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A ROLE FOR PROTEIN HNS IN CHROMOSOME STRUCTURE AND TRANSCRIPTIONAL SILENCING

by

VICTORIA PAULINE McGOVERN

A DISSERTATION

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ABSTRACT OF DISSERTATION
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The factors that shape the bacterial equivalent of eukaryotic chromatin, the nucleoid, must both compact a length of DNA thousands of times yet allow or participate in changes in gene expression. This dissertation defines new biochemical and genetic activities of HNS, one of the two most abundant nucleoid-associated proteins. Further, it shows HNS can work in opposition to the other abundant nucleoid associated protein, HU, and finally, it shows that the effects of the two proteins in the cell are dramatically different.

This study began with genetic characterization of a role for HNS in stabilizing bacteriophage Mu lysogens and biochemical studies indicating a role for HNS in increasing the binding of the Mu repressor to its binding site.

Next, biochemical studies showed that HNS stabilizes supercoils in vitro, an activity that had been thought unique to HU. Together with unwinding of the double helix by RNA polymerase, supercoiling stabilized by the two proteins

can explain most of the constrained supercoiling known to exist in bacteria.

Finally, studies of the effects of overexpressing the proteins in rapidly growing cultures showed that, while HU overexpression has little effect on the growth state of the cells, overexpression of HNS immediately stops growth of the culture and silences transcription. These differences in vivo, coupled with the biochemical antagonism of the proteins, suggest a model for the organization of the nucleoid into active and inactive regions, one governed by HU and the other by HNS.

Abstract Approved by: Committee Chairman Pat Higgins

Program Director Jeffrey H. Engbaugh

Date 9/22/92 Dean of Graduate School W. A. Fobley

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I asked my undergraduate advisors how to choose a graduate advisor: two of them were evasive and the third told me to tell him the answer in the morning. Later I realized they had answered the question- advisors are sneaky that way. I must thank the advisor I chose, Pat Higgins, who answered some questions more or less directly, taught me a number of interesting things, and paid for this adventure. I am indebted to Mark Winn for his critical reading of parts of this thesis and grateful to the members of my committee, Jeff Esko, Steve Harvey, Tim Townes and Chuck Turnbough for their advice and their time.

I also thank the people who have passed through the Higgins lab in my time here. The Townes and Turnbough labs, too, have been sources of information, materials, and people with good ideas: I appreciate their help.

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LIST OF ABBREVIATIONS

A	adenine
Amp	ampicillin
Ap	ampicillin
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
C	cytosine
cam	Chloramphenicol
Ci	curie
cpm	counts per minute
D	dimensional
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
FIS	factor for inversion stimulation
G	guanine
g	gram
g	acceleration due to gravity
h	hour
HMG	high mobility group
IHF	integration host factor
IPTG	isophenylthiogalactoside
kan	kanamycin
KGB	potassium glutamate buffer

LIST OF ABBREVIATIONS (Continued)

kn	kanamycin
LB	Luria broth
min	minute
M	molar
mg	milligram
mM	millimolar
mmol	millimole
mRNA	messenger RNA
N	any nucleotide
ng	nanogram
nM	nanomolar
nmol	nanomole
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pM	picomolar
pmol	picomole
r	resistant
RNA	ribonucleic acid
sec	second
SDS	sodium dodecyl sulfate
T	thymine
TCA	trichloroacetic acid
TBE	tris borate EDTA
Tet	tetracycline
Tris	tris (hydroxymethyl) aminomethane

LIST OF ABBREVIATIONS (Continued)

UV	ultraviolet
W	purine
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactoside
Y	pyrimidine
βgal	beta galactosidase
μg	microgram
μl	microliter
μM	micromolar
μM	micromole
σ	specific linking difference

INTRODUCTION

Prokaryotes and eukaryotes share the problem of organizing their genome so that it is compacted thousands of fold in length but remains accessible to regulatory and other factors. Both begin to solve this problem by maintaining their DNA in a supercoiled form. Supercoiling may be accomplished by distortion of the double stranded axis- overwinding or underwinding the DNA, or may reflect DNA writhing in space. Writhing may take the form of DNA wrapping around protein, but writhe can also be manifested in DNA turning around itself in space, creating an interwound structure. Investigation of the supercoiling state of the genome reveals a fundamental difference between prokaryotic and eukaryotic chromosomes: while eukaryotes constrain essentially all of this supercoiling (70) in the form of DNA writhe about the surface of the histones (reviewed in 26), about half of the supercoils in *Escherichia coli* are in an unconstrained interwound form (4) that can be relaxed by nicking the DNA. The remaining half of the supercoils in prokaryotes are somehow constrained (58,71). What are the factors responsible for this constraint?

Eukaryotes compact and constrain their DNA into chromatin, the DNA and associated proteins (reviewed in 21,26). However, the factors that shape the bacterial

chromosome are only beginning to be understood. While it has been useful to look to eukaryotic chromatin for the principles involved in building large DNA/protein complexes, searches for the bacterial equivalents of eukaryotic histones quickly found that the bacterial counterpart of chromatin is a more fragile structure than eukaryotic chromatin. Initial attempts to identify and purify the proteins of the nucleoid, the genome and its associated proteins, found six proteins(19).

The most abundant of these six proteins, HU, was named initially as a Histone-like protein from *Escherichia coli* strain U93, but contemporaneously purified as NS, a factor capable of making a ribosome fraction behave like a Native Subunit of the ribosome (75) and as HD, a histone-like DNA binding protein (2).

The far less abundant integration host factor (IHF), was named for its role in lambda biology (53). FIS, the factor for inversion stimulation in site specific recombination, was later discovered as another nucleoid protein involved in the life cycle of bacteriophage lambda (44).

HNS, the second most abundant nucleoid associated protein, was named as a chromosomal protein different from HU (Hetero-NS) and also found as a factor called H1 (72) and discovered again and named B1 (57). H, another abundant factor named for its histone-like character, was identified as an abundant protein that cross reacts with antibodies

against histone H2A (40). Histone-like protein 1 (HLP1) (47) was first genetically identified as *firA* (1), a factor that protects cells against rifampicin.

Later studies have shown that H and HLP1 are not truly nucleoid associated proteins: H was identified as ribosomal subunit S3 (7) and HLP1 as an outer membrane protein (1,12). Both of these proteins may have become associated with nucleic acids during cell lysis and later co-purified with the DNA binding proteins. Currently there are only four known major nucleoid associated proteins, IHF, HU, FIS and HNS (67,68).

HU and IHF have sequence properties in common and may be evolutionarily related. Both proteins are heterodimers encoded by unlinked genes. The two HU subunits share 70% homology at the amino acid level, and the IHF subunits have as much in common with the two HU proteins as they do with each other, suggesting that the two IHFs and one copy of HU, later duplicated, arose from a common ancestor (24). Together IHF and HU are referred to as the type II DNA binding proteins (24) and have been called "histone-like" proteins (19). Like the eukaryotic histones, these proteins are small: in *Escherichia coli* HU's subunits are 9.5 and 9.2 kilodaltons (62), while IHF's are 11.2 and 10.5 kilodaltons. Both proteins are basic and heat stable. Both have been highly conserved across species and through time. While *Escherichia coli* and *Salmonella typhimurium* both have heterodimeric HU proteins, some other bacteria have only one

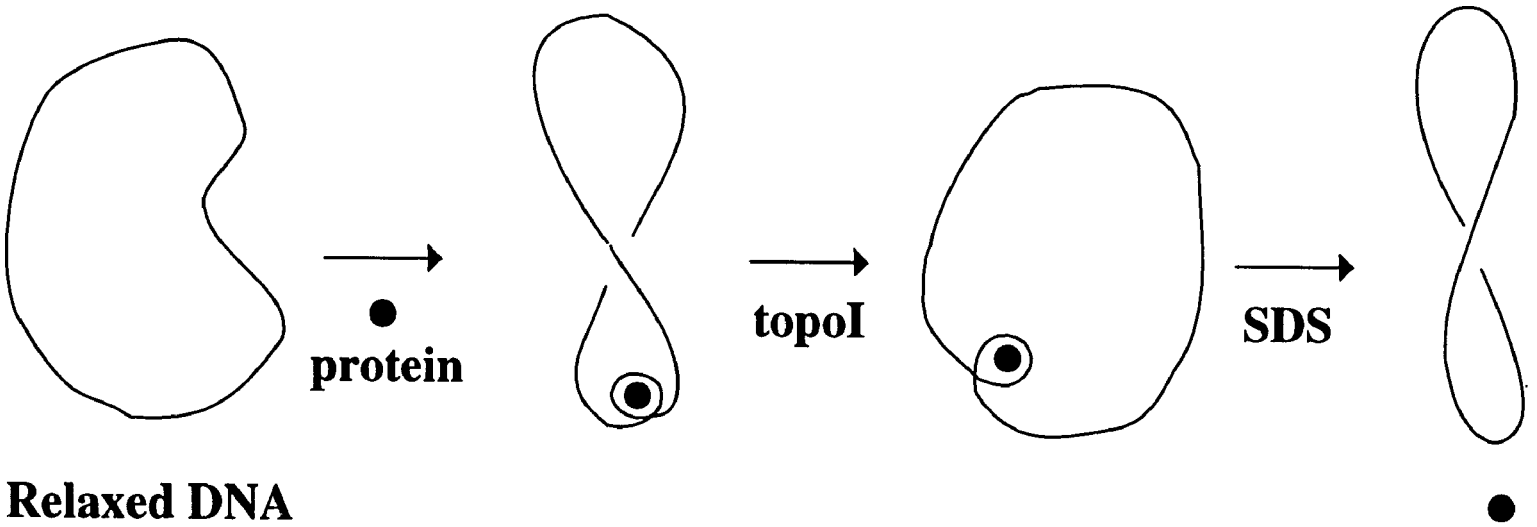
HU species, forming a homodimer (19). The two subunits of the HU heterodimer show striking sequence conservation between *Salmonella typhimurium* and *Escherichia coli*. In the 150 million years since the two species diverged, only one amino acid in the two HU subunits has been changed. Although these proteins are highly conserved, they are not encoded by essential genes (35).

HU, like IHF, has been shown to influence the curvature of DNA *in vitro* (37). Like the eukaryotic nucleosome, HU is able to stabilize supercoils in DNA *in vitro* (6,63). While HU is highly abundant, present at more than 30,000 copies per cell (14), IHF is less plentiful, present at about 1000 copies (68). Although HU has physical properties in common with IHF, a sequence specific DNA binding protein, and may bend DNA, it does not bind to a specific consensus sequence. A crystal structure for the homodimeric HU analog from *Bacillus stearothermophilus* has been determined, but the structure does not allow insight into how the protein binds to DNA (19). Though it is believed that alpha helical arms of the protein dimer interact with DNA, it is not clear whether the protein binds in the major groove or minor groove. Regions of conservation between the HUs of different species, and between HU and IHF, lie in the alpha helical arm regions and in the dimerization region.

Even though IHF and HU have properties in common with histones, protein-DNA complexes built with them only partly mimic the behavior of the protein-DNA structures formed with

true histones. HU, but not IHF, can stabilize supercoils *in vitro*, as histones can. This stabilization was demonstrated by a topological assay in which HU is bound to relaxed DNA (Figure 1). Protein binding can stabilize a supercoil by unwinding or overwinding the double helix, by wrapping the DNA or otherwise stabilizing DNA writhe, or by a combination of changing twist and writhe. Because the plasmid backbone is not broken by the binding, the plasmid compensates for the distortion by twisting the helix or writhing in space in a manner equal and opposite to that introduced by the protein binding. If the plasmid is then exposed to an enzyme that removes all strain on the DNA, the enzyme relaxes the compensating supercoils, leaving the protein-stabilized supercoils alone. When the proteins are stripped from the DNA by addition of SDS, the plasmid is left with a topology that reflects those changes in supercoiling associated with the previously bound protein. The effects on topology may be corroborated by the topology of plasmids isolated from *Salmonella typhimurium hupAhupB* double mutants, lacking both HU subunits. These cells show a loss of 10-15% of their supercoiling compared to wild type cells (36). HU bound to DNA can produce a nucleosome-like "beads on a string" pattern in transmission electron micrographs (32,63), but the "beads" observed are irregular in size and their appearance depends on how the sample is fixed for electron

Figure 1. A topological assay demonstrating stabilization of supercoils. Relaxed plasmid DNA is incubated with a factor that can stabilize supercoiling, either by altering the writhe or twist of the plasmid, or both (here shown as a left handed writhe about a protein). Because the plasmid backbone has not been broken, its linking number must remain unchanged. The topology of the plasmid compensates for the supercoil stabilized by the protein by writhing in space in an equal and opposite sense (shown as a right handed interwinding of the plasmid). Upon addition of topoisomerase I, this unbound writhe is relaxed, leaving the plasmid supercoiled by the continued binding of the supercoil-stabilizing protein. The topology of the plasmid can then be analyzed by agarose gel electrophoresis, allowing determination of both the magnitude of the supercoiling introduced and its positive or negative sign.


protein
topoI
SDS
Relaxed DNA

microscopy. *In vivo* antibody staining shows that the protein is found at the periphery of the nucleoid where transcription occurs (64), not at its core where a histone might be expected (20).

HU stimulates reactions involved in the site specific recombination activities of hin inversion (43) and Mu transposition (9), and also in replication of DNA from the chromosomal origin, oriC (14,56). The protein is a negative factor in the control of bacteriophage lambda replication (51). *In vitro*, it has been seen both inhibiting and stimulating transcription (2,25,49,62): it stimulates binding of purified CRP and purified lac repressor to DNA (25). HU has been shown to enhance bending of DNA and in its presence, ligase can circularize very small pieces of DNA (99 to 120 base pairs) that do not easily cyclize on their own (37). It can decrease the length of DNA needed between sites in systems that require separate regions of DNA to be brought together (42). It has been suggested that HU's range of activities reflects a role for the protein in making DNA more flexible (25).

Although HU participates in several DNA-oriented processes, comparison of its binding to various nucleic acids shows that it has a higher affinity for single stranded DNA and for RNA than for double stranded DNA (46).

IHF [reviewed in (27)], like HU, is a nonessential protein. It has been implicated as both a positive and negative factor in the transcriptional control of a number

of operons [reviewed in (27)]. While a consensus DNA sequence, YAANNNNTTGATW (29),has been determined for IHF binding, the protein does not bind every site that fits the consensus: it appears that context near the site is important. Consensus sequences located in AT rich regions are more often IHF binding sites than consensus sequences in less flexible regions of DNA (29). In many of the sites of IHF action studied, IHF interacts with other protein factors to change the local shape of the DNA, by bending the DNA or by forming multiprotein complexes around which the DNA is wrapped (34,59). IHF participates in lambda integration by helping to build such a complex. Another of the nucleoid associated proteins, FIS, participates in building a similar structure for the reverse reaction, lambda excision (77)

FIS is a homodimer of an 11.2 kilodalton protein. Estimates for the cellular level of FIS range from hundreds of copies (43) to thousands (77). The concentration of the protein drops significantly, about seventy fold, during stationary phase (77). Regulation of FIS transcription is sensitive to the growth phase of the cell and to the stringent response (55).

FIS is required for the assembly of the protein-DNA complex, the invertasome, involved in the hin and gin inversion site specific recombination reactions. It also interacts with an upstream activator sequence to transcriptionally activate several genes during the return of cells to rapid growth after stationary phase (54) but

works as a negative factor affecting its own transcription (55). FIS binds to a degenerate consensus sequence, (G/T)NNYRNN(A/T)NNYRNN(C/A) (38) and, like IHF, distorts the DNA backbone, bending it sharply (39).

HNS, the second most abundant nucleoid associated protein also binds to a consensus sequence (61), but the consensus, TNTNAN, is common. Like IHF, FIS and HU, it can bend DNA (83), and it shows an affinity for naturally bent regions of the chromosome (76). HNS is present at about 30,000 copies per cell and is most highly produced during stationary phase (73). It is encoded by a single gene, *hns*, and exists predominantly as a dimer in solution (22). It does not resemble the histones or the "histone-like" proteins in structure or charge. Though it compacts DNA *in vitro*, the physical properties of HNS are more reminiscent of the eukaryotic high mobility group protein HMG1 from yeast than they are of any histone (23). HNS is uncharged overall but contains a very high proportion of charged amino acids, forming acidic and basic patches. Protein isolations have shown that HNS is present in at least three forms, separable by two dimensional gel electrophoresis, that may reflect different modification states of the protein (73). The most abundant of these, originally called H1a, increases in concentration several fold during stationary phase (73).

HNS has recently been identified as the product of a single gene that has been discovered a number of times: HNS is defined as the product of the gene *hns*, located at 27

minutes on the *E. coli* chromosome but has been independently identified as a factor in the regulation of a number of transcriptionally repressed genes and operons (50). It has been named *bglY*, as a factor regulating sugar metabolism (10); *drdX*, as a locus involved in thermoregulation of pilliation (31), and as *virG* when regulating a family of other virulence factors in *Shigella* (17). It was named *pilG*, a locus playing a role in phase variation of fimbrae (74), as *cur*, a factor in cell clumping (13), and as *osmZ*, a regulator of the response of cells to changes in extracellular osmolarity (33).

In vitro, HNS has been seen to inhibit transcription from phage and *Escherichia coli* DNA. Its affinity for nucleic acids is different from that of HU: HNS binds more tightly to double stranded DNA than to other nucleic acids and prefers DNA overall to RNA. The affinity of HNS for DNA is higher than that of HU (22).

These four factors, along with the various specific regulatory factors, contribute to the architecture of the nucleoid. But the chromosome of a rapidly growing bacterial cell is a dynamic structure: it goes through changing patterns of gene expression in a life cycle that is orders of magnitude faster than that of most eukaryotic cells. Its DNA strands are constantly unwound, separated, and then resealed as transcriptional complexes pass by. How important are these changes in shaping the chromosome and making genes

available for expression, and how do the nucleoid associated proteins accommodate these changes?

Supercoiling in cells is not a passive force for DNA compaction. The level of supercoiling in cells appears to be carefully controlled. Bacteria contain the opposing enzymes gyrase and the ω protein, topoisomerase I. The activities of gyrase (reviewed in 8) include relaxing positive supercoils, creating negative supercoils, and in the absence of ATP, relaxing negative supercoils. Bacterial topoisomerase I only relaxes negative supercoils (80). Lethal topoisomerase I mutations can be suppressed by the acquisition of mutations in the genes for DNA gyrase, and cells carrying these secondary mutations have slightly decreased supercoiling (60).

