 65 | 685 | 235 |
| F133S | TTC to TCC | 165 | 60 | 780 | 195 |
| pLG339 control[d] | | 325 | 22 | 1,170 | 100 |

[a] M182Δ*lacX74 recA hns* and M182Δ*lacX74 recA hns*$^+$ cells carrying either the *proU* or the *5A6AgalP1* test promoter, cloned in pAA, were transformed with pLG H-NS and mutant derivatives. These cells were grown to mid-log phase, when the level of β-galactosidase activity was assayed as described in reference 22. Each value represents data from at least three independent measurements that differed by less than 10%.

[b] The *hnsΔ64C* allele is the consequence of an AT base pair deletion (indicated by the italicized A in *A*TG), resulting in a frameshift, the creation of an in-frame codon for Cys, and a stop codon (TGA).

[c] The *hnsΔ121* allele is the consequence of an AT base pair deletion between the codons for Lys-120 and Lys-121. This results in a frameshift and the creation of a new sequence encoding 12 amino acids (121-KKQWMRKVNPSTIS) before an in-frame stop codon (TGA).

[d] As controls for these measurements, we used the M182*hns*$^+$ and M182*hns* strains transformed with the vector-only plasmid pLG339.

## RESULTS

**Isolation of dominant negative H-NS mutants by using the *proU* promoter.** Random mutagenesis of the *hns* gene was followed by an in vivo genetic screen to identify dominant negative *hns*. Our screen examined the ability of the PCR-mutagenized *hns*, encoded on plasmid pLG H-NS, to interfere with the normal H-NS repression of the *proU* promoter. This was done by placing *cat* expression under the control of the *proU* promoter, and selection was based on enhanced resistance to the antibiotic chloramphenicol. Indeed, *hns*$^+$ bacteria containing the pKK*proU* construct have a Cm$^s$ phenotype, whereas *hns* strains carrying the same plasmid have a Cm$^r$ phenotype. We reasoned that in the presence of dominant negative H-NS, repression of the *proU* promoter would be weakened and result in these *hns*$^+$ cells acquiring a Cm$^r$ phenotype. Strain M182*hns*$^+$[pKK*proU*] was transformed with the mutagenized pLG H-NS plasmid DNA and plated on media containing ampicillin, kanamycin, and chloramphenicol. Thirteen colonies with an enhanced resistance to chloramphenicol were picked, and the pLG H-NS plasmid DNA was purified. Control experiments performed under the same conditions, but with either pLG339 or a nonmutagenized pLG H-NS plasmid, yielded no colonies. Quantitation of the activities of the H-NS mutants, initially selected by using the *proU-cat* fusion, was carried out with *lacZ* as the reporter gene downstream of the chosen promoters (Table 2).

**Sequences of dominant negative *hns* mutants.** The mutations responsible for the Cm$^r$ phenotype were located on the *Sal*I-*Eco*RI *hns* fragments in the pLG H-NS plasmids. This region of the plasmids was sequenced by using solid-phase sequencing techniques, and the amino acid sequences of the substituted H-NS proteins were inferred. One pLG H-NS derivative carried two substitutions, at positions 53 and 55, while two carried single base pair deletions resulting in the production of H-NS protein truncated after amino acids 64 and 121, respectively. The remaining seven pLG H-NS derivatives carried single amino acid substitutions at positions 26, 97, 110, 113, 116, 119, and 133 (Table 3 and Fig. 1). The mutation Y97C was isolated twice, in addition to two different changes at this position: Y97S and Y97H.

It is notable that all of the residues found to be altered in the mutant H-NS are highly conserved between *E. coli* H-NS and H-NS homologs from other bacterial species and the H-NS analog StpA (36). Interestingly, Spurio et al. previously reported that a four-amino-acid deletion in the loop 2 region of H-NS (including residues P-116 and G-113) produces a mutant with an impaired ability to compact the *E. coli* chromosome (30).

By using the published nuclear magnetic resonance structure of a 47-amino-acid C-terminal H-NS fragment involved in DNA binding (28), it is possible to locate the changes in 9 of the 12 different H-NS derivatives isolated (Fig. 1). Some of the changes result in either the complete (Δ64C) or partial (Δ121) loss of this region. The two remaining alterations (L26P and E53G T55P) affect residues in the N-terminal half of the protein.

**Determining the activities of the H-NS mutants.** To measure alterations in the repressor function of the mutant H-NS encoded on the pLG H-NS plasmids, the plasmid DNA was

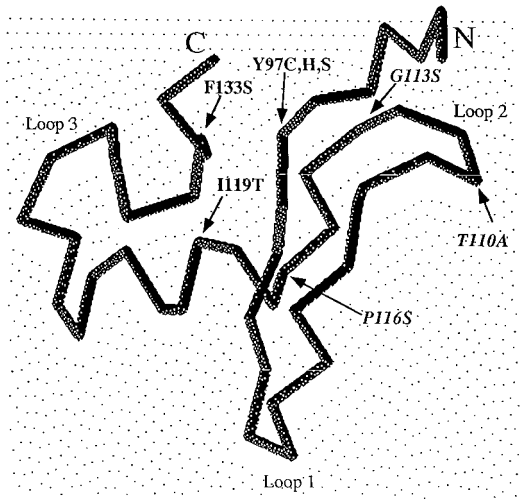


FIG. 1. The H-NS DNA binding fragment HNS47C (comprising amino acids 91 to 137) is represented as an α-carbon backbone trace. The locations and nature of the dominant negative substitutions are indicated, as are the N and C termini and the three loop regions of the fragment. The atomic coordinates for the HNS47C structure (28) were obtained from the Brookhaven Protein Data Bank file 1HNR (prerelease version communicated by H. Shindo and H. Nakamura). The figure was generated by using the RASMOL version 2.5 molecular renderer program.

introduced by transformation into M182*hns* tester strains containing either the pAA*proU* or pAA*5A6AgalP1 lac* expression vectors. Like *proU*, transcription from the semisynthetic *5A6AgalP1* promoter is considered to be directly repressed by the binding of H-NS (35). More precisely, the binding of H-NS to the 5A6A planar curve situated just upstream of *galP1* is required for promoter repression (25a, 43).

The data from the assays in the *hns*[pAA*proU*] strain show that in comparison with the native H-NS encoded on pLG H-NS, the mutants have a range of large (5.5- to 13-fold) defects in the ability to repress the *proU* promoter (Table 3). In the *hns*[pAA*5A6AgalP1*] host strain, the H-NS mutants are five- to ninefold defective in repressor function in comparison with native H-NS (Table 3). Hence, the data indicate that all of the H-NS mutants have substantial defects in repressor function at both the naturally occurring *proU* promoter and the semisynthetic *5A6AgalP1* promoter.

The residual repressor activities of the H-NS mutants at the *proU* and *5A6AgalP1* promoters were then examined to determine if the individual mutants had equivalent defects at both promoters. If the two promoters share a common mechanism of repression by H-NS, then the mutants would be expected to affect the two promoters similarly: some correlation would be expected between the residual repressor activity of a particular mutant at *proU* and at *5A6AgalP1*. The analysis reveals that there is little correlation between the residual repression of the *proU* and *5A6AgalP1* promoters by any given mutant (Fig. 2A). Hence, we believe it unlikely that H-NS uses identical mechanisms of repression at both promoters.

Second, although the mutants are all severely defective with respect to the wild type, it is perhaps significant that some have retained a greater ability to repress *5A6AgalP1* than *proU*, and vice versa. Hence, the 12 dominant negative mutants can be divided into two or three subclasses based on the observed residual activity at *proU* and *5A6AgalP1*. For the first class, there appears to be a parallel between the activities of the E53G T55P and L26P mutants, with the *5A6AgalP1* promoter being more effectively repressed than *proU*. In a second class, constituted by the majority of the C-terminal mutants (mutants inside the box in Fig. 2A), the *proU* promoter is more effectively repressed than *5A6AgalP1*. The remaining mutants

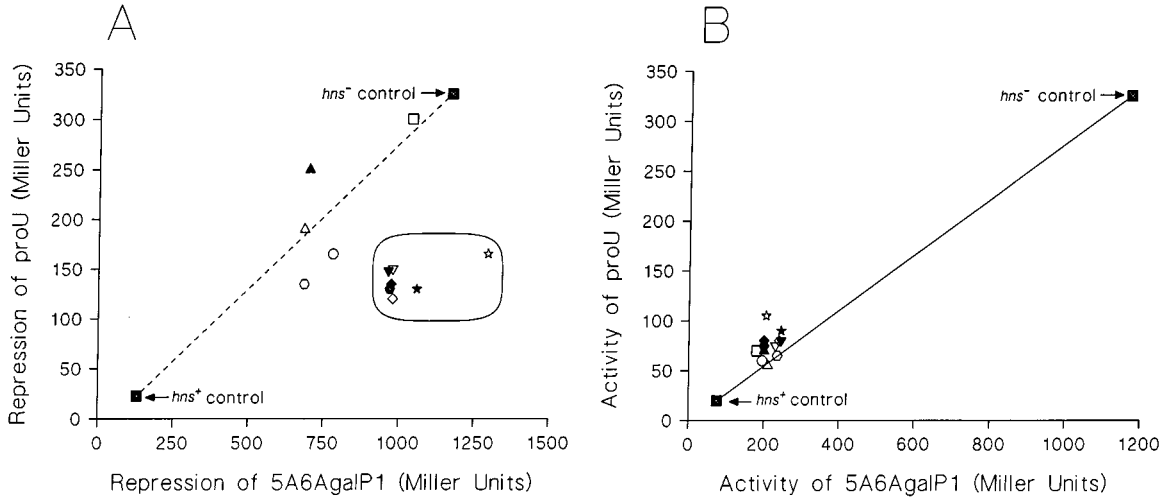


FIG. 2. (A) Plot of the residual repression activities of the 12 H-NS mutants at the *proU* and *5A6AgalP1* promoters. The data presented are taken from Table 3. The points indicating maximal repression and derepression of *proU* and *5A6AgalP1* are marked as *hns*$^+$ control and *hns*$^-$ control, respectively. The dashed line drawn between these two points indicates where the activities of mutants with equivalent defects at both promoters would be expected to fall. The mutants are represented by the following symbols: L26P, open square; E53G T55P, filled up triangle; Δ64C, open up triangle; Y97C, filled diamond; Y97H, open diamond; Y97S, filled down triangle; T110A, open down triangle; G113S, filled star; P116S, open star; I119T, filled hexagon; Δ121, open hexagon; F133S, open circle. The ovoid box surrounds the activity plots of H-NS mutants carrying alterations in the C-terminal domain, corresponding to class two mutants (see Results). Linear regression analysis of these data indicates that it does not fit a straight line ($r^2 = 0.2$). The equation of the straight line with the best fit was $y = 0.13x + 49.4$. (B) Plot of the dominant negative activities of the 12 H-NS mutants at the *proU* and *5A6AgalP1* promoters. The points indicating maximal repression and derepression of *proU* and *5A6AgalP1* are marked as *hns*$^+$ control and *hns*$^-$ control, respectively. The line drawn between these two points indicates where the activities of mutants with equivalent effects at both promoters would be expected to fall. The symbols are as in panel A. Linear regression analysis of these data indicates a good agreement with a straight line plot ($r^2 = 0.96$). Generation of these plots and their linear regression analysis were carried out with the program FigP version 6.0 (Biosoft).