Transcription of gyrase genes is sensitive to supercoiling: inhibition of gyrase with drugs, relaxation of template DNA and overexpression of topoisomerase I all lead to an increase in expression of the genes for both gyrase subunits (52). Transcription of the gene for topoisomerase I is stimulated by the opposite condition, an increase in negative supercoiling, and is inhibited in circumstances in which DNA becomes more relaxed (79). These linked and opposite effects of the two enzymes suggest that supercoiling is controlled by their action and not allowed to vary widely from some optimum level. Despite the effects of these opposed enzymes, genes have been identified whose transcription varies with template supercoiling (5,11,18).

Are these genes built to recognize and respond to variations in superhelicity away from this optimum level? The genes that have been identified as changing in expression have a variety of roles in the cell. It has been suggested that rather than maintain a single set point for supercoiling, bacteria use the level of supercoiling as a signal to activate transcription of genes whose promoters are especially sensitive to changes in supercoiling (15).

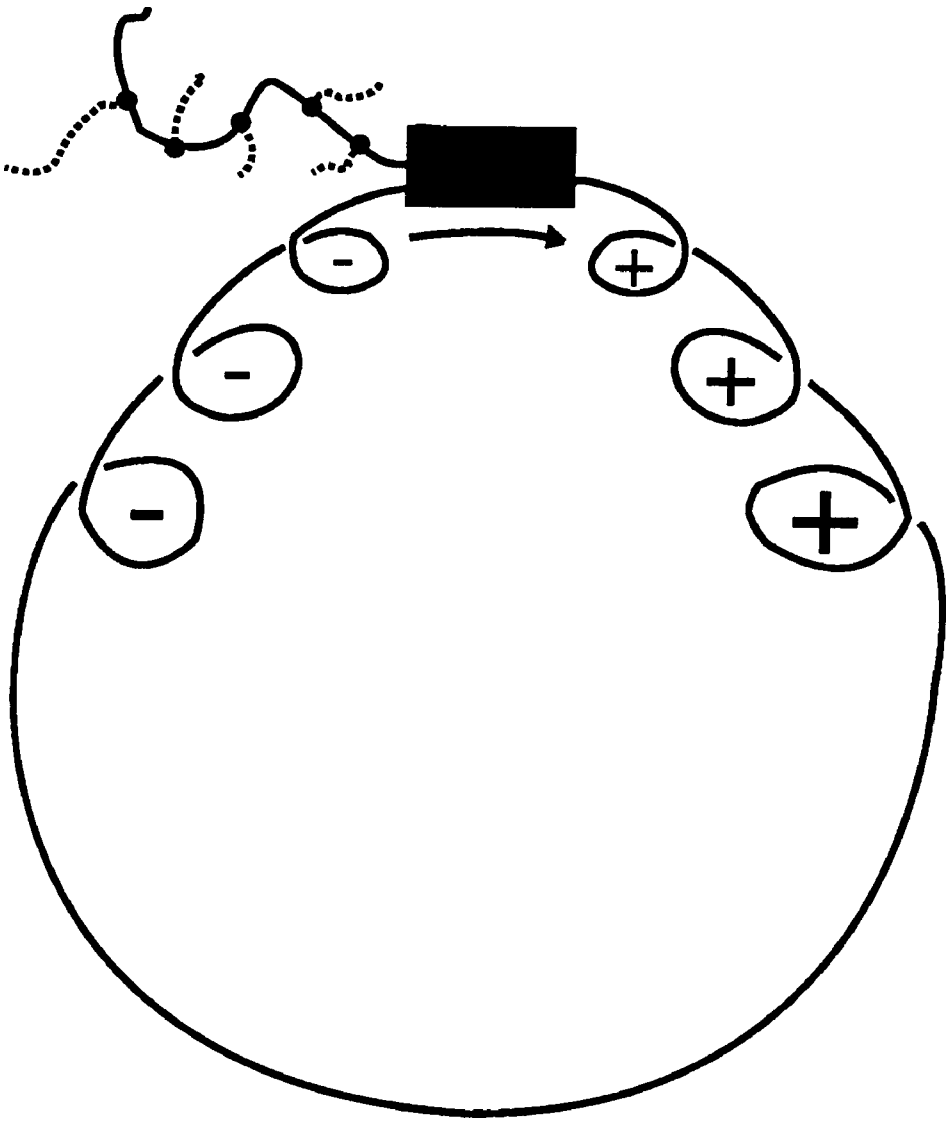
It also has been observed that some alterations in growth conditions including changes in incubation temperature, shifts from aerobic to anaerobic growth and changes in media composition can affect the supercoiling of plasmids isolated from cells or can affect the expression of genes in the chromosome (3,30,33). Factors other than the two opposed topoisomerases, then, may affect the superhelicity of DNA in the cell.

Liu and Wang pointed out another important factor affecting superhelicity (48): the movement of the transcription machinery along DNA is a generator of supercoiling. They proposed that because frictional drag will make it difficult for the large elongation complex to rotate in space through the cytosol separating the strands of the double helix along the DNA backbone as it passes, is a local positive supercoiling of the DNA ahead of the travelling polymerase and formation of a region of negative supercoiling behind it (Figure 2). Along with this dynamic formation of twinned domains of negative and positive

supercoiling, RNA polymerase also affects superhelicity just by the way that it binds DNA. The transcription complex separates the strands of the double helix over a length of about seventeen base pairs. This strand separation generates an unwinding of the DNA helix by about 580° (28,66). Since underwinding of the double helix is a form of negative supercoiling, RNA polymerase contributes to the local supercoiling in any region in two ways- by the strand separation that results from its binding and by the relative motion of the polymerase along the DNA backbone.

The Liu and Wang model suggests a reason for the use of two opposing topoisomerases: gyrase can remove positive supercoils ahead of the transcription machinery while topoisomerase I relieves the negative supercoils left in its wake. Several predictions from this model have been confirmed: transcription *in vitro* in the presence of topoisomerase I generates positive supercoils, which in the absence of topoisomerase I are annihilated by collision with negative supercoils (78). It has also been shown that stopping transcription in cells leads to a partial relaxation of reporter plasmids, supporting the idea that the transcriptional machinery is a supercoil generator (82). It has been shown that pBR322 grown in cells treated with the gyrase inhibitor novobiocin becomes positively supercoiled, and that this supercoiling is dependent on

Figure 2. The twin domain model of DNA supercoiling [figure after (78)]. When RNA polymerase (shown as a black box trailing a nascent RNA chain) separates DNA strands and travels through a gene, either the polymerase and its associated nascent RNA must swivel through the cytosol around the transcribed DNA, or else two domains of supercoiling must be created in the vicinity of the moving transcription complex: one, a zone of positive supercoiling or overwinding, travels ahead of the complex, while the other, a region of underwound or negatively supercoiled DNA trails behind it.



transcription from the strong promoter of the plasmid's tetracycline resistance gene (82).

Given that the transcriptional complex is a machine that makes supercoils, it is difficult to say whether changes in DNA topology during environmental shifts are the consequence of changes in transcription in the cell or are a signal, generated by some other mechanism, to activate a new set of promoters.

A model supporting the latter idea has been proposed concerning the role of H-NS in the chromosome. Rather than supporting a scheme in which the cell maintains its supercoiling near one set level, this model suggests that variations in DNA supercoiling levels seen in response to environmental changes allow the cell to use the chromosome as a "pleiotropic physiological barometer" (15). Such a model is most attractive because it gives the cell a way of alerting broad and different classes of genes to physiological changes, acting as a new global regulatory signal. It has been suggested that HNS plays an active role in determining the superhelicity of the chromosome, acting to establish the set point of the cell's supercoiling (41).

Given that promoter activities are changed by gyrase inhibitors (45, 65, 69), that some genes, including those for gyrase, use supercoiling as a cue for transcriptional regulation, and that promoter strengths *in vitro* can vary with supercoiling (45, 52), it is clear that some promoters are able to sense DNA topology. The question is whether the

cell uses this potential source of information in a global control system.

Physiological changes in DNA supercoiling increase and decrease the superhelicity of topological markers in the cell (16, 30, 33, 84), but the direction of changes in response to the same stimuli varies among closely related species (15, 30). The change in supercoiling is transient, with the average superhelicity of plasmids rapidly rebounding to a fairly constant level (3). DNA isolated from hns^- cells has been reported to have different topology than wild type cells, but these changes in topology have been variable, and changes have occurred both increasing and decreasing plasmid supercoiling (17, 33, 41).

The work in this thesis suggests a different model for the role of HNS in cells. In the first paper, we find a new role for this pleiotropic regulatory factor. In a series of genetic experiments carried out by N. Patrick Higgins, HNS was detected as a factor that stimulates Mu transposition (*smt*) by approximately two fold. A series of biochemical experiments done by Maurizio Falconi showed that small amounts of HNS increase the binding of Mu repressor to its binding site in the Mu early operator region. I demonstrated that the supercoiling of a topological marker plasmid in the *hns* strain is unaffected by the lack of HU. We conclude that the effect of HNS on a Mu lysogen in the cell is to stabilize repression of the Mu lytic functions by Mu repressor. In the second paper, I discuss stabilization of

supercoils *in vitro* by HNS, an activity that had been overlooked in earlier biochemical studies of the protein. The discovery that HNS can order supercoils may explain a major fraction of the constrained supercoiling not accounted for by binding of HU and RNA polymerase. I also demonstrate for the first time a biochemical interaction between HNS and HU, and find that they are antagonistic in their interaction with DNA. In the third paper, time course experiments done by Adam Jaworski and I examine, simultaneously, cell growth, protein production, DNA topology and transcription in cells that are overexpressing either of the two abundant supercoil stabilizing proteins, HU or HNS. We find that the consequences of their expression are drastically different. The series of experiments presented in this thesis leads me to propose a fundamentally different model for the role of HNS in chromosome organization.

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MUTATIONS ALTERING CHROMOSOMAL PROTEIN H-NS INDUCE MINI-MU TRANSPOSITION

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ABSTRACT

Bacteriophage Mu is one of the most efficient transposons known, capable of moving a hundred viral copies to new positions in the bacterial chromosome in an hour. Mu also forms stable lysogens. In bacteria lysogenic for the defective protein fusion-forming phage Mu*d*II1681, which can transpose and replicate but does not encode genes for DNA packaging and cell lysis, the frequency of transposition changes as colonies age. To find host genes that alter the spontaneous Mu transposition frequency, we used a genetic screen with mini-Mu*dlacZ* fusion formation as an assay. H-NS (also called H1a and B1) is an abundant non-specific DNA binding protein localized to the bacterial chromosome. H-NS has an unusual structure of interspersed patches of acidic and basic residues reminiscent of eukaryotic HMG proteins. Mutations in *hns* caused an increase in Mu-specific transcription and a dramatic increase in Mu*d*II1681 transposition rates when cells were put under certain growth conditions. Purified H-NS stabilized Mu repressor-DNA complexes *in vitro*, suggesting that H-NS contributes to the organization of transcriptionally inactive DNA *in vivo*.

INTRODUCTION

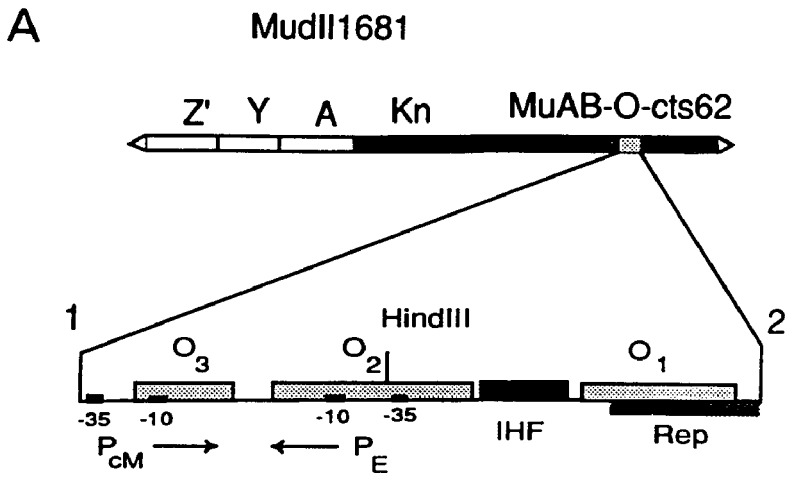
The movement of transposable elements is a readily observed genetic phenomenon in virtually all organisms (see Berg and Howe, 1989). Only a few regulatory mechanisms are known that influence transposition frequencies *in vivo* and these mechanisms affect individual transposons in very

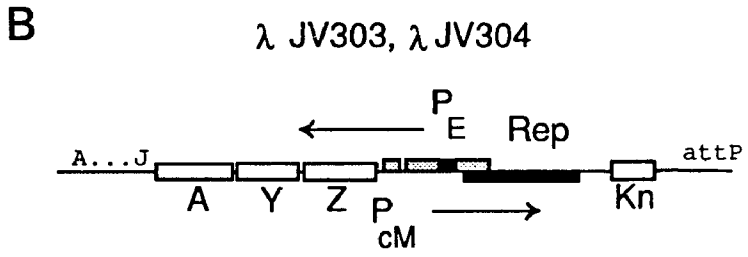
specific ways (Kleckner, 1990). No general regulatory system influencing more than one element is known.

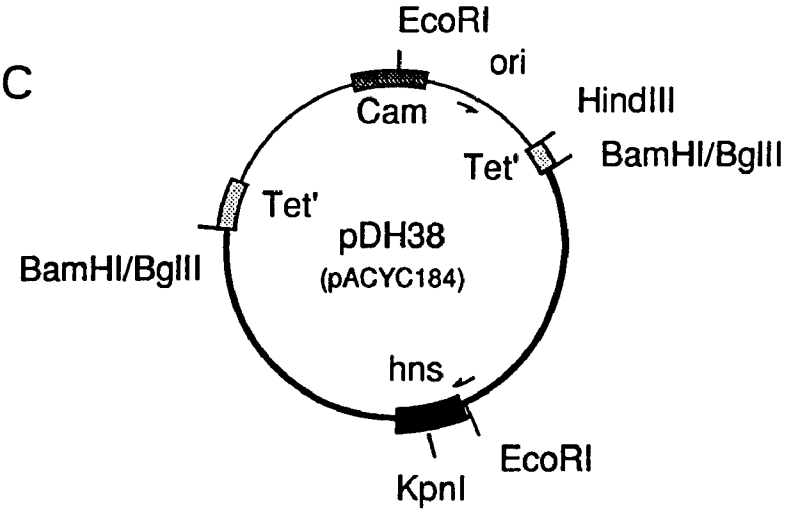
Mu is a transposon with a viral lifestyle, and its transposition mechanism is closely related to one that is also used by eukaryotic retroviruses (Fujiwara *et al.*, 1988). Mu is one of the most efficient transposons known, as it can hop to new chromosomal sites 100 times per hour under ideal conditions (Pato, 1989). Since transposition is linked to efficient replication, Mu is ideal for studying regulation of transposition. One method to visualize new transposition events in growing bacterial colonies employs a protein-fusion version of mini-Mu*dlac*, Mu*d*II1681 (Figure 1A; Castilho *et al.*, 1984). Mu*d*II1681 is proficient for transposition and contains a promoterless *lacZ* operon positioned at its right end. Synthesis of a *lacZ'*-fusion protein occurs when Mu hops into the correct reading frame of a gene that is being transcribed and translated. Cells undergoing transposition stain blue if XGal is present in the medium, and this method can be used to locate and quantitate transposing cell populations in bacterial colonies growing on solid media (Shapiro and Higgins, 1989).

Shapiro and Higgins demonstrated that wild type strains of *E. coli* frequently develop genetically distinct sectors with high transposition activity (1989). Using Mu*d*II1681 transposition as a screen, several genetic changes have been found that cause a stimulated Mu transposition (Smt)

Figure 1. Physical maps of plasmids and phage sequences. A. Mu*dII1681* contains the Mu A and B gene products, the RepTs62, the transposase binding sites, and a "protein-fusion" form of the *lac* operon that lacks sequences for transcription initiation and for translation initiation. At the center of this transposon is the neomycin phosphotransferase gene of Tn903. Stippled boxes indicate three operator sites for binding Mu repressor, O_1, O_2, and O_3, together with the two convergent promoters that regulate lytic and lysogenic development. Repressor binding at O_2 blocks RNA polymerase -35 and -10 contacts with promoter P_E (Krause and Higgins, 1984). Repressor bound to O_3 blocks the P_{cM} -10 contact (Krause and Higgins, 1986). A densely stippled box between O_1 and O_2 shows the binding site for IHF. The coding frame for RepTs62 starts within O_1. For repressor binding assays, the operator region was subcloned; restriction sites for *Eco*R1 and *Bam*H1 were located at positions marked 1 and 2, respectively. B. Specialized plaque forming λ transducing phage used to quantitate Mu transcription included a promoter-less *lacZYA* operon oriented so that the P_E promoter modulates synthesis of βGal. Mu repressor (Rep) is autoregulated from P_{cM} as it would be in a Mu prophage (Krause and Higgins, 1986). λJV303 contains a Mu fragment with the RepTs45 repressor and λJV304 contains RepTs62. λ genes *A*, *J*, and *attP* (not to scale) are shown positioned at the ends. C. Plasmid pDH38 contains a 6kb *Bgl*II fragment from *Salmonella* inserted at the *Bam*HI site on pACYC184. The gene encoding H-NS (transcribed clockwise on this plasmid) is located near the center of this insert. Also shown is the position of the *Kpn*I restriction site that was used to disrupt *hns* by insertion of a neomycin resistance cassette.


A
MudII1681
Z'
Y
A
Kn
MuAB-O-cts62
1
2
HindIII
O3
O2
O1
-35
-10
-10
-35
IHF
Rep
PcM
PE


B
λ JV303, λ JV304
PE
Rep
A...J
attP
A
Y
Z
P
cM
Kn


C
EcoRI
ori
Cam
HindIII
BamHI/BglII
Tet'
Tet'
pDH38
(pACYC184)
BamHI/BglII
hns
EcoRI
KpnI

phenotype. Both complex genetic rearrangements and simple point mutations can cause an Smt phenotype in *E. coli* and *Salmonella*. Sonti and Roth (1989) discovered one recurring duplication that includes the genes between minute 45 and minute 80 in *Salmonella*. This duplication (and a similar duplication in *E. coli*) appears as a fast-growing sector on agar media containing several different single carbon sources (Sonti and Roth, personal communication). Strains carrying this duplication have an Smt phenotype (Sonti and Roth, personal communication). Point mutations that give an Smt phenotype map near minute 27 and minute 72 (Higgins, unpublished).