(Δ121 and F133S) have activities which fall between those in the first two classes and hence could constitute a third class.

The dominant negative activity of each of the H-NS mutants was measured by transformation of pLG H-NS plasmid DNA into M182*hns*$^+$ tester strains containing either the pAA*proU* or pAA*5A6AgalP1 lac* expression vector. In the *hns*$^+$[pAA*proU*] strain, the presence of the H-NS mutants resulted in a 2.5- to 4.5-fold increase in the level of β-galactosidase activity compared with that seen in the *hns*$^+$[pAA*proU*][pLG339] control cells (Table 3). In the *hns*$^+$[pAA*5A6AgalP1*] host strain, the presence of the mutant H-NS resulted in a two- to threefold increase in the level of β-galactosidase activity compared with that measured in the *hns*$^+$[pAA*5A6AgalP1*][pLG339] control cells (Table 3). Hence, the results demonstrate that the H-NS mutants can all interfere with the repression normally exerted by native H-NS at both the *proU* and *5A6AgalP1* promoters.

Measurements of promoter activity were also made after overnight growth and in the *hns*$^+$ strain GJ74 carrying a chromosomal *proU-lacZ* fusion (12). The results were equivalent to those obtained in the exponential growth phase in assays using the plasmid reporter constructs. Hence, the H-NS mutants apparently do not affect promoter activity via alterations in the copy number of the plasmid reporter constructs or in a manner dependent on growth phase (results not shown).

The dominant negative activities of the H-NS mutants at the *proU* and *5A6AgalP1* promoters were then examined to determine if the individual mutants had comparable effects at the two promoters. The analysis (Fig. 2B) reveals that there is good correlation between the dominant negative activities of the individual mutants at both the *proU* and *5A6AgalP1* promoters. This finding is in sharp contrast to the previously observed variability in the mutants' repressor function at the two promoters: compare a best fit with $r^2 = 0.2$ for the data in Fig. 2A with $r^2 = 0.96$ for the data in Fig. 2B. We interpret this good correlation, together with the observation that the dominant negative activity falls within a fairly narrow range, as evidence that our genetic screen was effective.

In summary, the results indicate that the changes disrupt a property, or properties, of H-NS important for its proper functioning at both the natural *proU* and the semisynthetic *5A6AgalP1* promoters. As outlined in the introduction, a simple explanation for these effects is that mixed multimers with compromised function can be formed between the mutant and native H-NS protein in the cell, resulting in the inhibition of the native H-NS activity.

**Construction and analysis of dominant negative StpA mutants.** To observe the activity of native StpA and examine the effects of StpA mutants, it is necessary to modify the strategy used above for the analysis of H-NS and the dominant negative H-NS mutants. First, since multicopy StpA and H-NS share many activities, it is necessary to use a genetic background which is *hns* to reveal the activity of StpA. Second, since *stpA* is usually only poorly expressed from the chromosome (in both *hns*$^+$ and *hns* backgrounds), *stpA* genes must all be placed on multicopy plasmids (41). To observe the activity of StpA, we used the TP2600Δ*lacX74 hns* host strain transformed with the *lac* expression vector pRW*proU*. In this background, the repressor activity of native multicopy *stpA* from plasmid pD StpA can be observed at the *proU* promoter. These cells can then be cotransformed with a second plasmid encoding the StpA derivatives, in this case the compatible plasmid pYC. In this system, any inhibition of native StpA activity at the *proU* promoter by the mutant StpA can be determined.

The extent of the homology between H-NS and StpA was probed by constructing N- and C-terminally altered StpA mutants, predicted to be dominant negative on the basis of our findings for H-NS. The StpA derivatives constructed for study were StpA L26P, StpA Δ65C, StpA Y97C, and StpA P116S.

TABLE 4. Sequences and in vivo activities of the four StpA mutants

| pYCStpA derivative | Codon change | Expression of the *proU* promoter in TP2600[a] (U of β-galactosidase activity) | |
|---|---|---|---|
| | | pDU9 | pDStpA |
| Wild type | None | 28 | 25 |
| L26P | CTG to CCG | 260 | 65 |
| Δ65C[b] | ΔORF codons 66–136 | 190 | 50 |
| Y97C | TAT to TGT | 240 | 45 |
| P116S | CCA to TCA | 265 | 40 |
| pACYC184 control[c] | | 250 | 30 |

[a] TP2600Δ*lacX74 hns* cells carrying the *proU* promoter, cloned in pRW, were transformed with either pDU9 or pD StpA. The pDU9- and pD StpA-containing cells were then transformed with pYC StpA and mutant derivatives. These cells were grown to mid-log phase, and assays were performed as described in Materials and Methods.

[b] The *stpA*Δ65C allele has a deletion of the *stpA* ORF from codon 65 to the *stpA* stop codon. The mutagenic primer used was designed to create simultaneously the large deletion and the insertion of a C-terminal cystine at position 66. This alteration was made to mimic that found in the H-NSΔ64C mutant. The one amino acid difference in length is a consequence of the difference in positions of the methionine residues in StpA and H-NS; StpA has M-65, whereas H-NS has M-64.

[c] As a control for the measurement of the StpA repressor activity, we determined the β-galactosidase activity in TP2600*hns*[pRW*proU*] cells transformed with the vector-only plasmids pACYC184 and pDU9. As a control for the measurement of StpA dominant negative activity, we used TP2600*hns*[pRW*proU*] cells transformed with plasmids pACYC184 and pD StpA.

Native, multicopy StpA represses transcription from the *proU* promoter ninefold, as demonstrated by the lower level of β-galactosidase activity in the TP2600Δ*lacX74 hns*[pRW*proU*][pD StpA][pACYC184] cells than in the control cells containing pDU9 and pACYC184 (Table 4). In comparison with native StpA, the StpA mutants have a sevenfold-reduced ability to repress *proU* (Table 4). Therefore, the mutations have all affected the normal activity of multicopy StpA and thus parallel the known effects of the mutations in H-NS.

The normal activity of native multicopy StpA at *proU* is inhibited in the presence of the StpA mutants. The data in Table 4 indicate a 1.2- to 2.2-fold increase in β-galactosidase activity in the TP2600Δ*lacX74 hns*[pRW*proU*][pD StpA] cells containing the pYC StpA mutants in comparison with the activity observed in the control cells. This inhibition of native StpA activity demonstrates that all of the StpA mutants are dominant negative. We conclude that mutations originally found to cause dominant negative activity in H-NS have similar effects when introduced into the new context of multicopy StpA.

**Dominant negative H-NS mutants disrupt the activity of native StpA.** The results presented above establish that by using coexpression from multicopy plasmids, dominant negative effects can be detected between native StpA and the StpA mutants. It occurred to us that if the StpA mutants were substituted by the H-NS mutants, we could then check whether the altered H-NS also affected promoter repression by native StpA.

The TP2600[pRW*proU*] host strain was transformed with either pD StpA or pDU9. In turn, these cells were transformed with pLG H-NS or its mutant derivatives. The data from measurements made in the TP2600[pRW*proU*][pDU9] cells carrying the H-NS mutants (Table 5) indicate that, as before, the

TABLE 5. In vivo activities of the 12 dominant negative H-NS mutants in the presence of wild-type StpA

| pLG H-NS derivative | Expression of the *proU* promoter in TP2600[a] (U of β-galactosidase) | |
|---|---|---|
| | pDU9 | pDStpA |
| Wild type | 25 | 19 |
| L26P | 190 | 65 |
| E53G T55P | 165 | 55 |
| Δ64C | 155 | 80 |
| Y97C | 170 | 35 |
| Y97H | 190 | 30 |
| Y97S | 190 | 40 |
| T110A | 180 | 30 |
| G113S | 200 | 50 |
| P116S | 220 | 65 |
| I119T | 210 | 55 |
| Δ121 | 240 | 50 |
| F133S | 230 | 50 |
| pLG339 control[b] | 290 | 26 |

[a] TP2600Δ*lacX74 hns* cells carrying the *proU* promoter, cloned in pRW, were transformed with either pDU9 or pD StpA. The pDU9- and pD StpA-containing cells were then transformed with pLG H-NS and mutant derivatives. These cells were grown to mid-log phase, and assays were performed as described in Materials and Methods.

[b] As a control for the measurement of H-NS repressor activity, we determined the β-galactosidase activity in TP2600*hns*[pRW*proU*] cells transformed with the vector-only plasmids pLG339 and pDU9. As a control for the measurement of H-NS disruption of StpA activity, we used TP2600*hns*[pRW*proU*] cells transformed with plasmids pLG339 and pD StpA.

TABLE 6. In vivo activities of the four StpA mutants in the presence of wild-type H-NS

| pD StpA derivative | Expression of the *proU* promoter in TP2600[a] (U of β-galactosidase) | |
|---|---|---|
| | pLG339 | pLG H-NS |
| Wild type | 28 | 18 |
| L26P | 180 | 30 |
| Δ65C | 165 | 40 |
| Y97C | 140 | 35 |
| P116S | 170 | 85 |
| pDU9 control[b] | 230 | 24 |

[a] TP2600Δ*lacX74 hns* cells carrying the *proU* promoter, cloned in pRW, were transformed with either pLG339 or pLG H-NS. The pLG339- and pLG H-NS-containing cells were then transformed with pD StpA and mutant derivatives. These cells were grown to mid-log phase, and assays were performed as described in Materials and Methods.

[b] As a control for the measurement of StpA repressor activity, we determined the β-galactosidase activity in TP2600*hns*[pRW*proU*] cells transformed with the vector-only plasmids pLG339 and pDU9. As a control for the measurement of StpA disruption of H-NS activity, we used TP2600*hns*[pRW*proU*] cells transformed with plasmids pLG H-NS and pDU9.

H-NS mutants have a greatly reduced ability to repress the *proU* promoter. Strikingly, the data from measurements made in the TP2600[pRW*proU*][pD StpA] cells containing the H-NS derivatives (Table 5) show that in the presence of the H-NS mutants, the repressor activity of the multicopy native StpA is inhibited. This is demonstrated by the 1.5- to 3-fold increase in β-galactosidase activity in the cells containing mutant H-NS in comparison with the control cells containing pD StpA and the control plasmid pLG339. The observed inhibition of StpA activity by the H-NS mutants indicates that they have the ability to interfere with the action of StpA at the *proU* promoter. A simple explanation for this observation is that the H-NS mutants directly affect native StpA activity via protein-protein interactions, resulting in the formation of a hybrid species with reduced function. In short, native StpA appears to be unable to discriminate between itself, its mutated derivatives, and homologous H-NS mutants.