The product of a single gene mapping near minute 27, *hns*, has recently been recognized as the factor responsible for a number of different independently discovered phenotypes in enteric bacteria (May et al. 1990). The gene has been named over and over again. As *bglY*, it is known for a complex phenotype that allows cells to express a cryptic operon, allowing growth on β-glucosides (Defez and De Felice, 1980; Mahadevan *et al.*, 1987; Schnetz *et al.*, 1987). As *bglY*, the gene was also noted to greatly stimulate the rate of spontaneous chromosomal deletions (Lejeune and Danchin, 1990). As virulence gene *drdX*, it is known for its ability to alter thermoregulation of a pilus gene cluster (Goransson et al. 1990). It was called *ozmZ* when an effect on osmoregulation of *proU* was recognized (Higgins et al. 1988) and named *pilG* when it was found regulating the rate of phase

variation in fimbrae (Spears et al.1986). In *Shigella*, it was discovered again, changing the temperature regulation of a group of plasmid-borne genes (Dorman et al. 1990). The protein H-NS is known on its own virtue as an abundant protein of the bacterial nucleoid and is thought to play a key role in the large scale organization of the chromosome (Spaasky et al., 1984; Gualerzi et al., 1986; Pon et al., 1988).

We've found that strains with *hns* mutations have significantly elevated rates of mini-Mu transposition and weakened Mu transcriptional repression *in vivo*. Transposition activity was strongly dependent on growth conditions. Shifting cells from growth on rich LB medium to minimal medium containing glucose or malate as a sole carbon source significantly increased mini-Mu transposition. This change in growth condition also caused a change in the SDS-polyacrylamide gel electrophoretic pattern of proteins reacting with anti-H-NS antibodies. In addition to these *in vivo* observations, we found that purified H-NS stabilizes Mu repressor binding to operator DNA *in vitro*. These discoveries may indicate a regulatory circuit wherein H-NS, altered according to the growth history of the cell, modulates transcription levels in several different operons and transposons.

RESULTS

The *bgl* operon is silent in wild type *E. coli* strains, yet it encodes a group of proteins that allow the cell to

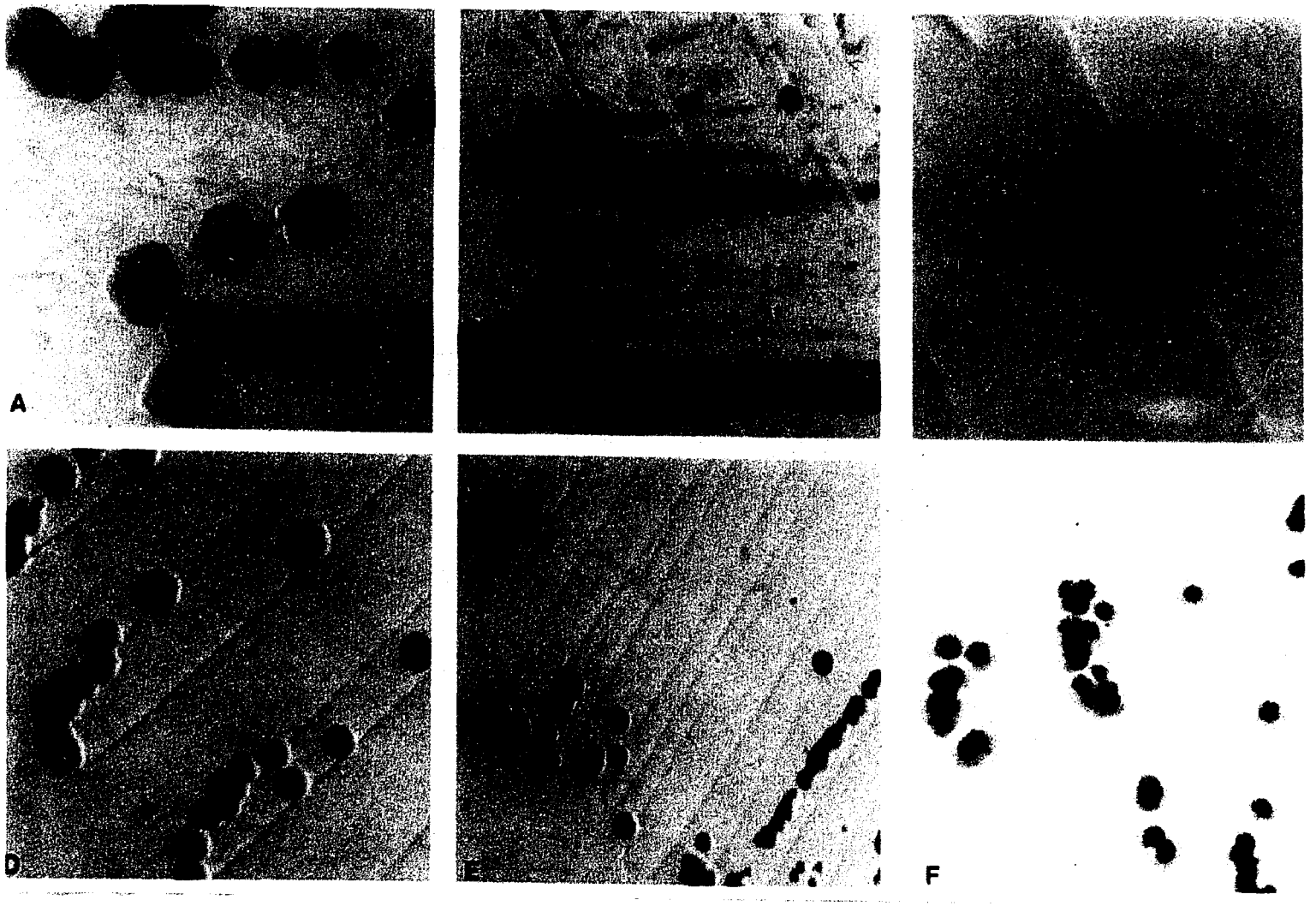
efficiently utilize salicin as a sole carbon source. At least two types of mutations can activate *bgl* expression (Defez and De Felice, 1980; Reynolds *et al.*, 1986). One mutant class is caused by insertion of a transposon into the 5'-flanking region near the *bgl* promoter (Reynolds *et al*, 1986). Movement of several different transposons to this location has been shown to be quite efficient (Reynolds *et al*, 1986). A second class of mutations that activates *bgl* transcription is associated with a gene called *bglY* (Defez and De Felice, 1980; Mahadevan *et al.*, 1987; Schnetz *et al.*, 1987), which encodes H-NS (May et al. 1990). To isolate new H-NS mutations, we used a MacConkey plate screening strategy of Defez and De Felice (1980). The strategy is based on the observation that mutations that allow utilization of salicin can be detected by growth and fermentation of the mutant sectors.

E. coli B178 was incubated for several days at 37° on MacConkey base plus 0.5% salicin plates. Red papillae appeared. Some of these sectors had a mucoid phenotype when cells were grown on plates incubated at 23°. In several cases, the mutation causing a mucoid phenotype and an ability to grow on salicin were found to be linked to a *tyrTβ*::Tn10 (see below). The mutations allowing growth on NCE-salicin were moved by P1 transduction into NH801, selecting for linkage to the Tet^R in *tyrTβ*::Tn10. NH801 has an *arg-lac* deletion and a single copy of Mud*II*1681 linked to *thyA*. When Tet^R transductants were plated on LB medium containing

5-bromo-4-chloro-indolyl-β-D-galactoside (XGal) to record MudII1681 transposition activity in the colony, there was not much color change. When Tet^R transductants that retained a mucoid phenotype at 23° were plated on NCE-malate with XGal, the plating efficiency was low and the colonies that formed were small and dark blue, due to very active MudII1681 transposition (Figure 2 B). Inspection of the small dark colonies showed that β-galactosidase was being leached into the surrounding agar, so some cell lysis was occurring (Figure 2 C). A similar phenotype was observed when these cells were grown on NCE-glucose or M9-glucose plates containing XGal. Salicin utilizing mutants that did not have the mucoid phenotype showed no dramatic Mu phenotype (data not shown); these mutations are likely to be the expected class that maps near the *bglF* operon (Mahadevan, et al. 1987).

One mutation was mapped by P1 transduction using the mini-Tn10 collection of Singer *et al.*(1989). The Smt phenotype was about 20 % linked to the tetracycline resistance gene (Tet^R) in CAG12028 at 28.50 min, 90% linked to Tet^R of strain CAG12169 near *bglY* at 27.25 min and very loosely linked to CAG 12016 at 26.75 min. The Smt locus was also close (>95% linked) to a mini-Tn10 in LB364, which is in *tyrT*β at min 27.3. Thus, the gene for Smt maps very close to *bglY/osmZ*, which is at about 27.4 min (Bachmann, 1990). Strain NH757 was constructed carrying both Tet^R from LB364 and the Smt phenotype.

Figure 2. Colony transposition phenotype of NH801, NH802, TT15891, and NH776. Cells were plated on agar media and incubated for 5 days at 37°. A, NH801 on NCE-malate at 2.5 X magnification; B, NH802 on NCE-malate at 2 X magnification; C, isolated colony of NH802 on NCE-malate at 10 X magnification; D, TT15891 on NCE-glucose medium at 2.5 X magnification; E, NH776 on NCE-glucose medium at 2.5 X magnification; F, NH776 on NCE-malate medium at 2.5 X magnification.


A
D
F

Mutations in hns cause an Smt phenotype in E. coli and Salmonella. To test whether Smt was caused by mutation in *hns*, plasmid pDH38 (see Figure 1C) carrying a cloned copy of *hns* from *Salmonella typhimurium* was introduced into *E. coli* strain NH757. Introduction of pDH38 into NH757 caused it to grow as white colonies on MacConkey-salicin plates (data not shown). Western blot analysis was then carried out using antibodies to purified H-NS as a probe. Strain NH788, carrying the Smt phenotype and linked *tyrTβ*::Tn10, showed significantly reduced staining at the position in the gel corresponding to the band of purified H-NS protein (Figure 3). Due to the location of the gene conferring the Smt phenotype, its salicin growth phenotype, its complementation with a clone of Salmonella *hns*, and the deficiency of H-NS protein detected by Western blot analysis, this mutation was named *hns*651.

To test whether a *Salmonella hns* mutation would give the Smt phenotype, we examined the Mu*dII*1681 transposition pattern in derivatives with a disrupted allele of *hns*. Since mutations in *Salmonella hns* do not allow utilization of salicin as a sole carbon source (Higgins and Hillyard, unpublished), an *hns* mutation was created by cloning the neomycin phosphotransferase gene of Tn903 into the *Kpn*I site near the center of *hns* on a plasmid (see Figure 1C), and this allele (*hns*1::Kn^R) was moved onto the *Salmonella* chromosome by homologous recombination (Hillyard and Marsh,

Figure 2.A. Two dimensional gel analysis of the products of HNS supercoiling. The standard supercoiling reaction was carried out as previously (Figure 1 legend), then reaction products were separated by 2 dimensional electrophoresis through 1% agarose in TBE. In the first dimension the gel was run in the absence of chloroquine at 2.5 V/cm for 18 hours. The gel was then equilibrated in 40 μM chloroquine, turned 90° and then run in the second dimension in the presence of 40 μM chloroquine in TBE at 2.5 V/cm for 40 hours. Following extensive washing in water, gels were stained with ethidium bromide. Topological changes were quantified by determining the center of the topoisomer distribution in each reaction. A schematic of the two dimensional gel is shown beneath the gel. Marker lanes are numbered. Arrows indicate the centers of topoisomer distribution. 1, relaxed plasmid; 2, supercoiled plasmid; 3, topoisomer ladder generated from pHK09 by relaxation of the plasmid with topoisomerase I after addition of several concentrations of ethidium bromide; 4, supercoiled DNA relaxed in the reaction cocktail; A, 28 pM HNS; B, 21 pM HNS; C, 14 pM HNS; D, 7 pM HNS; E, 3 pM HNS; F, 0 HNS.

c.

H-NS

NH787 LB

NH788 LB

NH787 Malate

NH788 Malate

H-NS

LT2 LB

NH685 LB

in preparation). An Smt phenotype was observed whenever the *hns*1::Kn^R was introduced by P22 transduction into TT15891, which has a single copy of Mu*d*II1681 oriented in the white configuration in the xylose operon (Figure 2). After five days of growth at 37° on NCE-glucose-XGal medium, Mu*d*II1681 formed new *lacZ'*-protein fusions that produced dark-staining, well-separated individual colonies and pale blue crowded colonies (Figure 2 E). An isogenic strain with a wild type *hns* gene developed very little color in five days (Figure 2 D).

In *Salmonella* strains carrying *hns*1::Kn^R, the Mu transposition phenotype was dramatic but not as severe as in *E. coli* strains carrying *hns*651. *Salmonella* colonies that formed on NCE-glucose and NCE-malate plates were stained blue (Figure 2E-F), whereas LB-grown colonies were white (not shown). Slightly more transposition was observed when cells were plated on NCE-malate-XGal compared to NCE-glucose-XGal. But the blue dye resulting from XGal cleavage was confined to the colonies rather than spreading into the agar, as was seen in *E. coli* (Figure 2, compare C and F), and the plating efficiency was normal. To ensure that the transposition phenotype in *Salmonella* was not a unique property of *xyl*543::Mu*d*II1681, *hns*1::Kn^R was moved by P22 transduction into TT15882 and TT15893. Both strains carry a single white Mu*d*II1681 at a different chromosomal location. When the *hns*1::Kn^R allele was transduced, each new strain produced nearly colorless colonies on LB-XGal medium but dark blue

colonies after 3-5 days of incubation on NCE malate-XGal plates (not shown). So although the *hns*1::Kn^R mutation did not confer on *Salmonella* the ability to grow on salicin as a sole carbon source, it derepressed MudII1681 transposition.

*hns*651 changes Mu transcription but not plasmid supercoiling. To examine Mu transcription in cells with *hns*651, lysogens of λJV304 and λJV303 were constructed (Figure 1B). λJV304 contains the left end of Mu*cts*62 cloned so that the temperature sensitive (Ts) Mu repressor (RepTs62) regulates *lacZ* expression from the early promoter (P_E, Figure 1A) that normally modulates synthesis of the transposase mRNA. λJV303 is similar, except that it contains a Mu fragment with the RepTs45 repressor regulating *lacZ*. Four strains were constructed. Two λJV304 lysogens were isogenic except for the region near 27 min; NH787 is wt and NH788 is a recombinant carrying the genes closely linked to Tet^R from NH757. NH789 and NH790 are the corresponding λJV303 lysogens carrying wt genes and the Tet^R region from NH757, respectively. NH787 and NH788 cultures were grown overnight at 32°, diluted 50-fold into fresh medium, grown at 32°C, 37°C, or 42°C to a density of 50 Klett units, and then β-galactosidase (βGal) assays were performed on cell extracts. At 32° there was no measurable difference between the two strains--27 and 30 units of βGal were produced, respectively (Table II). However, at 37° the *hns* mutant NH788 made 4890 units of βGal compared to 2300 units in the wt NH787. The experiment was repeated with NH789 and NH790, which has

Table 1. Regulation of two Mu Ts repressors is altered by H-NS651

	β-gal expression in λJV lysogens grown at					
	32°C		37°C		42°C	
Repressor	*hns*$^+$	*hns*651	*hns*$^+$	*hns*651	*hns*$^+$	*hns*651
RepTs62	27	30	2320	4890	>9000	>9000
RepTs45	210	253	4250	9300	>9000	>9000

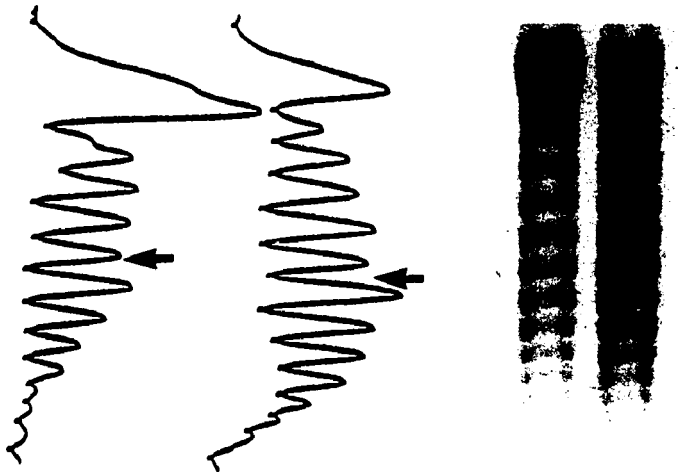
RepTs45 instead of RepTs62. A similar phenotype was also found when RepTs45 was present. At 37° the *hns* mutant NH790 made 9300 units and wt NH789 made 4300 units of βGal. Thus, at 37° transcription regulation by two different Ts repressors was about half as stringent in the presence of *hns*651 as it was when the wt allele was present.

One explanation that has been given for the ability of the H-NS protein to alter gene expression in diverse genetic systems is that this protein is linked to changes in DNA supercoiling (Dorman et al., 1990). The Mu operator region contains two convergent promoters that are sensitive to supercoiling (Higgins et al., 1989). Therefore, we examined supercoiling of pBR322 in NH801 and NH802 growing in LB or minimal-glucose medium. Cells containing pBR322 were grown in LB to a density of 50 Klett units and harvested. To see if supercoiling was more responsive under conditions that caused mini-Mu induction, we also grew cells in NCE-glucose medium to a density of 50 Klett units and harvested. Plasmid DNAs from both cells under both growth conditions were isolated for supercoiling analysis. In both media, NH802 cultures grew at roughly half the rate of NH801, but we found only a very slight difference in plasmid supercoiling (Figure 4). Plasmid DNAs were run in gels containing several concentrations of chloroquine. This analysis confirmed the fact that the DNA was negatively supercoiled (data not shown). The shift in topoisomer distribution found in the *hns*$^-$ strains moved the plasmid population by only about 1/2

Figure 4. Analysis of plasmid supercoiling. Strains NH801 and NH802 were transformed with pBR322 and grown in LB (left) or NCE-glucose (right) to a density of 50 Klett units. Growth was stopped by chilling to 4° and cells were harvested, and plasmids were isolated according to the alkaline-SDS method described by Maniatis (1982). Topoisomers were separated in 1% agarose gels run in Tris borate EDTA buffer in the presence of two different levels of chloroquine to ensure that bands represented negative supercoiling. The gels in this photograph were run with 4 μM chloroquine. After staining the gel with ethidium bromide the bands were photographed and peaks were quantitated with a Bio Rad densitometer. Centers of the topoisomer distributions, marked by an arrow, were determined by integration of peak areas.