**A reciprocal effect: dominant negative StpA mutants disrupt the activity of native H-NS.** Conversely, are the dominant negative StpA mutants also able to effect the activity of native H-NS? To test this, the TP2600[pRW*proU*] host strain was first transformed with plasmid pLG H-NS encoding native H-NS. These cells were then transformed with pD StpA and its mutant derivatives. As a control for the activity of the *stpA* alleles, they were also transformed into a strain in which pLG H-NS was substituted by the blank control plasmid pLG339.

The data in Table 6 show that as before, the StpA mutants have a greatly reduced ability to repress the *proU* promoter in comparison with wild-type multicopy StpA. The data also show that in the presence of the mutant StpA, the normal repression of *proU* expression by H-NS is disrupted. This is demonstrated by the 1.2- to 3.5-fold increase in β-galactosidase activity in cells containing the StpA mutants in comparison with control cells containing pLG H-NS and the pDU9 vector. In addition, the StpA mutants were observed to disrupt *proU* repression in the *hns*$^+$ *proU-lacZ* fusion strain, GJ74 (data not shown). Hence, the mutant StpA is apparently able to inhibit the activity of native H-NS. A simple explanation for this observation mirrors the one suggested above: a hybrid species with reduced function could form between native H-NS and the mutant StpA.

**Cross-regulation of the *hns* and *stpA* promoters.** Native H-NS and StpA are capable of both negative autogenous control and cross-repression (9, 34, 41). We anticipated that the dominant negative mutants could inhibit this repression; therefore, we compared the quantity of native *hns* and *stpA* mRNA transcripts present in cells containing either a dominant negative mutant or a control plasmid bearing a wild-type gene. The results of this primer extension analysis demonstrate that the *hns* and *stpA* promoters are, respectively, four- and twofold

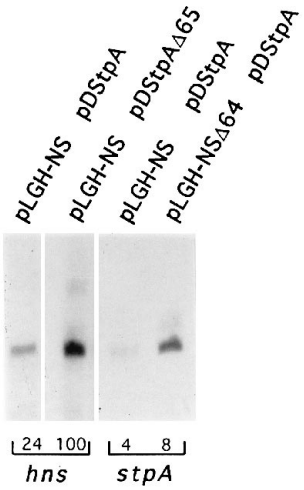


FIG. 3. Relative quantitation of *hns* and *stpA* mRNA transcripts by primer extension. The left half shows the cDNA product corresponding to the *hns* mRNA transcript in TP2600[pRW*proU*][pLG H-NS] cells cotransformed with either wild-type *stpA* (lane 1) or the *stpAΔ65C* dominant negative allele (lane 2). The right half shows cDNA product corresponding to the *stpA* mRNA transcript in TP2600 [pRW*proU*][pD StpA] cells cotransformed with either wild-type *hns* (lane 3) or the *hnsΔ64C* dominant negative allele (lane 4). The radioactivity in each cDNA product (expressed as arbitrary units) is shown at the bottom. Exposure of the autoradiograph of the *stpA* cDNA (lanes 3 and 4) was three times longer than that for the *hns* cDNA (lanes 1 and 2).

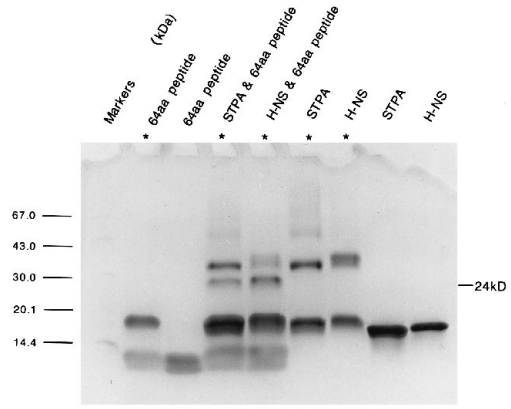


FIG. 4. Coomassie blue-stained SDS-polyacrylamide gel showing the analysis of protein-protein cross-linking reactions. An asterisk indicates addition of cross-linking reagents: Lane (from left to right): low-molecular-weight protein markers (Pharmacia); H-NSΔ64 $His_6$ peptide (8 kDa) cross-linked (the 8-kDa band corresponds to the 64-amino-acid [64aa] peptide, and the more slowly migrating species corresponds to a protein with a molecular mass of 16 kDa); H-NSΔ64 $His_6$ peptide, no cross-link; StpA $His_6$ and H-NSΔ64 $His_6$ peptide cross-linked; H-NS $His_6$ and H-NSΔ64 $His_6$ peptide cross-linked (the expected position for a ≈24-kDa species is indicated on the right); StpA $His_6$ cross-linked; H-NS $His_6$ cross-linked; StpA $His_6$, no cross-link; H-NS $His_6$, no cross-link.

more active in the presence of a dominant negative mutant (Fig. 3). For the *hns* promoter, this is apparent when the quantities of *hns* transcript in lanes 1 (pLG H-NS and pD StpA) and 2 (pLG H-NS and pD StpAΔ65C) are compared. The presence of the StpAΔ65C mutant causes an enhancement in the expression of wild-type *hns* mRNA. A similar effect is apparent at the *stpA* promoter when the quantities of *stpA* transcript in lanes 3 (pD StpA and pLG H-NS) and 4 (pD StpA and pLG H-NSΔ64C) are compared. The presence of the H-NS mutant enhances expression of wild-type *stpA* mRNA.

Importantly, these results indicate that the dominant negative properties of the mutants at *proU* and *5A6AgalP1* could not originate via an enhanced repression of the *hns* or *stpA* promoter. The mutants clearly do not increase the repression of these promoters, and so they would be very unlikely to cause a reduction in the quantity of native H-NS or StpA protein present in the cell. These results again lead to the interpretation that dominant negative activity occurs at the level protein-protein interaction and hybrid species with compromised function are formed.