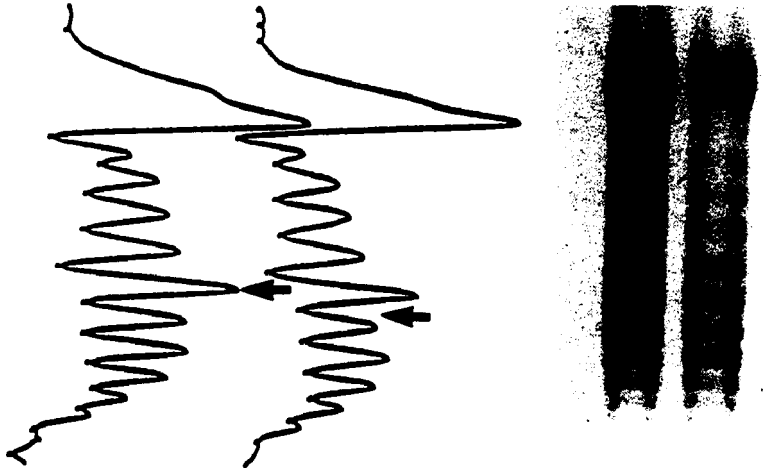
NCE Glucose

802 801



L Broth

802 801



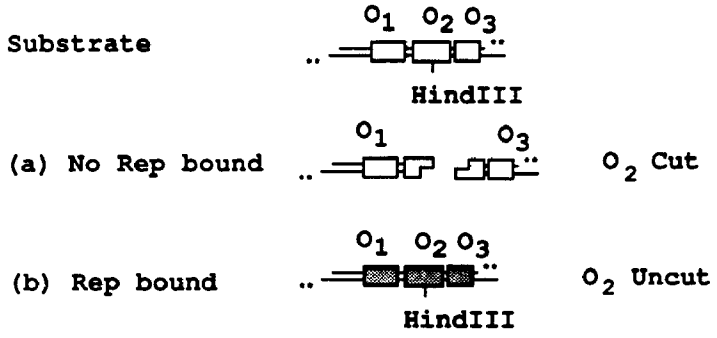
topoisomer toward relaxed position (Figure 4). The topological displacement associated with the *hns*651 mutation is minuscule compared to that which has been measured in topoisomerase mutants (Pruss et al. 1982; Steck et al., 1984) or in mutants lacking protein HU (Hillyard et al., 1990).

H-NS stabilizes Mu repressor-DNA complexes. Since *hns*651 did not alter plasmid supercoiling to a significant extent, we were led to look for more direct effects of H-NS on Mu repressor-DNA interactions. H-NS can alter RNA polymerase transcription reactions even after RNA polymerase is bound to a promoter by inhibiting open complex formation (Spassky et al., 1984). Therefore, if H-NS binds close to other proteins, it could alter their activity on DNA as well.

The effect of purified H-NS on RepTs62-Mu DNA complexes was examined *in vitro*. The Mu regulatory region has 3 operators and two convergent promoters (Figure 1A). Purified RepTs62 binds cooperatively to three blocks of DNA designated O_1, O_2, and O_3 (Krause and Higgins, 1984; Krause and Higgins, 1986). At O_2, Rep binding prevents RNA polymerase from initiating transcription at P_E, which is the control point of a polycistronic operon encoding transposition and regulatory functions (Krause et al., 1983). Rep binding also blocks access to the cleavage site of *Hin*dIII restriction enzyme (Krause and Higgins, 1984; Figure 1). *Hin*dIII restriction is a simple, sensitive, quantitative assay for repressor binding efficiency (see Figure 5). To test whether H-NS affects RepTs62-DNA interactions directly, *Hin*dIII digestion of

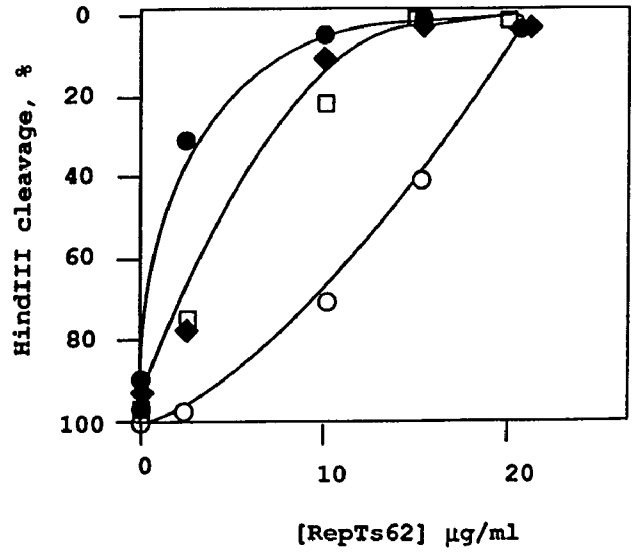
Figure 5. Effect of H-NS on Mu repressor binding reactions *in vitro*. Reactions carried out as described in Materials and Methods contained: A, no RepTs62; B, 2.5µg/ml RepTs62; C, 10µg/ml RepTs62; D, 15µg/ml RepTs62; E, 20 µg/ml RepTs62; F, 5 µg/ml H-NS; G, 2.5 µg/ml RepTs62 plus 5 µg/ml H-NS; H, 10 µg/ml RepTs62 plus 5 µg/ml H-NS; I, 15 µg/ml RepTs62 plus 5 µg/ml H-NS; J, 20 µg/ml RepTs62 plus 5 µg/ml H-NS; K, 10 µg/ml H-NS; L, 2.5 µg/ml RepTs62 plus 10 µg/ml H-NS; M, 10 µg/ml RepTs62 plus 10 µg/ml H-NS; N, 15 µg/ml RepTs62 plus 10 µg/ml H-NS; O, 20 µg/ml RepTs62 plus 10 µg/ml H-NS; P 20 µg/ml H-NS; Q, 2.5 µg/ml RepTs62 plus 20 µg/ml H-NS; R, 10 µg/ml RepTs62 plus 20 µg/ml H-NS; S, 15 µg/ml RepTs62 plus 20 µg/ml H-NS; T, 20 µg/ml RepTs62 plus 20 µg/ml H-NS. After incubation with repressor and HindIII restriction endonuclease as described in methods, the DNA molecules were resolved on polyacrylamide gels. Bottom. The fraction of DNA cleaved was measured by densitometry using a BioRad Video densitometer. Plots are given for reactions carrying the indicated amounts of Mu RepTs62 and no H-NS (open circles), 5 µg/ml H-NS (open boxes), 10 µg/ml H-NS (diamonds), or 20 µg/ml H-NS (closed circles).

A



A B C D E F G H I J K L M N O P Q R S T

B



complexes formed between Mu repressor and an EcoRI-BamHI Mu operator fragment (see Figure 1A) was examined in the presence of different levels of H-NS (Figure 5). The presence of 5 μg/ml H-NS caused RepTs62 (at concentrations of 3-10 μg/ml) to protect 2-3 times as much Mu operator DNA from *Hin*dIII digestion as in the absence of H-NS. The stimulation of Rep-DNA binding could not be duplicated by replacing H-NS with bovine serum albumin or HU protein. RepTs62 binding was more efficient at 20 μg/ml H-NS, but H-NS may bind Mu operator DNA weakly by itself since H-NS gave some protection at concentrations above 10 μg/ml. Nonetheless, even at 20 μg/ml H-NS, where there is about 10% inhibition of HindIII cleavage activity, 90% of the DNA was susceptible to cleavage in the absence of added repressor. We also found a similar but smaller stabilization of wt repressor (data not shown). The concentration of wt repressor that was necessary to observe protection was 10-20 fold lower due to the better intrinsic binding activity of wild type repressor. Thus, pure H-NS doubles RepTs62 binding efficiency *in vitro*, which is close to the measured *in vivo* difference caused by the *hns*651 mutation observed for Mu transcription in LB grown cells (Table II).

SDS-PAGE behavior of bands staining with anti-H-NS antibodies changes with growth condition. The Western blot analysis of proteins from the wild type strain NH787 grown in LB showed two bands reacting with the anti-H-NS antiserum in the 15-20% gradient acrylamide SDS gel system (Figure 3).

The purified H-NS, which was isolated from an overproducing strain, also had two bands with the most dense staining at the position of faster mobility. Conversely, proteins isolated from the wt NH787 grown in LB showed a pattern in which the most dense stain was associated with the slightly slower migrating species.

The Smt phenotype was much more dramatic when cells were grown on minimal medium containing glucose or malate as a carbon source compared to when they were plated on LB. To see if the proteins detected with our anti-H-NS antibodies would change in response to growth conditions, we blotted an equal amount of protein from NH787 grown in NCE-malate liquid. Malate grown cells showed three striking differences from LB-grown cells. First, malate grown cells lacked the predominant band of LB-grown cells. Second, they contained relatively more of the band migrating at the position of the fastest band found in LB grown cells. Third, malate grown cells had a new band that migrated well behind the other two bands. When NH788, the *hns*651 mutant, was grown on malate, it showed significantly reduced staining at the fast mobility position but not much difference in the slowest migrating band (Figure 3). Thus, mini-Mu transposition analysis and the Western blot profiles of proteins detected with anti-H-NS antisera both showed a phenotype that was substantially different in cells growing in LB broth compared to NCE-malate liquid.

Two simple explanations would account for these results. First, our antisera may contain antibodies detecting more than one protein, which could result from shared epitopes or contamination of the H-NS protein used to elicit the immunological response. The protein most likely to be confused with H-NS is the slower migrating band of the doublet. This band is close to, but not perfectly aligned with the strongest band in pure H-NS. However, this strong band disappeared when the *hns*651 mutation was introduced (NH788 LB) and it also disappeared when cells were shifted to NCE-malate medium (NH787 Malate). Thus, if this band is a contaminant, it must be be under the influence of the *hns*651 mutation and responsive to growth conditions that make H-NS critical for Mu regulation. The second possibility is that H-NS can be modified so that it moves with more than one mobility during SDS-PAGE analysis. We first saw the doublet on a standard SDS-gel blot, but the behavior became more reproducible when we used longer gradient acrylamide SDS-gels. Several different protein modifications might lead to altered mobility of this magnitude. Interestingly, Spassky et al. reported that H-NS can be isolated as three variants forming discrete spots on isoelectric focusing gels (1984). The third band that appears only when cells are grown on malate is likely to be different from H-NS because its behavior was not altered by introduction of the *hns*651 mutation.

Salmonella LT2 grown on LB showed three different bands. In addition to the most prominent band that migrates slightly behind the main peak of pure *E. coli* H-NS, there was a fast moving band and a slow moving band (Figure 3, LT2 LB). The fast-moving band was present in strain NH685, which contains a *hns* gene cut in half by a cloned Kn^R cassette, while the slow moving band was greatly reduced. Thus, *Salmonella* also shows two bands strongly affected by the introduction of an *hns* mutation and one band that was not strongly affected. An important experiment that needs to be done is to isolate H-NS from malate grown cells to test its gel mobility and effect on Mu repressor binding reactions in vitro.

DISCUSSION

H-NS silences transcription. *hns* mutations were discovered and given different names because they cause a complex phenotype in several unrelated genetic systems (May et al., 1990). Information about H-NS effects on transcription is available for four operons. H-NS silences transcription of *bglF* (Defez and De Felice, 1980; Lejeune and Danchin, 1990; Mahadevan et al., 1987), *proU* (Higgins et al., 1988; May et al., 1990), and *pap* (Goransson et al., 1990). In addition, we've shown here that H-NS strengthens Mu repression, both *in vivo* and *in vitro*.

One mechanism that has been proposed to explain how H-NS affects gene expression is focused on DNA supercoiling. If H-NS modulates supercoiling, then systems sensitive to the influence of supercoiling would be subject to H-NS control

(Dorman et al., 1990). However, we found no significant change in plasmid supercoiling in strains carrying the *hns*651 mutation, even under growth conditions that promote significant induction of Mu transposition (Figure 4).

An alternative model has been proposed based on direct specific binding of H-NS to DNA sequences, which occurs at concentrations of 3μM (about 50 μg/ml) in the *lac* and *gal* control regions (Rimsky and Spassky, 1990). At this concentration, H-NS inhibits RNA polymerase bound to promoters (Spaasky, 1984), so H-NS might alter regulation by directly competing with other regulatory proteins. However, the effects we found in the Mu RepTs62-DNA interactions occur at concentrations about one tenth that necessary to see direct binding of H-NS in the *lac* and *gal* footprinting studies (Rimsky and Spassky, 1990).

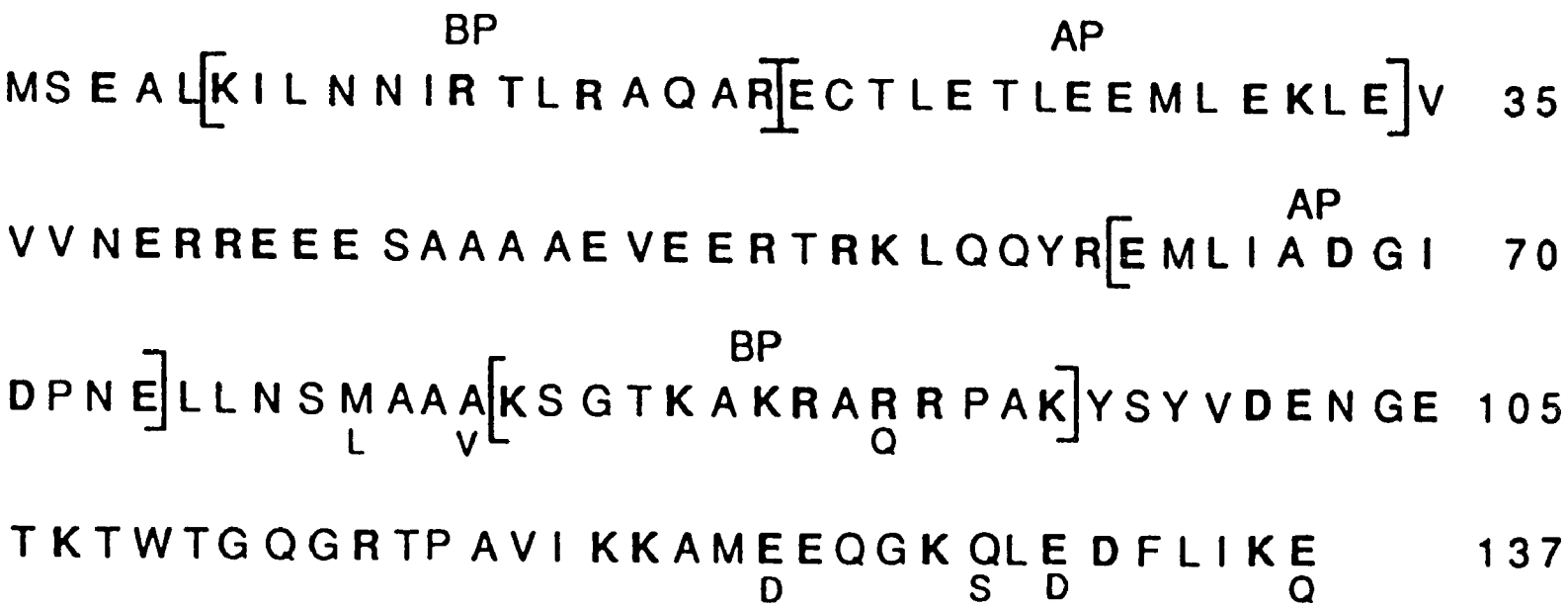
We suggest that H-NS binding may silence transcription in two ways that have not been envisioned by previous models. First, physical interaction between H-NS and repressor may extend and strengthen specific repressor-DNA complexes, providing not only enhanced repression but also a mechanism for specificity in H-NS mediated silencing. In a second model, H-NS dimers may stabilize a DNA loop. A strong IHF binding/bending site probably allows the operator to fold into a three dimensional loop in which repressors are bound to two sites, O_1 and O_2, separated by IHF's sharp bend (Higgins et al., 1989). H-NS dimers may stabilize repressor-DNA complexes by crosslinking interwound DNA arms of the loop

at points that are brought sufficiently close in the folded structure.

A protein that fortifies DNA loops or that stabilizes specific protein-DNA complexes would be useful in silencing gene expression. Looping is an important element of repression in two well characterized systems--the galactose operon (Adhya, 1989) and the arabinose operon (Schleif, 1990). Regarding the ability to interact specifically with other regulatory elements, H-NS has an unusual structure and amino acid composition (Figure 6). Thirty percent of the side chains are charged, but acidic and basic residues are balanced so the pI of the protein is near 7.1. The acidic and basic residues occur in "patches", and we note a similarity in two regions to the HMG-1 protein of yeast (Haggren and Kolodrubetz. 1988) (Figure 6). In yeast, transcription silencing occurs through the action of as yet unspecified proteins acting on specific DNA sequences (Brand et al., 1985). Some HMG proteins are essential but the mechanism through which these proteins exert their effect is not well understood (Haggren and Kolodrubetz, 1988). They may associate with active and inactive chromatin through contact with other chromosomal proteins rather than through recognition of specific DNA sequences.

H-NS effects change in different growth media. The second noteworthy behavior is the sharp dependency of growth medium on the H-NS influence of Mu transposition. When cells were grown on minimal salts with glucose or malate as the sole

Figure 6. H-NS--an unusual protein. The one letter code is given for *Salmonella* H-NS, as deduced from its DNA sequence (Marsh and Hillyard, 1990; Hulton et al., 1990). Acidic and basic residues are in bold print with patches indicated in brackets. Amino acids that differ in *E. coli* H-NS (Pon et al., 1988) are shown below the *Salmonella* sequence. Two segments of H-NS that show structural similarity to yeast HMG1 are indicated below the *Salmonella* sequence. The first region of similarity is aligned with H-NS amino acid 72 matched to HMG1 residue 133; there are 8 matches within this stretch of 17 amino acids. The second region of similarity is aligned with HMG1 residue 110 matched to H-NS residue 200; there are 6 identities and 12 similarities within this segment of 24 residues. Identical amino acid residues are shaded, chemically similar residues are marked with two dots, and evolutionarily favored substitutions identified by the FASTA program are indicated by one dot, respectively.


BP
AP
MSEALKILNNIRTLRAQARECTLETLEEMLEKLEV 35
AP
VVNERREEESAAAAEVEERTRKLQQYREMLIADGI 70
BP
DPNELLNSMAAAKSGTKAKRARRPAKYSYVDENGE 105
L V Q
TKTWTGQGRTPAVIKKAMEEQGKQLEDFLIKE 137
D S D Q

carbon source, H-NS was a much more critical determinant of Mu activity. Coinciding with the change in Mu transposition rate was a difference in the behavior of proteins reacting with anti-H-NS antibodies. In *E. coli*, one band disappeared and another band appeared when cells were shifted from LB to NCE-malate liquid. Although we have not yet proven the identity of each protein band detected, two of the anti-H-NS reacting proteins were drastically altered when the *hns*651 mutation was introduced.

Could this behavior of H-NS be significant in adaptations that bacterial cells make for growing on simple carbon sources? There are several reports of transposon movement that occurs with very high efficiency in colonies growing with carbon source liminations. One example is in the *bgl* operon where transposition into the region just upstream of the first gene occurs with high efficiency on plates containing salicin (Reynolds et al. 1986). Another example is the efficient transposition of an IS element out of the *bglF* gene when a selection for salicin utilization is applied (Hall, 1988). And Shapiro demonstrated an astounding efficiency of Mu-driven rearrangements that form *ara-lacZ* fusions on plates that contain arabinose and lactose (Shapiro, 1984). In addition to these observations on transposition, Cairns and Hall have described an amazing efficiency in the occurrence of point mutations when selective forces are applied to colonies (Cairns et al., 1988; Hall, 1990). It has not escaped our attention that a

modifiable chromosome element that can change the frequency of rare and potent genetic events under selective conditions would be useful in shaping the genetic structure of bacterial populations.

CONCLUSIONS

Regulation of mini-Mu transposition in *E. coli* colonies is under genetic and physiological control. Mutations in the H-NS protein greatly elevate the Mu*d*II1681 transposition rate, especially when cells are grown on agar plates with glucose or malate as the sole carbon source. Correlated with increased transposition activity was a change in the Western blot profiles of proteins reacting with anti-H-NS antisera. H-NS is a prime candidate for mediating the increase in the rate of phage Mu genetic activity observed when cell populations are starved for a carbon source (Shapiro, 1984; Shapiro and Higgins, 1989). Further biochemical and genetic studies of H-NS are needed to identify the operons under H-NS control and to describe the mechanism by which H-NS alters chromosome activity under different growth conditions.

MATERIALS AND METHODS

Growth of bacteriophage and bacterial strains. Bacterial strains are listed in Table 1. Bacteria were grown in LB (5g NaCl, 10g tryptone, 5g yeast extract) or NCE (Berkowitz et al., 1968) plus either 0.2% malate, 0.2% glucose, or 0.5% salicin. NCE salts were made as a 50-fold concentrate containing (per liter) 197g KH_2PO_4, 325g K_2HPO_4, 175g Na $(NH_4)HPO_4$-H_2O. NCE solid media were made by mixing 1 liter

Table 2. Strains of E. coli and S. typhimurium used in these studies

Strain	Description	Reference or source
Escherichia coli		
B178	W3110 *galE*	C. Georgopolus
LB364	*tyrT*β::Tn10	C. Georgopolus
NH756	B178 *hns*651	Red sector of B178
NH757	B178 *hns*651 *tyrT*β::Tn10	NH756 × LB364
CAG12169	*zch*/506::Tn10	(Singer, 1989)
CAG12016	*zcg*/3060::Tn10	(Singer, 1989)
CAG12028	*zci*/233::Tn10	(Singer, 1989)
NH801	*thi* Δ(*argF-lac*)U169 Mud*I*I1681 (near *thyA*)	
NH802	NH801 *hns*651 *tyrT*β::Tn10	NH801 × NH757
MC4100	F⁻ *araD* Δ(*argF-lacZYA*) *U*169 *rpsL relA1 flbB deoC ptsF rbsR*	M. Casadaban
NH787	MC4100 λJV304	MC4100 + λJV304
NH788	NH787 *hns651 tyrT*β::Tn10	NH787 × NH757
NH789	MC4100 λJV303	MC4100 + λJV303
NH790	NH789 *hns*651 *ytrT*β::Tn10	NH789 × NH757
Salmonella typhimurium		
LT2	Wild type	J. Roth
NH685	*hns*1::Kn^R	D. Hillyard
DH3136	*hns*1::Kn^R *zde*/5410::Tn10	D. Hillyard
TT15882	*ara*664::Mud*I*I1681	R. Sonti
TT15891	*xyl*543::Mud*I*I1681	R. Sonti
TT15893	*xyl*545::Mud*I*I1681	R. Sonti
NH775	*hns*1::Kn^R *ara*664::Mud*I*I1681 *zde*/5410::Tn10	TT15882 × DH3136
NH776	*hns*1::Kn^R *xyl*543::Mud*I*I1681 *zde*/5410::Tn10	TT15891 × DH3136
NH777	*hns*1::Kn^R *xyl*545::Mud*I*I1681 *zde*/5410::Tn10	TT15893 × DH3136

of sterile H_2O, 40 ml 50 X NCE concentrate, and 2 ml 1 M $MgSO_4$. Then, an equal volume of sterile molten 3% agar was added followed by the carbon source. P22 phage transductions were carried out as described by Davis et al. (1980) and P1 transductions were done according to Miller (1972). DNA manipulations and transformation of bacterial strains with plasmid DNA were as described (Maniatis et al. 1982).

Preparation of crude extracts and analysis of H-NS by Western blotting. Five ml aliquots of cells grown to a density of 150 Klett units in appropriate media were concentrated by centrifugation, and the pelleted cells were resuspended and boiled for 3 min in 300 µl of SDS-gel loading buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 30% glycerol, 1mM β-mercapto-ethanol). To analyze the proteins, 5 µl aliquots from the 300 µl mixture of cells suspended in SDS-gel buffer were separated on acrylamide gels (15-20% acrylamide gradient resolving gels with a 4% stacking gel) and transferred to Schleicher and Schuell BA85 nitrocellulose membranes. Transfer was performed electrophoretically for 3 h at 25 mA in 25 mM Tris buffer (pH 8.3) containing 192 mM glycine and 20% v/v methanol. Membranes were saturated with 1% BSA before overnight incubation with rabbit polyclonal antibody raised against purified H-NS at 1/5000 dilution in PBS buffer supplemented with 1% BSA and 0.01% Tween 80. The antibody response was amplified with a secondary biotinylated antibody raised against rabbit immunoglobulins and a biotin-streptavidin complex coupled with peroxidase. The blot was

developed using 3-3' diamino-benzidine from Sigma at 0.2 mg/ml in Tris-HCl, pH 7.6.

Protein puricatión. RepTs62 was purified from hyperproducing cells (Vogel et al., submitted) by the method of Krause and Higgins (1984). The purified protein was >95% pure as judged by SDS-polyacrylamide gel electrophoresis and was found to be free of single or double stranded DNase activity, using linear and supercoiled DNA substrates. H-NS protein was purified as described (Falconi et al., 1988; Gualerzi et al. 1986). The protein was prdoduced in an E. coli strain containing a plasmid with H-NS placed under the control of the λ p_L promoter (M. Falconi R. Calogero and C. Gualerzi, in prepreparation).

Mu repressor-DNA binding reactions. Repressor binding reactions were assembled at 34°C with $5.5x10^{-11}$M ^{32}P-labeled pHK09 operator fragment, which was labeled by filling in EcoR1 and BamH1 sites indicated in Figs. 1 and 5. Repressor binding reactions containing 10mM Tris-HCl,pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 1mM DTT, 50 50µg/ml bovine serum albumin, and 50µg/ml calf thymus DNA were incubated with purified Mu repressors for 30 min. After addition of *Hin*dIII, incubation was continued for 1 hour. DNA fragments were resolved by electrophoresis in a 7.5% acrylamide gel after addition of 1/5 volume of 15% Ficoll, 0.25% bromophenol blue,and 0.25% xylene cyanol. Bands were visualized by autoradiography and quantitated using a BioRad Video densitometer.

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DNA SUPERCOILING BY THE NUCLEOID ASSOCIATED PROTEIN HNS

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(Submitted to SCIENCE)

ABSTRACT

HNS, genetically implicated in transcriptional regulation and a plentiful protein associated with the bacterial chromosome, stabilizes negative supercoils in vitro. Based on in vitro reactions with plasmid DNA, the estimated intracellular quantity of HNS, HU, and RNA polymerase can account for most of the constrained supercoils in bacterial chromosomes. While both HU and HNS form negative supercoils, each protein's supercoiling action is antagonistic to that of the other. Properties of these proteins suggest a model for organizing transcriptionally inactive and active regions in bacterial genomes.

One of the vexing problems in molecular biology is understanding how a DNA molecule, which is a thousand to hundreds of thousands times longer than the diameter of its cellular compartment, is shaped into a functional chromosome inside living cells. In both prokaryotes and eukaryotes, the first level of DNA compaction is the formation of negative supercoiling. However, prokaryotic chromosomes, unlike eukaryotic chromosomes, are maintained under continual negative superhelical tension. About half of the supercoils of prokaryotic DNA are in an interwound, energetically stressed conformation that is lost when the chromosome is nicked multiple times with gamma radiation (1). The remaining half of supercoils appear to be constrained. The

factors that are responsible for constraining half of the chromosome's supercoils are not understood.

The search for proteins that shape the prokaryotic chromosome has looked for structural and functional equivalents of the eukaryotic histones, proteins that both compact and supercoil DNA and make it available for gene expression and genetic recombination. However, finding the histone equivalents in bacteria has been difficult. While eukaryotic chromatin, the genetic material and associated proteins, is a fairly stable structure, bacterial chromatin, the nucleoid, is fragile (2). The compact nucleoid has a short lifetime outside the cell, making even electron microscopy of the structure difficult.

The biochemical approach to studying protein framework components of the nucleoid has yielded mixed results. Some DNA-binding proteins have turned out to have other roles in the cell. For example, the H protein, which was shown to cross react immunologically with histone H2a (3), was ultimately discovered to be ribosomal subunit S3 (4). Another protein, called skp or HLP1, (5) turned out to be localized in the outer membrane of viable cells. These proteins must have become chromosome-associated after cell lysis, and they were mistaken for proteins with histone-like properties because of their DNA binding activity. On further study, only four proteins have proven by genetic and biochemical experiments to be elements of chromosome architecture in vivo (6). These four proteins are HU,

integration host factor or IHF, the factor for inversion stimulation or FIS, and HNS, which has also been called H1a and B (7). Each one has a dimeric subunit structure [see reviews by Schmid (6)].

HU and IHF are closely related heterodimeric proteins, each with two subunits encoded by unlinked genes in Escherichia coli and Salmonella typhimurium (8). In some species, HU is a homodimer composed of a single polypeptide chain (9). The HU protein's primary sequence is highly conserved among widely divergent bacterial species (10). HU, present at 30,000 dimers per cell, is the most abundant nucleoid protein and has shown no sequence specificity in DNA binding. However, IHF and FIS are sequence specific DNA binding proteins that bend DNA at their sites of action (11), Surprisingly, none of the chromosomal proteins is essential for cell viability.

The E. coli chromosome has 4.7×10^6 base pairs and is underwound by an amount that is equivalent to about 23,000 supercoils. Half of these supercoils are maintained under torsional stress, so what constrains the remaining equivalent of 11,000-12,000 supercoils? Searches for abundant proteins that create a linking number deficit upon binding to DNA have turned up only two things--RNA polymerase and HU. RNA polymerase unwinds the Watson-Crick helix by 1.7 turns (580°) when it binds to a promoter in a pre-initiation complex and while it is in the act of transcribing genes (12). Since there are 3,000 RNA polymerase molecules in the cell, most of

which may be engaged in transcription (13), the unwinding caused by these bound molecules explains a linking deficit equivalent to 5,000 supercoils, leaving 6,000-7,000 constrained supercoils unaccounted for.

HU is often called a histone-like protein because it has a low molecular weight, has a basic pI and carries a high charge density, is heat stable, and immunologically cross-reacts with nucleoid proteins from the cyanobacteria and chloroplasts (14). More importantly, HU compacts DNA (15), and if HU is incubated with relaxed closed circular DNA in the presence of a topoisomerase that removes all torsional strain, the DNA becomes negatively supercoiled. These supercoils represent DNA writhe associated with the bound HU protein (16). Ten HU dimers are required to make one supercoil in vitro. Thus, 30,000 HU molecules present in living cells could restrain 3,000 supercoils. Consistent with this estimate, plasmids from cells lacking HU exhibit a supercoil loss of 10-12% (17), which, if plasmid behavior reflects the status of the cell genome, extrapolates to 2,300-2,800 supercoils in the chromosome. This leaves 3,000-4,000 supercoils unassigned and raises the question, do other proteins organize chromosome writhe?

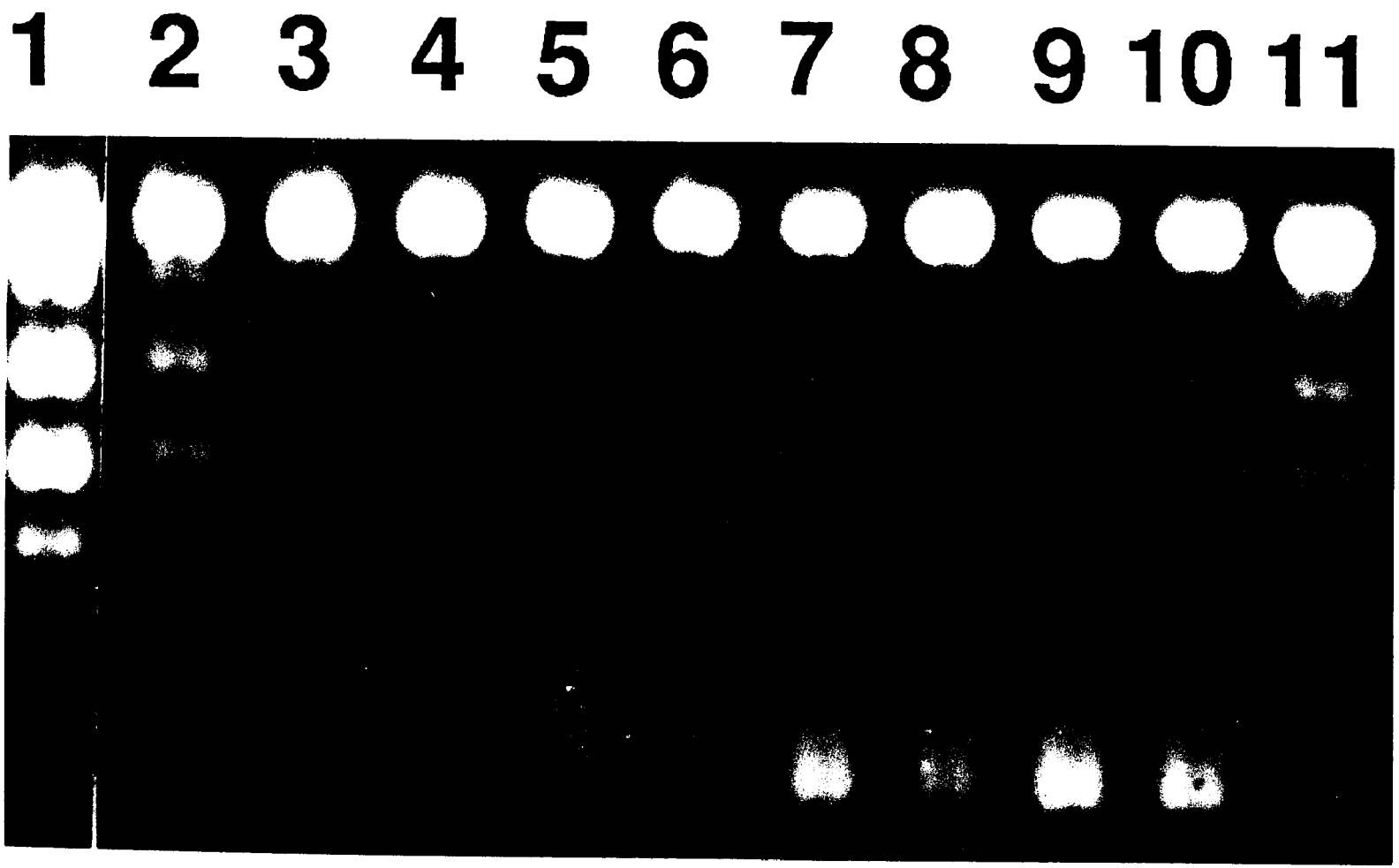
The second most abundant nucleoid-associated protein, HNS, is structurally unrelated to HU, IHF, or FIS. HNS is composed of two identical 15 kd subunits, which makes this molecule twice the size of HU or IHF proteins. It is highly charged, but contains equal numbers of acidic and basic

residues, resulting in a pI near 7. In primary structure, HNS does not resemble a histone. Rather, HNS is similar to a high mobility group protein, like the yeast HMG 1 protein (18). HNS is present and abundant throughout the cell cycle. Twenty thousand dimers are present in log phase cells and this number increases several fold in stationary phase (19).

In the course of studying HU-mediated supercoiling, we observed that the addition of glutamate made supercoiling reactions more reliable. Potassium glutamate buffer or KGB (100 mM potassium glutamate, 10 mM magnesium acetate, 25 mM Tris acetate, 50 μg/ml bovine serum albumin, and 0.5 mM β-mercaptoethanol) was developed as a general physiological reaction buffer (22). Many buffers contain Na^+ and Cl^-, while the dominant cation in the cell is potassium and the dominant anion is glutamate (23). Restriction enzymes and DNA and RNA polymerases work efficiently in KGB, despite their widely differing salt and pH optima in sodium chloride based buffers. Potassium glutamate also stimulates tubulin assembly (24). One explanation for the effect of glutamate is that macro-molecular assembly is driven by an entropic process in which protein-protein (or protein-DNA) interactions are aided by the destabilization of glutamate that is bound to the surface of a protein.