**Detection of H-NS hybrids in vitro.** At low concentrations, native H-NS has been shown by chemical cross-linking to be a dimer with an apparent molecular mass of ≈32 kDa (14, 29). To demonstrate the existence of hybrid H-NS and StpA species in vitro, we used the truncated dominant negative H-NSΔ64 mutant. The significant difference in mass between the H-NSΔ64 mutant (8 kDa) and full-length H-NS and StpA (≈16 kDa) suggests that an H-NSΔ64–H-NS or H-NSΔ64–StpA hybrid could be detected by looking for a cross-linked species with a molecular mass of ≈24 kDa. StpA, H-NS, and the H-NSΔ64 peptide were purified by using a His tag procedure (see Materials and Methods) and used in EDC-NHS-catalyzed cross-linking reactions (Fig. 4). Intact fusion proteins were used in these experiments, as DNase I footprinting and gel retardation studies in vitro, as well as in vivo complementation studies, have failed to reveal differences between the recombinant and native proteins (data not shown).

In the absence of additional proteins, the 8-kDa H-NSΔ64C peptide can be cross-linked into a 16-kDa species, indicating that the peptide has the capacity to interact with itself and form dimers (lane 2). In contrast, when either full-length H-NS or StpA is mixed with this 8-kDa peptide, a ≈24-kDa species is apparent (lanes 4 and 5). As this 24-kDa species is not present when the peptide is cross-linked to itself or when H-NS or StpA alone is cross-linked (lanes 6 and 7), it is most likely the result of full-length H-NS or StpA interacting with the 8-kDa N-terminal peptide of H-NS and forming a cross-linkable 24-kDa hybrid species. This 24-kDa hybrid species is clearly detectable, as its molecular mass allows it to migrate to a position on the gel different from those of the 8-, ≈16-, and ≈32-kDa bands also produced.

The results of these in vitro protein-protein cross-linking experiments provide firm support for the idea that the dominant negative activity of the H-NS and StpA mutants can occur at the level of protein-protein interaction and that H-NS and StpA form hybrid species. In addition, the finding that the N-terminal 64 amino acids of H-NS can be cross-linked into an 16-kDa species suggests that the N terminus of H-NS contains a dimer interface.

## DISCUSSION

The results presented in this report allow us to (i) propose possible molecular mechanisms explaining the dominant negative effects of the H-NS mutants, (ii) clarify the origin of the functional similarity between H-NS and StpA at the molecular level, and (iii) establish that cross-talk between native H-NS and StpA does not occur solely at specific promoters but also functions by protein-protein interaction and the formation of hybrid species.

The most likely explanation for the dominant negative activity of the mutants involves the formation of hybrid species with compromised function. Consistent with this idea are the results of the protein-protein cross-linking experiments, which directly demonstrate the existence of such mutant–wild-type hybrids in vitro (Fig. 4). In addition, there is no correlation between the residual abilities of the mutants to repress either of the test promoters and their rather uniform dominant negative activity. Hence, their dominant negative activity cannot be explained solely by an alteration in the ability to bind DNA target sites (Fig. 2). Possible alternative explanations for dominant negative activity have been investigated and shown to be lacking. First, aberrant regulatory effects at the *hns* and *stpA* promoters by the mutants can be excluded, as demonstrated by the primer extension analysis of *hns* and *stpA* mRNAs (Fig. 3). Second, and consistent with the results from the primer extension analysis, the mutants have only a residual ability to repress the *proU* and *5A6AgalP1* promoters; these promoters are clearly not subject to an enhanced repression by the mutants (Table 3).

The nature, locations, and effects of the mutations characterized indicate that H-NS most probably contains two domains. Evidence for the two-domain structure of H-NS comes from (i) the isolation, characterization, and cross-linking of the H-NSΔ64C dominant negative mutant, consisting of amino acids 1 to 64, and (ii) the nuclear magnetic resonance structural study of a stable C-terminal H-NS fragment, consisting of amino acids 91 to 137 (28) (Fig. 1). Mutations resulting in dominant negative activity have been isolated in both domains.

Both curved DNA binding (43) and higher-order protein multimerization have been implicated in the normal repressor activity of H-NS (11, 20, 21), and thus disruption of either could result in dominant negative activity. Our data are compatible with the idea that the majority of the C-terminal mutations affect a single H-NS property (Fig. 2A). As they fall in

a region of H-NS implicated in DNA binding, it is probable that they disrupt this H-NS property (Fig. 1). Hence, a likely molecular mechanism explaining the dominant negative activity of these mutants is that they form hybrid species with the native H-NS in the cell and so compromise the ability of H-NS to bind target sites. The N-terminal substitutions (L26P and E53G T55P) fall far from the supposed DNA binding domain and, in addition, have effects at the *proU* and *5A6AgalP1* promoters different from those of the C-terminally substituted H-NS (Fig. 2A and reference 36a). Hence, we consider that these mutations are unlikely to directly effect the H-NS DNA binding activity. We propose that these alterations affect the ability of H-NS to oligomerize and so could inhibit the formation of high-order protein assemblies.

The differential effects of the C- and N-terminal mutants at the *proU* and *5A6AgalP1* promoters can be tentatively explained if the most important H-NS property required for *proU* repression is formation of higher-order multimers, while in contrast at *5A6AgalP1*, promoter repression might have a higher requirement for curved DNA recognition by H-NS. Indeed, the known characteristics of the *5A6AgalP1* and *proU* promoters indicate that this could be the case. Upstream curved sequences have been shown to modulate repression of the *galP1* promoter (43), and extended nucleoprotein structures have been demonstrated at the *proU* promoter (20). In addition, no obviously curved DNA sequences have been detected in the primary H-NS target site in the *E. coli proU* promoter (20). It is possible that these two apparently separable H-NS properties vary in importance, depending on the promoter context, and hence could be exploited for the differential regulation of promoter activity.