Two highly purified nucleoid associated proteins, IHF and HNS, were tested in constrained supercoiling assays using KGB buffer. While IHF, at high or low concentrations, showed no significant reaction (data not shown), HNS (25) introduced

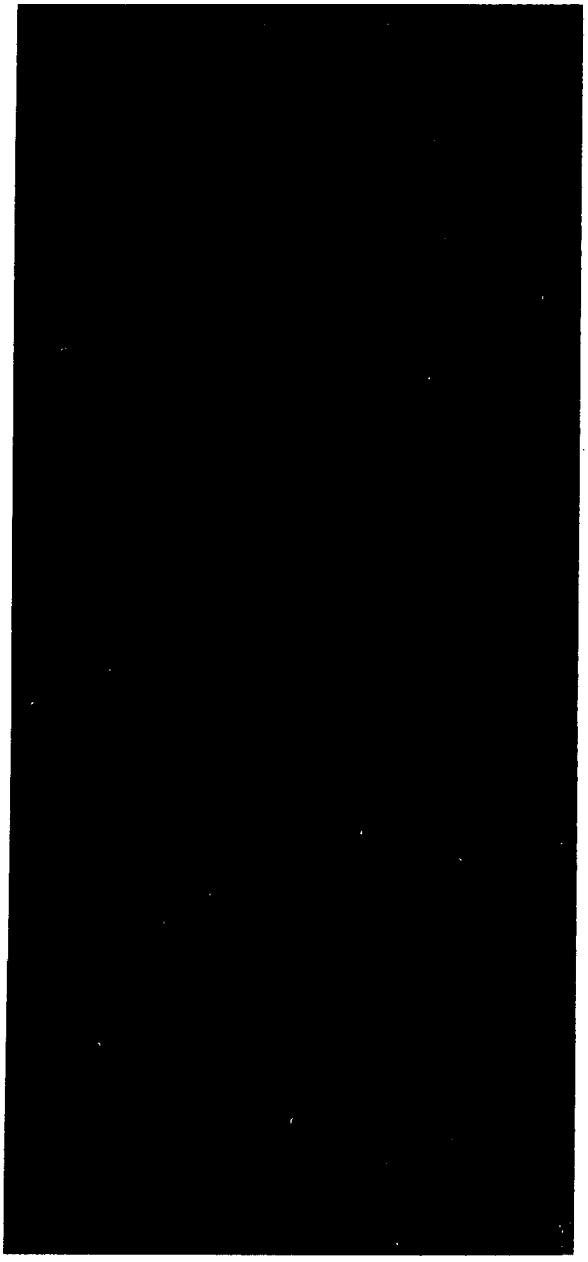
Figure 1. Supercoiling of plasmid DNA in the presence of HNS. 200 ng (0.1 pM) of relaxed plasmid pHK09 (3320 bp) was incubated with varying concentrations of HNS in a reaction volume of 10 µl for 30 minutes at 37°. Following preincubation, calf thymus topoisomerase was added for 30 minutes at 37°. Reaction products were separated on a 1% agarose gel in Tris borate EDTA buffer at 2.5 V/cm. Lane 1, relaxed DNA; lane 2, 3 pM HNS (10 µg/ml); lane 3, 7 pM HNS; lane 4, 14 pM HNS; lane 5, 21 pM HNS; lane 6, 28 pM HNS; lane 7, 35 pM HNS; lane 8, 42 pM HNS; lane 9, 49 pM HNS; lane 10, 56 pM HNS; lane 11, no HNS.


1 2 3 4 5 6 7 8 9 10 11

high negative supercoiling into DNA (Fig 1). Supercoiling was quantitated through a range of protein concentrations by resolving the topoisomer products in two dimensional agarose gels. The first dimension was without chloroquine and the second dimension was run in the presence of 40 μM chloroquine (Figure 2a). Centers of the gaussian topoisomer distribution were determined and the quantity of supercoils introduced was compared to the HNS dimer concentration added (Figure 2b). From the slope near the steepest portion of this curve, the number of HNS dimers required to form one supercoil was estimated to be 6-10, which is similar to the 10 dimers per supercoil that has been measured for HU (16). The supercoiling curve of HNS may have a sigmoid character, which would indicate cooperativity, something not observed in HU binding. Maximal supercoiling was reached at about 30 pM HNS, or at an HNS:DNA weight ratio of 5:1, wherein nineteen supercoils were introduced into the 3.3 kilobase plasmid pHK09. This represents one supercoil per 174 base pairs and a $-\sigma$ =0.057. Thus, on a molar basis, HNS is as efficient as HU at constraining negative supercoils. Supercoiling was inhibited at very high HNS concentrations, probably because HNS saturation of the DNA can block topoisomerase I binding.

Because HU is a common contaminant of purified DNA binding proteins, we examined our purified HNS by western blot analysis with a polyclonal anti-HU antibody. When 10 μg HNS was analyzed we saw no staining at the HU position in the gel (data not shown). From these data, our purified HNS is

Figure 3. Western blot analysis of H-NS. Cells were grown to 150 Klett units at 37° in either LB or NCE-malate. Aliquots from cells indicated in the figure were run in lanes 1-4, 6 and 7. 50 ng of purified H-NS was loaded in lane 5.



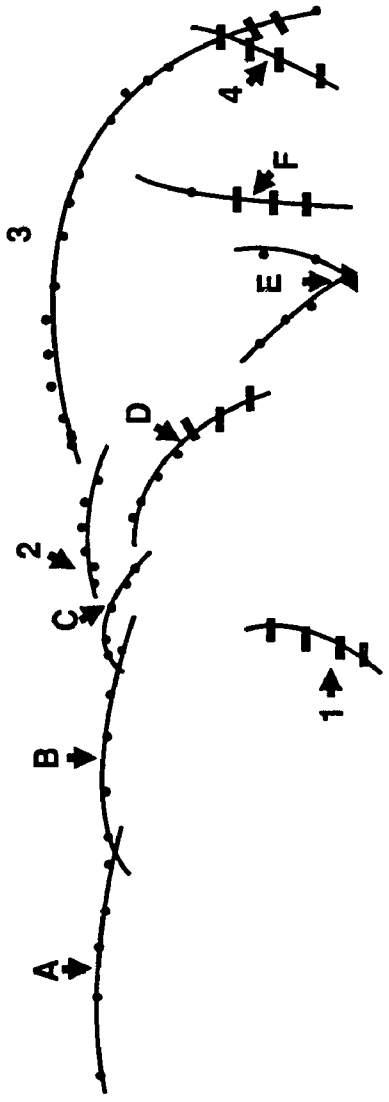
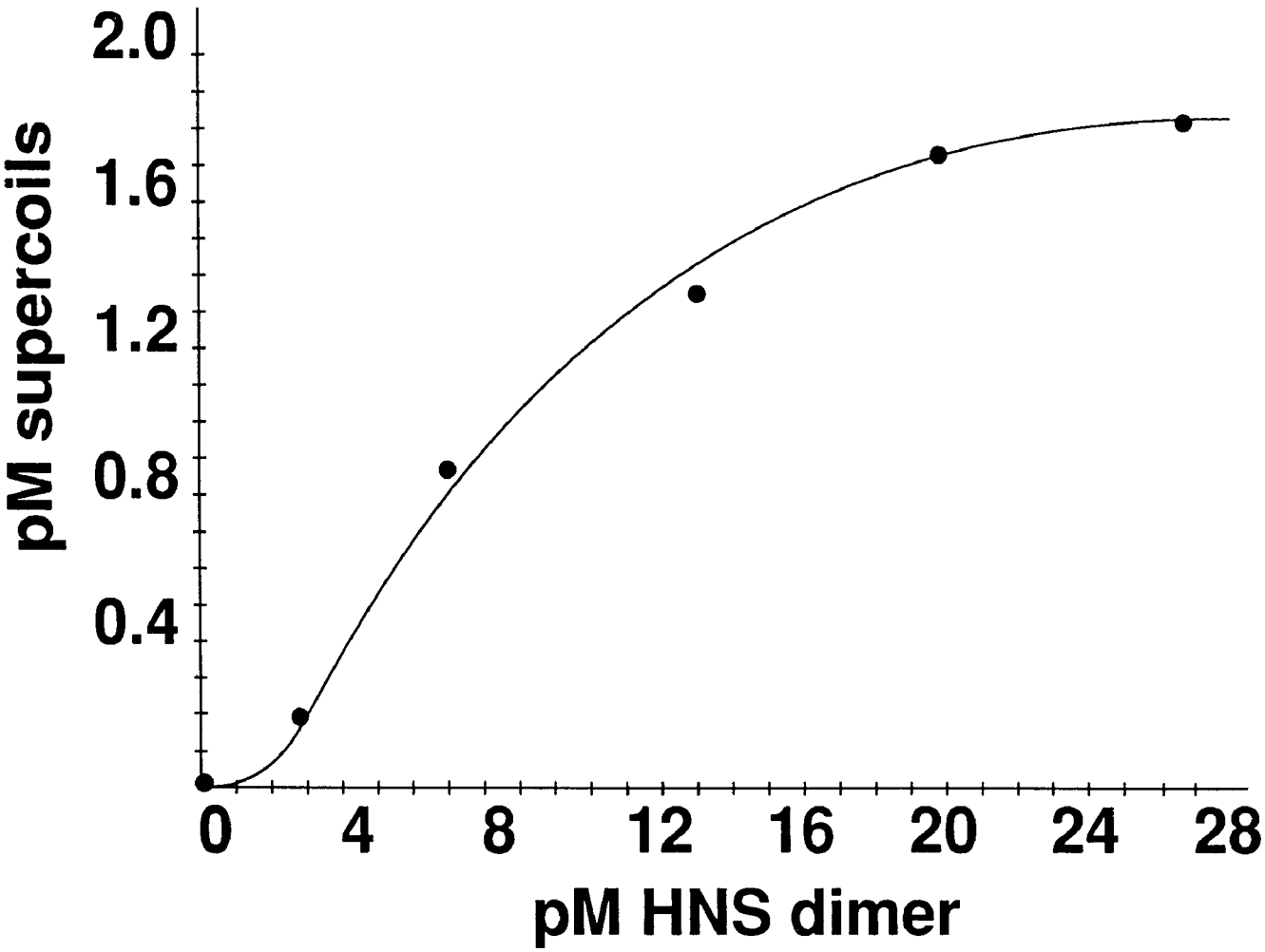

A
B
C
D
E
F
1
2
3
4

Figure 2.B. Graphical analysis of 2D gel. Plasmid pHK09 was incubated with varying concentrations of HNS, then relaxed with topoisomerase I as previously and separated by 2 dimensional electrophoresis (see Figure 2a). The quantity of supercoils introduced at each concentration (in picomoles) is compared to amount of HNS dimer added to the reaction.

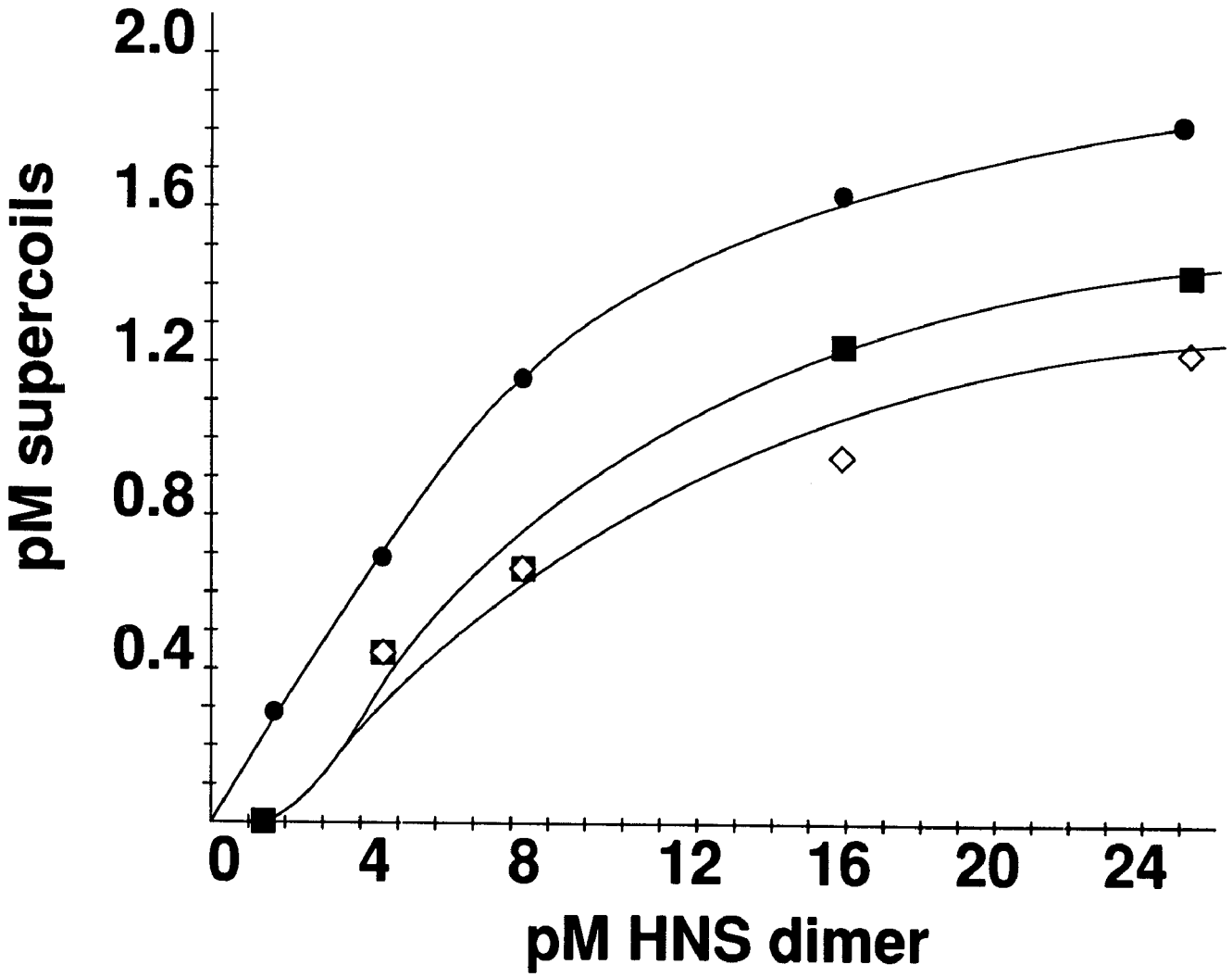

2.0
1.6
1.2
0.8
0.4
pM supercoils
0
4
8
12
16
20
24
28
pM HNS dimer

judged to be less than 1% contaminated by HU. This is insufficient HU to support the observed supercoiling reactions. However, there is another reason why HU contamination does not explain the HNS-dependent supercoiling reaction.

To determine whether HU and HNS work synergistically, we studied the consequences of adding both proteins to the same reaction mixture (Figure 3). Rather than causing an increase, HU diminished HNS-induced supercoiling. The effect was seen when HNS protein was added before HU protein (Figure 3), when this order was reversed, or when the the proteins were added simultaneously (data not shown). Addition of 2.8 pM HU to reactions containing 26 pM HNS reduced the supercoiling by about 20% and 5.5 pM HU reduced the supercoiling by 30%. The HU effect was also seen at lower HNS concentrations (Figure 3, 16.6 pM and 8.3 pM), so the disruption of supercoils is not a reflection of competition between the two proteins for scarce binding sites on the DNA.

With the discovery of the HNS supercoiling activity, recognizing that estimates of the superhelical constraint by these proteins were made under different conditions, most of the constrained chromosomal supercoiling can be assigned to proteins: RNA polymerase creates underwinding equivalent to 5,000 supercoils, HU controls 3,000 supercoils, and HNS controls 2,000-3,000 supercoils. Two reasons can be given for why HNS supercoiling activity has previously been missed. First, potassium glutamate buffers improve the supercoiling

Figure 3. HU destabilizes supercoils formed by HNS. HNS was preincubated with pHK09 for 30 minutes at 37°, HU then was added at 3 pM/reaction (5 µg/ml) (closed squares), 6 pM/reaction (open diamonds), or not added (closed circles); after further incubation for 30 minutes at 37°, topoisomerase I was added and incubation continued for 30 minutes at 37°. Reaction products were separated by 1% agarose gels in one dimension in the presence of three different chloroquine concentrations to ensure separation of topoisomers. Changes in topology were evaluated by determining the center of the supercoil distribution. The number of supercoils introduced at each concentration is compared to the amount of HNS dimer added to the reaction.


2.0
1.6
1.2
0.8
0.4
pM supercoils
0
4
8
12
16
20
24
pM HNS dimer

reactions. Second, because HU is more abundant and also interferes with HNS-dependent supercoils, HU would mask the HNS activity. Other proteins, like helicases, DNA polymerases, and DNA repair enzymes, are possible candidates for underwinding DNA. But none of these are as abundant as RNA polymerase, HU, and HNS in log phase cells, so their contribution is not expected to be as great.

The mixing experiment raises a question. Both HU and HNS constrain negative supercoils, but HU disrupts supercoils introduced by HNS, and HNS disturbs HU dependent supercoils. Why are their actions non-complementary? The answer may lie in the physiological roles that these two proteins play in the cell. In genetic experiments, HNS has been shown to modulate transcription. HNS is most abundant during stationary phase when cells stop growing. Moreover, mutants deficient in HNS are diminished in transcriptional repression of genes involved in unrelated biochemical pathways. Operons that become partially derepressed in HNS mutants include *proU*, *pap*, *bgl*, and the early genes of bacteriophage Mu (18,26). No similar phenotype is known for HU. The cellular response to too much of these proteins is also informative. HU overexpression causes no significant change in transcription, whereas HNS overexpression in growing cells triggers an immediate stoppage of transcription and causes growth arrest (27). HNS blocks transcription in vitro, even after RNA polymerase has been bound to a promoter (28).

We suggest that HU and HNS have distinct chromosomal roles. In regions where HU is the dominant protein, transcription proceeds efficiently. Regions where HNS is most abundant remain in a supercoiled un-transcribed state. How could one supercoiling protein mechanically counteract another? Much more work needs to be done on these two proteins to determine an answer. However, one possibility is that HU forms solenoidal DNA writhe reminiscent of histones (15), while HNS creates interwound supercoils that physically block RNA polymerase movement. In fact, this regulatory strategem has a parallel in eukaryotes, where genes silenced in heterochromatin can be expressed only after changes in the protein components arrayed on DNA create euchromatin.

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25. HNS, produced in an E. coli strain containing a plasmid with HNS placed under the control of the inducible λ p_L promoter was purified as described (18). HU was isolated from E. coli N99. Pelleted cells lysed by sonication were cleared of debris, then successively centrifuged at 37,000 and 150,000 x **g**. Supernatant after the 150,000 **g** step was brought to 50% ethanol. Precipitated material was removed by centrifugation and the supernatant was dialyzed and further purified by chromatography as in the method of N. E. Dixon, A. Kornberg, *Proc. Natl. Acad. Sci. USA.* 81, 424-428 (1984).

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HNS SILENCES TRANSCRIPTION IN VIVO

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(to be submitted to Genes and Development)

ABSTRACT

Two bacterial nucleoid associated proteins, HNS and HU, have been shown to constrain supercoils in DNA *in vitro*. Overexpression of HU and of HNS has markedly different effects, suggesting that the cellular roles of the two supercoil-stabilizing proteins are different. HU overproduction has little physiological effect, but HNS overexpression blocks transcription and stops cell growth. Though a natural high level of expression of HNS occurs during stationary phase, HNS participates in regulation of transcription throughout the cell cycle, helping to maintain genes in a non-transcribed state. The consequences of HNS overexpression and its effects on a number of transcriptionally controlled genes suggest a role for HNS in transcriptional silencing throughout the cell cycle. The differential effects of HU and HNS overexpression suggest that HNS can do more than enhance repression: HNS may organize DNA into transcriptionally inactive domains while HU interacts with genes that can be transcribed.

INTRODUCTION

In vivo studies suggest that prokaryotic chromosomes are organized differently than eukaryotic ones: the DNA of *Escherichia coli* is less constrained than that of eukaryotes (Pettijohn, 1980; Sinden, 1980). While eukaryotic chromosomes appear to exist with little torsional strain in the DNA, (Sinden, 1980), prokaryotic chromosomes are held under continual net negative superhelical tension.

Only about half of their supercoils are constrained.

Despite this basic difference, the search for bacterial factors responsible for compacting the chromosome while leaving it available for replication and transcription has led to the characterization of a handful of proteins that strongly associate with the chromosome, forming a loosely defined structure, the nucleoid. (Reviewed in Drlica, 1987;Schmid, 1990; Schmid, 1991)

Among the proteins of the nucleoid, the DNA binding protein HU has been a good candidate for the role of a histone (Drlica, 1987). The genes for HU have been maintained through time and conserved across species, like those for histones in eukaryotes. Small, basic and heat stable like a eukaryotic histone protein, HU is a heterodimer of 9.5 and 9.2 kilodalton proteins, and is present at high copy number, about 30,000 (Dixon, 1984) dimers per cell. HU's similarity to another of the nucleoid associated proteins, the sequence-specific DNA binding and bending protein integration host factor (IHF), suggests a role for HU in altering the shape of DNA. HU has been shown to influence the curvature of DNA *in vitro* (Hodges-Garcia, 1989), and like the eukaryotic nucleosome, HU is able to to constrain supercoils in DNA *in vitro* (Broyles, 1986; Rouviere-Yaniv, 1979). Unlike the eukaryotic histones, HU is a nonessential protein (Higgins, 1988).

Another small nucleoid associated protein, HNS, does not resemble IHF and HU in structure or in charge. HNS is a

dimer of 15 kilodalton subunits, twice the size of HU and IHF. The physical properties of HNS are more reminiscent of the eukaryotic high mobility group protein HMG1 than of histones (Falconi, 1991). HNS is the product of a gene located at 27 minutes on the *E. coli* chromosome. This gene has been independently identified in different genetic systems a number of times as a factor without which transcriptionally repressed operons become more easily activated (May, 1990). It has been named *bglY*, as a factor regulating β-glucoside metabolism (Defez, 1980); *drdX*, as a locus involved in thermoregulation of pilliation (Goransson, 1990), as *virG* when regulating a family of other virulence factors in *Shigella* (Dorman, 1990), and as *cur* when it was identified as a factor in cell aggregation (Diderichsen, 1980). It has also been identified as *pilG*, a locus playing a role in phase variation of fimbrae (Spears, 1986) and as *osmZ*, a regulator of the response of cells to changes in extracellular osmolarity (Higgins, 1988).

Recently, while studying host genes that influence the movement of transposable elements, we identified genetic loci involved in the stability of bacteriophage Mu. (Falconi, 1991). Using a mini-Mudlac that is readily transposable and produces lacZ protein fusion when it lands in frame behind promoters in expressed genes, we detected three classes of mutations that stimulated the transposition of Mu, as reflected by intense staining of colonies grown on the β galactosidase substrate X-gal. We named this

phenotype stimulation of Mu transposition (*smt*). One class of mutations causing the smt phenotype mapped to 27 minutes. This class was caused by mutations in *hns*.

A role has been suggested for HNS in organizing the bacterial chromosome (Dorman, 1991; Hulton, 1990). Studies of the interaction of HNS with the Mu regulatory regions suggest that the effect of HNS in this system reflects the ability of HNS to stabilize and enhance the interaction of Mu repressor with its binding site (Falconi, 1991). The cooperative binding of repressor to its defined sites is improved by low concentrations of HNS. At higher concentrations, HNS, like HU, stabilizes negative supercoils in plasmid DNA. (McGovern and Higgins, submitted).

Like HU, HNS is an abundant chromosome associated protein. It is present at about 30,000 copies and is most highly produced during stationary phase (Spassky, 1984).

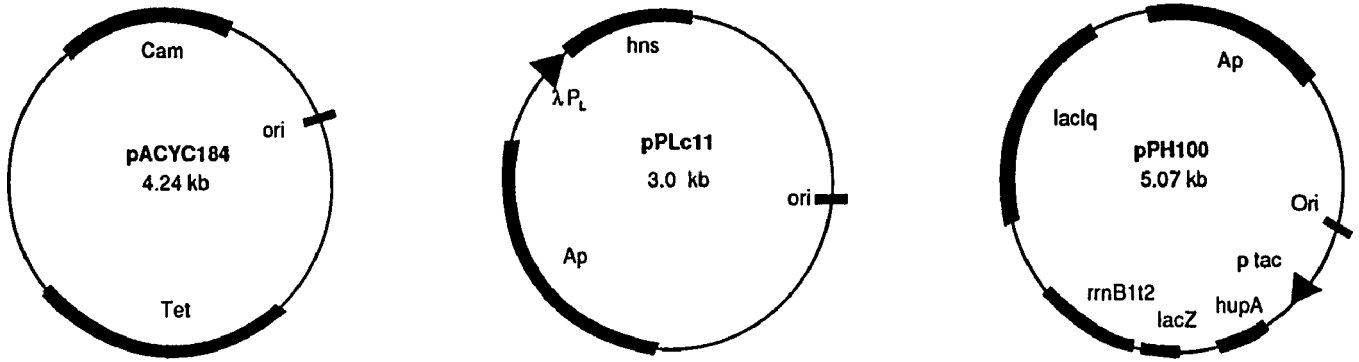
To better understand why bacteria maintain two nonessential abundant nucleoid associated supercoiling proteins, we undertook an *in vivo* study of the effects of the two proteins.

Using plasmids that allow us to induce production of large amounts of either of the two proteins, we have studied their effects on cell growth and transcription. The two proteins had markedly different effects.

Table 1. Strains of E. coli used in these studies

Strain	Description		Reference/Source
N99	str galK F-		H. Nash
NH270	N99	pPH100	Higgins 1988
NH1169	NH270	pACYC184	This work
NH1205	N99	pACYC184	This work
NH753	N99	pPLc11	C. Gualerzi
NH1177	NH753	pACYC184	This work
NH2177	N99	pPLc2833	This work

Figure 1. Plasmids used in this study. A. pACYC184, carrying resistance to tetracycline and chloramphenicol, was used as a selectable plasmid marker unlinked to the overexpressed proteins HU and HNS, and also as a topological marker. B. pPLc11 bears an ampicillin resistance gene and the gene for *E coli* HNS under the control of the temperature inducible promoter from lambda, λp_L. C. pPH100 contains an ampicillin resistance gene and the gene for *Salmonella typhimurium* HU2 (*hupA*) under the control of the pTac promoter, inducible by addition of the lac inducer IPTG.


Cam
ori
pACYC184
4.24 kb
Tet
hns
λ P_L
pPLc11
3.0 kb
ori
Ap
Ap
laclq
pPH100
5.07 kb
Ori
p tac
rrnB1t2
lacZ
hupA

A B C

EXPERIMENTAL PROCEDURES

Plasmids and Strains. *E. coli* strains used in this study are listed in Table 1. Plasmids employed are shown in Figure 1. HU was overexpressed by induction of the *Salmonella typhimurium hupA* gene from strain NH270, carrying the plasmid pPH100 (Higgins, 1988), a derivative of the expression vector pTTQ18. pPH100 carries a copy of the $lacI^q$ repressor to regulate the inducible pTac promoter that drives HU expression. Overproduction of HU was begun by addition of the isophenylthiogalactoside (IPTG), a gratuitous inducer of the lac operon, to 250 mM.

HNS was overproduced by induction of the *E. coli* HNS gene from pPLc11, a derivative of the expression vector pPLc2833 in which the structural gene for HNS is driven by a thermoinducible λ pL promoter (Spurio, 1992).

pACYC184 was included as an independent topological marker and also provided a selectable marker, chloramphenicol resistance, not linked to the HU or HNS gene.

Overproduction of HU. *E. coli* NH270 carrying pPH100 was grown to an absorbance at 600 nm (A_{600}) of approximately 0.5 in well aerated cultures at 32° C in L broth containing 50 μg/ml ampicillin, then divided into two fractions. One fraction was induced by the addition of IPTG to 250 μM. The second fraction was maintained as an uninduced control culture.

Overproduction of HNS. *E. coli* N99 carrying pPLc11 was grown at 32° C in L broth containing 50µg/ml ampicillin. When the culture reached an A_{600} of 0.5, the culture temperature was rapidly brought to 42° C by the addition of one half volume of media at 65°. Cultures were incubated at 42° to induce, then harvested by centrifugation and resuspension in media at 32°. A control culture for HNS overexpression, strain N99 carrying the expression vector with no HNS gene present, was subjected to the same treatment to determine the effects of temperature shifts.

Transcription Assay. The transcription assay was developed after the method of Pato and von Meyenburg (Pato, 1970). Cultures were sampled for transcription before and after induction with heat or IPTG. One milliliter aliquots of the growing culture were transfered to 30 ml scintillation vials prewarmed to the growth temperature of the cells. 10 µl of 5-^{3}H Uridine (26.9 Ci/mM, 1 mCi/ml) was added immediately after transfer of the culture to the vial, then over a 2 minute time course, 100 µl of the material in the vial was removed to 500 µl of lysis buffer (0.1M NaCl, 0.01M tris-Cl, pH 7.5, 0.02 M EDTA, 0.5% SDS) at 95° C and incubated at 95° C for 5 minutes. 100 µl of the lysis mix was spotted onto Whatman GFC filters. The filters were thoroughly dried, then subjected to TCA precipitation. The TCA perceptable counts were determined by counting for 5 minutes in EcoScint scintillar. Counts incorporated by this method were more than 90% resistant to the replication-stopping drug enoxacin

and more than 95% of counts incorporated were rifampicin sensitive (data not shown), so total incorporation of counts reflects transcription.

DNA topology. 10 mls of growing culture were pelleted, and plasmids were extracted by the alkaline lysis method (Maniatis, 1982). Topoismers were separated on agarose gels containing 5 μM chloroquine. Changes in DNA topology were determined by the band counting method (Keller, 1975).

Protein extraction and separation. To obtain proteins for visualization by protein electrophoresis, 10 ml of growing culture were pelleted and resuspended in 3 x cracking buffer (0.15 M tris HCl, pH 7.0; 6% SDS; 2.1 M 2-mercaptoethanol; 40% glycerol), then boiled for 10 minutes. The lysates were then analyzed using 15 or 18% SDS-polyacrylamide gels. The gels were stained with coomassie blue.

RESULTS

HU and HNS can be reliably overproduced using plasmids in which their genes are fused to inducible promoters. Using the plasmid pPH100 (Higgins, 1988), in which the *Salmonella typhimurium hupA* gene is under the control of the pTac promoter we overexpressed HU in cells at mid-log phase. Because the phenotypes of mutants lacking one or the other HU subunit are subtle (Hillyard, 1990), because some bacterial species naturally have only one HU subunit (Drlica, 1987) and because *Salmonella* HU2 can replace *Escherichia coli* HU heterodimer in several biochemical assays *in vitro* (V.McGovern, unpublished observation), we

Figure 2. Protein HU overproduction profile of strain NH1169. Lane M: markers- apparent molecular weights 2.7, 8.1, 13.7, 18.2, 30.4, 42.1. A sample was taken before induction (0), then IPTG was added and samples were removed at times shown. The arrowhead marks the position of HU.


M
0'
15'
30'
1h
3h

posit that that overexpressing a *Salmonella* HU homodimer for these investigations is not unreasonable. Cells were grown to an A_{600} nm of 0.5, then the lac inducer iso-phenyl-thiogalactoside (IPTG) was added to 250μM. A large increase in the concentration of the 9k HU-2 protein was observed on coomassie blue stained 18% polyacrylamide gels (FIGURE 2). Western blotting developed with a biotin-streptavidin detection system and a polyclonal anti HU antibody, provided by George Chaconas, indicated that a ~40 fold overproduction of the protein had been achieved (data not shown).

HNS overproduction from the thermoinducible overproducing plasmid pPLc2883 was induced at an A_{600}= 0.5 by the addition of an appropriate volume of 65° medium, rapidly bringing the culture temperature to 42°. The induction of HNS was observed by coomassie staining of a 15% polyacrylamide gel (Figure 3). Western blotting with a polyclonal HNS antibody demonstrated an overproduction similar to that of HU, ~40 fold more protein than found in an uninduced control (data not shown).

The growth of the cultures after induction was monitored by following their A_{600}. Overexpression of HU produced a modest decrease in the growth rate, increasing the doubling time by a factor of 1.8. Although the growth rate decreased, the induced culture achieved the same optical density at stationary phase as the uninduced control (Figure 4).

Figure 3. Protein HNS overproduction profile of strain NH1177. First lane: preinduction. Second lane: After heating at 42° for 15 minutes. The culture was returned to 32° and samples were removed at times shown. Lane M: markers- apparent molecular weights 2.7, 8.1, 13.7, 18.2, 30.4, 42.1. Position of HNS is marked with an arrowhead.

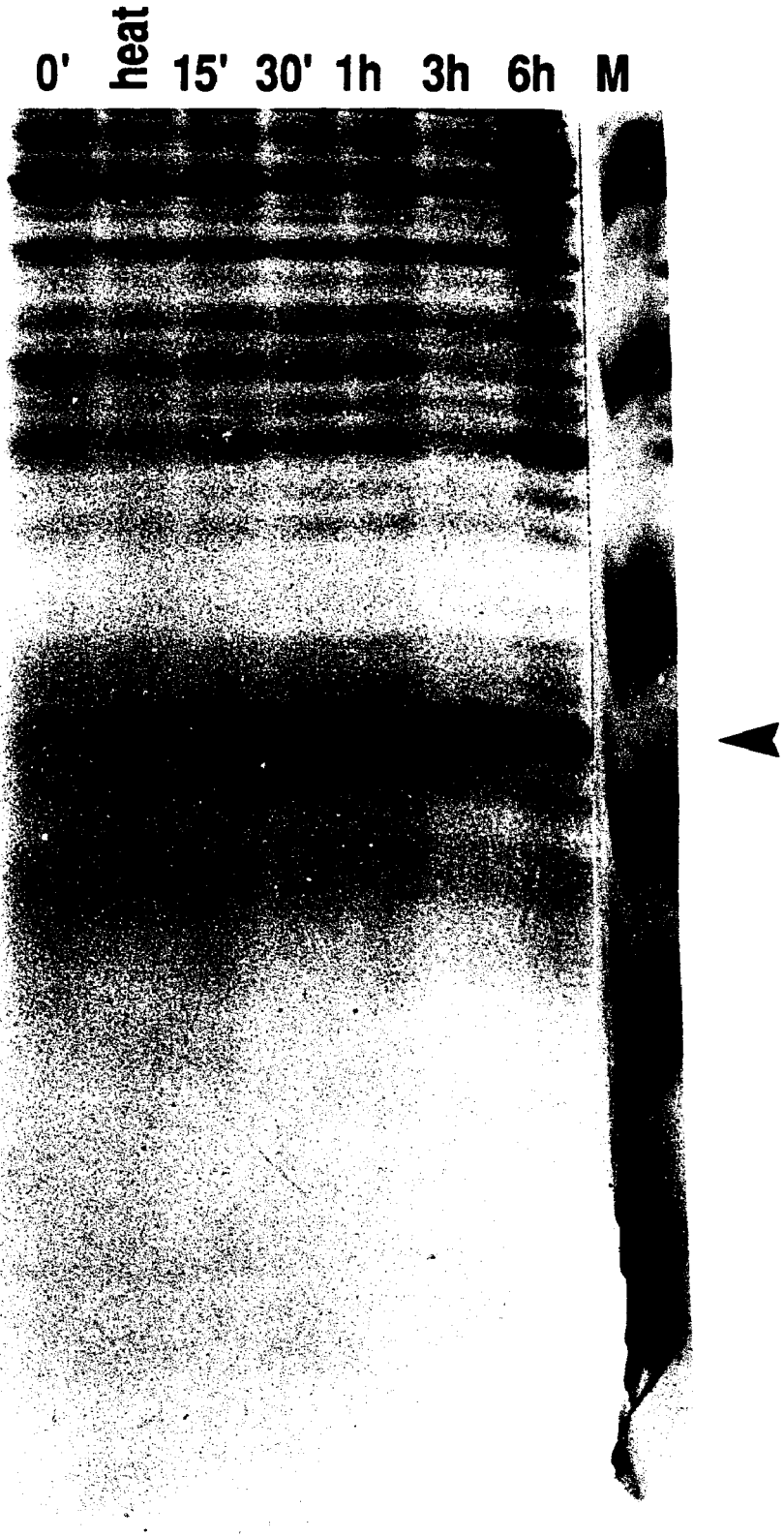

0'
heat
15'
30'
1h
3h
6h
M

Figure 4. Growth profile after overexpression of HU. Growth of the overproducing strains was monitored by A_{600}. *E. coli* NH1169, was grown in well aerated flasks at 32° C to A_{600}=0.5. At this absorbance, the culture was split and one aliquot (solid circle) was induced, by addition of IPTG, to overproduce HU. The other (Solid box) was left uninduced. Growth of uninduced and induced cells was monitored until a stable plateau was achieved.