The comparative analysis of native H-NS and StpA has revealed that the proteins have many functional and mechanistic parallels (27, 41). The ability of native H-NS and StpA to cross-talk was first apparent from the simultaneous cross-regulation of the *hns* and *stpA* promoters. It was possible to extend these simple cross-regulatory loops to the regulatory circuits governing the expression of many other genes. The master controller of many of these circuits appears to be H-NS, with StpA often serving as a molecular backup. However, the analysis of the global effects of StpA and H-NS indicate that this is not always the case, with a subset of genes being regulated exclusively by the combination of H-NS and StpA (41). One scenario which could account for this observation is that a hybrid H-NS–StpA species is strictly required in certain circumstances. In these situations, the specific function contributed by the StpA protein could be related to its RNA processing activity: a hybrid H-NS–StpA molecule could have different nucleic acid binding properties to either of the parent proteins and hence have a distinct biological role.

The StpA protein was examined for the presence of structural and functional similarities to H-NS. The results of our analysis of dominant negative StpA derivatives show that alterations originally found in H-NS retain an equivalent effect when introduced into the new context of StpA (Table 4). This finding indicates that the domain organization and function in StpA are closely related to those in H-NS. It follows that the origins of the dominant negative activity in H-NS and StpA are homologous. We propose that in StpA, as in H-NS, that dominant negative activity arises through the formation of hybrid species with specific defects in DNA binding and high-order protein oligomerization.

Further evidence for the existence of structural and functional homologies between H-NS and StpA comes from their inability to discriminate between one another. This is demonstrated by the promiscuous activity of both the H-NS and StpA dominant negative mutants in vivo (Tables 5 and 6) and the cross-linking of the H-NSΔ64C peptide to StpA in vitro (Fig. 4). Together with their high level of sequence identity, these findings suggest that they have functionally interchangeable protein-protein docking structures. One consequence of this in the cell should be that under the appropriate conditions, StpA and H-NS form a stable hybrid species. This could account for the existence of a subset of genes which require the simultaneous presence of both H-NS and StpA for their regulation and be of general importance for any global regulatory network involving H-NS and StpA (41).

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## REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology. John Wiley & Sons, New York.
2. **Barnes, W.** 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proc. Natl. Acad. Sci. USA **91:**2216–2220.
3. **Barry, T., S. Geary, S. Hannify, C. MacGearailt, M. Shalloo, D. Heery, F. Gannon, and R. Powell.** 1992. Rapid mini-preparations of total RNA from bacteria. Nucleic Acids Res. **20:**4940.
4. **Bell, A., K. Gaston, R. Williams, K. Chapman, A. Kolb, H. Buc, S. Minchin, J. Williams, and S. Busby.** 1990. Mutations that alter the ability of the *Escherichia coli* cyclic AMP receptor protein to activate transcription. Nucleic Acids Res. **18:**7243–7250.
5. **Bracco, L., D. Kotlarz, A. Kolb, S. Diekmann, and H. Buc.** 1989. Synthetic curved DNA sequences can act as transcriptional activators in *E. coli*. EMBO. J. **8:**4289–4296.
6. **Busby, S., D. Kotlarz, and H. Buc.** 1983. Deletion mutagenesis of the *E. coli* galactose operon promoter region. J. Mol. Biol. **167:**259–274.
7. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
8. **Dattananda, C. S., K. Rajkumari, and J. Gowrishankar.** 1991. Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and a negative regulatory element within the first structural gene. J. Bacteriol. **173:**7481–7490.
9. **Dersch, P., K. Schmidt, and E. Bremer.** 1993. Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth phase control and autoregulation. Mol. Microbiol. **8:**875–889.
10. **Finlay, C. A., P. W. Hinds, and A. J. Levine.** 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell **57:**1083–1093.
11. **Göransson, M., B. Sondén, P. Nilsson, B. Dagberg, K. Forsman, K. Emanuelsson, and B. Uhlin.** 1990. Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. Nature (London) **344:**682–685.
12. **Gowrishankar, J.** 1985. Identification of osmoresponsive genes in *Escherichia coli*: evidence for participation of potassium and proline transport systems in osmoregulation. J. Bacteriol. **164:**434–445.
13. **Gowrishankar, J.** 1989. Nucleotide sequence of the osmoregulatory *proU* operon of *Escherichia coli*. J. Bacteriol. **171:**1923–1931.
14. **Gualerzi, C. O., M. A. Losso, M. Lammi, K. Friedrich, R. T. Pawlik, M. A. Canonaco, G. Gianfranceschi, A. Pingoud, and C. L. Pon.** 1986. Proteins from the prokaryotic nucleoid. Structural and functional characterisation of the *Escherichia coli* DNA binding proteins NS(HU) and H-NS, p. 101–134. *In* C. O. Gualerzi and C. L. Pon (ed.), Bacterial chromatin.
15. **Grabarek, Z., and J. Gergely.** 1990. Zero-length crosslinking procedure with the use of active esters. Anal. Biochem. **185:**131–135.
16. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature (London) **329:**219–222.
17. **Lauder, S. D., and S. C. Kowalczykowski.** 1993. Negative co-dominant inhibition of RecA protein function. J. Mol. Biol. **234:**72–86.
18. **Lejeune, P., and A. Danchin.** 1990. Mutations in the *bglY* gene increase the frequency of spontaneous deletions in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **87:**360–363.

19. **Lodge, J., R. Williams, A. Bell, B. Chan, and S. Busby.** 1990. Comparison of promoter activities in *Escherichia coli* and *Pseudomonas aeruginosa*: use of a new broad-host-range promoter-probe plasmid. FEMS Lett. **67:**221–226.
20. **Lucht, J. M., P. Dersch, B. Kempf, and E. Bremer.** 1994. Interactions of the nucleoid-associated DNA binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. J. Biol. Chem. **269:**6578–6586.
21. **McGovern, V., N. P. Higgins, R. S. Chiz, and A. Jaworski.** 1994. H-NS over-expression induces an artificial stationary phase by silencing global transcription. Biochimie **76:**1019–1029.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Miller, J. H.** 1978. The operon, p. 31–88. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Oppenheim, A., and D. Noff.** 1975. Deletion mapping of *trans*-dominant mutants of the lambda repressor. Virology **63:**553.
25. **Perrin, S., and G. Gilliland.** 1990. Site-specific mutagenesis using asymmetric polymerase chain reaction and a single mutant primer. Nucleic Acids Res. **18:**7433–7435.
25a. **Rimsky, S.** Unpublished data.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Shi, X., and G. N. Bennett.** 1994. Plasmids bearing *hfq* and the *hns* like gene *stpA* complement *hns* mutants in modulating arginine decarboxylase gene expression in *Escherichia coli*. J. Bacteriol. **176:**6769–6775.
28. **Shindo, H., T. Iwaki, R. Ieda, H. Kurumizaka, C. Ueguchi, T. Mizuno, S. Morikawa, H. Nakamura, and H. Kuboniwa.** 1995. Solution structure of the DNA binding domain of a nucleoid-associated protein, H-NS, from *Escherichia coli*. FEBS Lett. **360:**125–131.
29. **Spassky, A., and H. Buc.** 1977. Physico-chemical properties of a DNA binding protein: *Escherichia coli* factor H1. Eur. J. Biochem. **81:**79–90.
30. **Spurio, R., M. Dürrenberger, M. Falconi, A. La Teana, C. Pon, and C. Gualerzi.** 1992. Lethal overproduction of the *Escherichia coli* nucleoid protein H-NS: ultramicroscopic and molecular autopsy. Mol. Gen. Genet. **231:** 201–211.
31. **Stoker, N., N. Fairweather, and B. Spratt.** 1982. Versatile low copy number plasmid vectors for cloning in *Escherichia coli*. Gene **18:**335–341.
32. **Thein, S. L., and J. Hinton.** 1991. A simple and rapid method of direct sequencing using Dynabeads. Br. J. Haematol **79:**113–115.
33. **Tupper, A. E., T. A. Owen-Hughes, D. W. Ussery, S. S. Santos, D. J. P. Ferguson, J. M. Sidebotham, J. C. D. Hinton, and C. F. Higgins.** 1994. The chromatin-associated protein H-NS alters DNA topology *in vitro*. EMBO. J. **13:**258–268.
34. **Ueguchi, C., M. Kakeda, and T. Mizuno.** 1993. Autoregulatory expression of the *Escherichia coli hns* gene encoding a nucleoid protein: H-NS functions as a repressor of its own transcription. Mol. Gen. Genet. **236:**171–178.
35. **Ueguchi, C., and T. Mizuno.** 1993. The *E. coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. **12:**1039–1046.
36. **Ussery, D. W., J. C. D. Hinton, B. J. A. M. Jordi, P. E. Granum, A. Seirafi, R. J. Stephen, A. E. Tupper, G. Berridge, J. M. Sidebotham, and C. F. Higgins.** 1994. The chromatin associated protein H-NS. Biochimie **76:**968–980.
36a. **Williams, R.** Unpublished data.
37. **Yamada, H., T. Yoshida, K. Tanaka, C. Sasakawa, and T. Mizuno.** 1991. Molecular analysis of the *Escherichia coli hns* gene encoding a DNA binding protein, which preferentially recognizes curved DNA sequences. Mol. Gen. Genet. **230:**332–336.
38. **Yamada, H., S. Muramatsu, and T. Mizuno.** 1990. An *Escherichia coli* protein that preferentially binds to sharply curved DNA. J. Biochem. **108:**420–425.
39. **Zhang, A., and M. Belfort.** 1992. Nucleotide sequence of a newly-identified *Escherichia coli* gene, *stpA*, encoding an H-NS like protein. Nucleic Acids Res. **20:**6735.
40. **Zhang, A., V. Derbyshire, J. Galloway Salvo, and M. Belfort.** 1995. *E. coli* protein StpA stimulates self-splicing by promoting RNA assembly. RNA **1:**783–793.
41. **Zhang, A., S. Rimsky, M. Reaban, H. Buc, and M. Belfort.** 1996. *Escherichia coli* protein analogs StpA and H-NS: regulatory loops, similar and disparate effects on nucleic acid dynamics. EMBO J. **15:**1340–1349.
42. **Zhou, Y., X. Zhang, and R. Ebright.** 1991. The random mutagenesis of gene sized DNA molecules by use of PCR and *Taq* DNA polymerase. Nucleic Acids Res. **19:**6052.
43. **Zuber, F., D. Kotlarz, S. Rimsky, and H. Buc.** 1994. Modulated expression of promoters containing upstream curved DNA sequences by the *Escherichia coli* nucleoid protein H-NS. Mol. Microbiol. **12:**231–240.