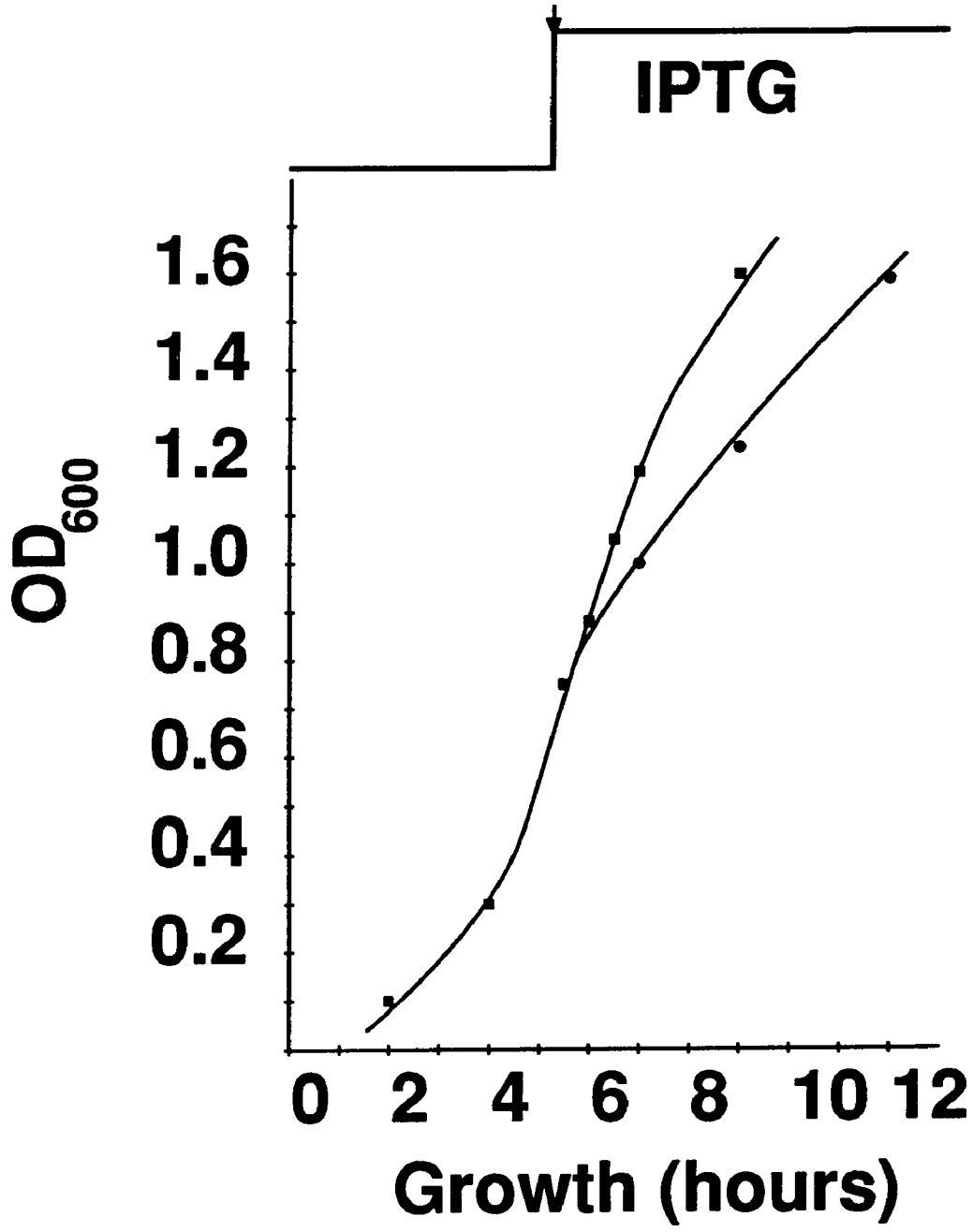
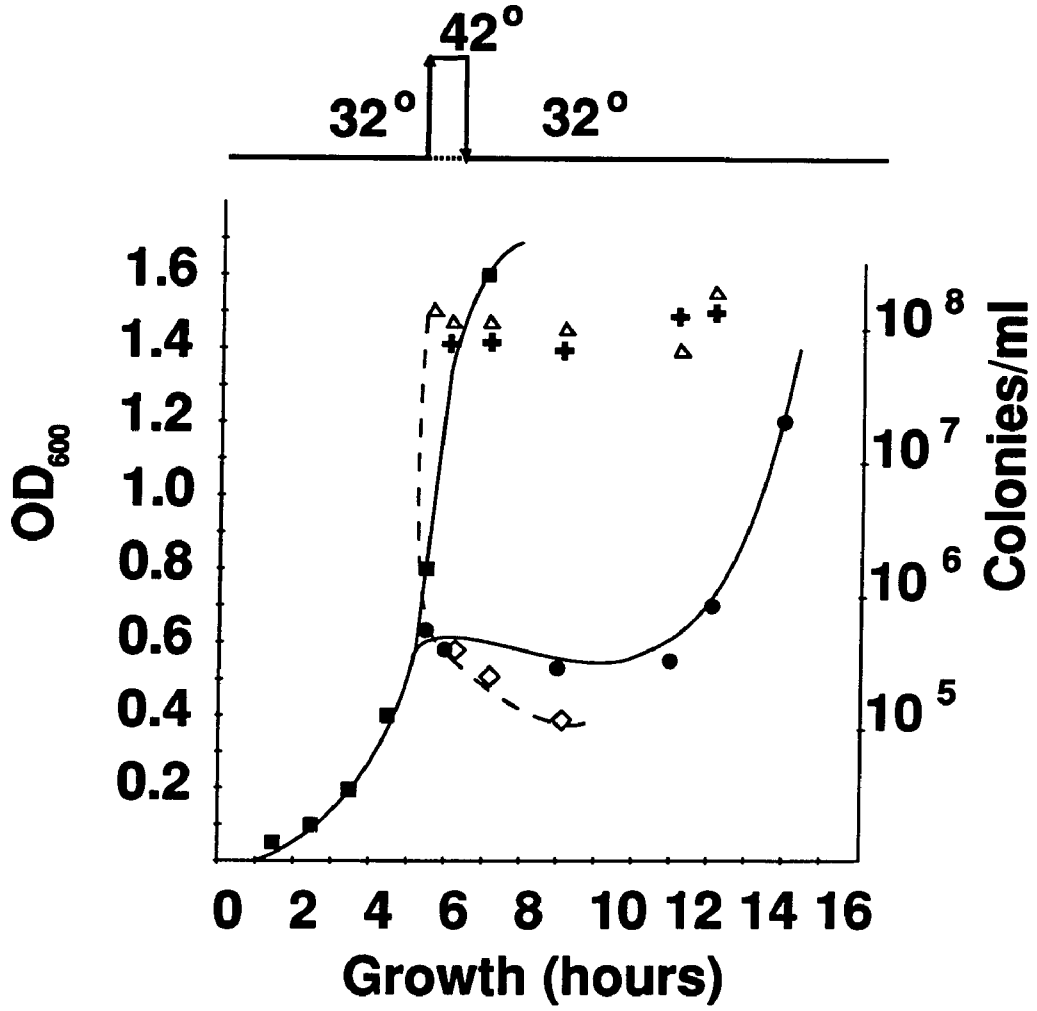

IPTG
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
OD600
0 2 4 6 8 10 12
Growth (hours)

Figure 5. Growth profile after overexpression of HNS. *E. coli* NH1177 (solid circles) or the expression vector pPL2883 (solid boxes) were grown at 32° C to =0.5, then shifted to the inducing temperature, 42° C, by addition of one half volume of media at 65°. After 15 minutes at 42°, cultures were brought back to 32° and further growth was monitored until the A_{600} reached a stable plateau. Aliquots from the induced culture were removed during growth and plated on selective media to determine the viability of cells in culture: cells per ml plated on L broth (solid crosses); Cells per ml plated on LB with 30 μg/ml chloramphenicol (open triangles); Cells per ml plated on 50 μg/ml ampicillin (open diamonds).

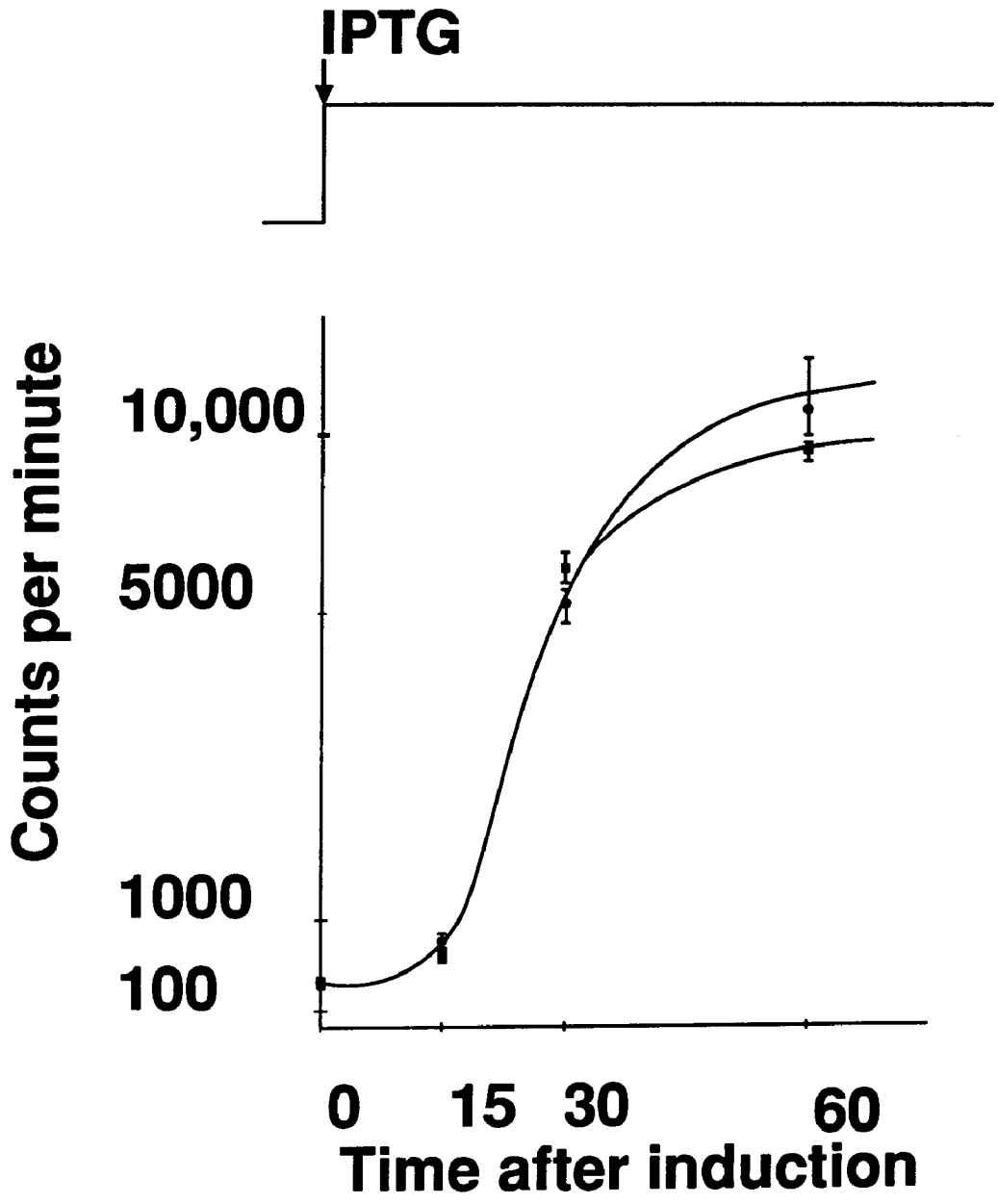

42°
32°
32°
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
OD600
10 8
10 7
10 6
10 5
Colonies/ml
0 2 4 6 8 10 12 14 16
Growth (hours)

When HNS was induced, an immediate cessation of growth was observed. The induced culture failed to increase in optical density for a period of several hours, then recovered (Figure 5).

To determine whether the change in growth of the induced cultures was due to death of the growing cells (Spurrio, 1992), we plated aliquots of the cultures on selective and nonselective media. When HU was overproduced, the number of colonies observed on plating the induced cultures reflected continued growth of the cells and continued expression of the amp gene carried on pPH100 and the cam gene on a second plasmid, pACYC184, present in the strain as a topological marker (data not shown).

When HNS was overproduced, plating on L broth showed that the cells were viable during the period in which they did not grow. Thus the recovery in growth was not due to cell death followed by repopulation by rare survivors (Figure 3). Plating on chloramphenicol indicated that the cells stably maintained the marker plasmid during the long lag period. Cells picked from these plates could reliably be reinduced and maintained the growth characteristics of the parent culture (data not shown). There was a 99.99% decrease in the viability of cells in the presence of the drug. Colonies from L broth plates or chloramphenicol plates, were resistant to ampicillin. The ampicillin sensitivity may be caused by anomalous cell wall synthesis

Figure 6. Transcription profile of HU overproducing cells. Transcription in cells overproducing HU was monitored as described in materials and methods in an induced HU overproducing culture of NH1169, (solid squares) and in an uninduced control culture (solid circles).


IPTG
Counts per minute
10,000
5000
1000
100
0
15
30
60
Time after induction

in HNS overproducing cells during the lag period, making the cells vulnerable to the the action of ampicillin.

Because HNS silences transcription in several specific operons (Falconi, 1991; Goransson, 1990; Spassky, 1984), we compared the effects of overexpression of HU and HNS on transcription. When HU was overexpressed, transcription was virtually unaffected (Figure 6). A culture bearing the HNS overproducer was grown to mid log phase and then induced by addition of 65° media. After fifteen minutes of induction at 42° the cells were returned to 32°. Transcription was assayed by measuring the incorporation of tritiated uridine into TCA precipitable counts (Figure 7). In a control experiment using cells containing the parent plasmid pPLc2833 without the HNS gene present, transcription increased after the initial heating step. When HNS was overproduced the cells exhibited a rapid loss of transcription that lasted several hours.

To see how overexpression of HU and HNS affected supercoiling, agarose gel electrophoresis was carried out, allowing examination of the supercoiling of the reporter plasmid pACYC186. During starvation (Jaworski, 1991) or inhibition of transcription (Wu, 1988) , reporter plasmids become more relaxed than when they are growing rapidly, possibly because of the loss of transcription driven supercoiling on the plasmid.

On HU overproduction, the center of the topoisomer distribution of the marker plasmid was virtually unchanged

Figure 7. Transcription profile of HNS overproducing cells. Transcription in cells overproducing HNS was monitored as in Figure 6. in an induced culture of NH1177 (solid squares) and in NH2177,(solid circles).

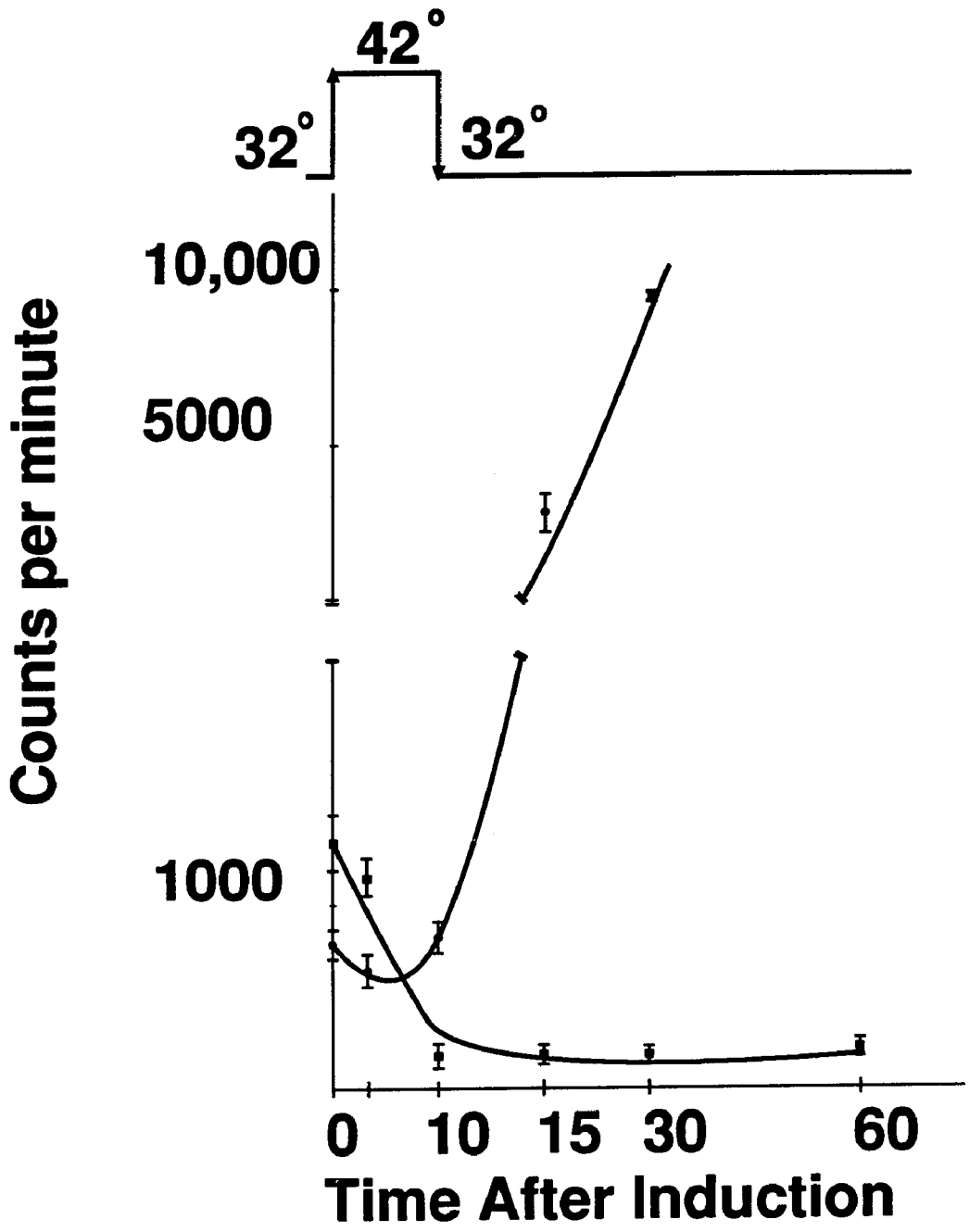

42°
32°
32°
10,000
5000
1000
Counts per minute
0
10
15
30
60
Time After Induction

Figure 8. Topology of plasmid DNA in cells overproducing HU. DNA was extracted from 1., induced NH1169 or 2., NH1205 at times 1, before addition of IPTG; 2, 15 minutes postinduction; 3, 30 minutes postinduction; 4, 60 minutes postinduction and 5, 3 hours post induction and separated on a 1% agarose gel containing 5μM chloroquine. NH1169 contain the overproducing plasmid pPH100 as well as the topological marker pACYC184.

B

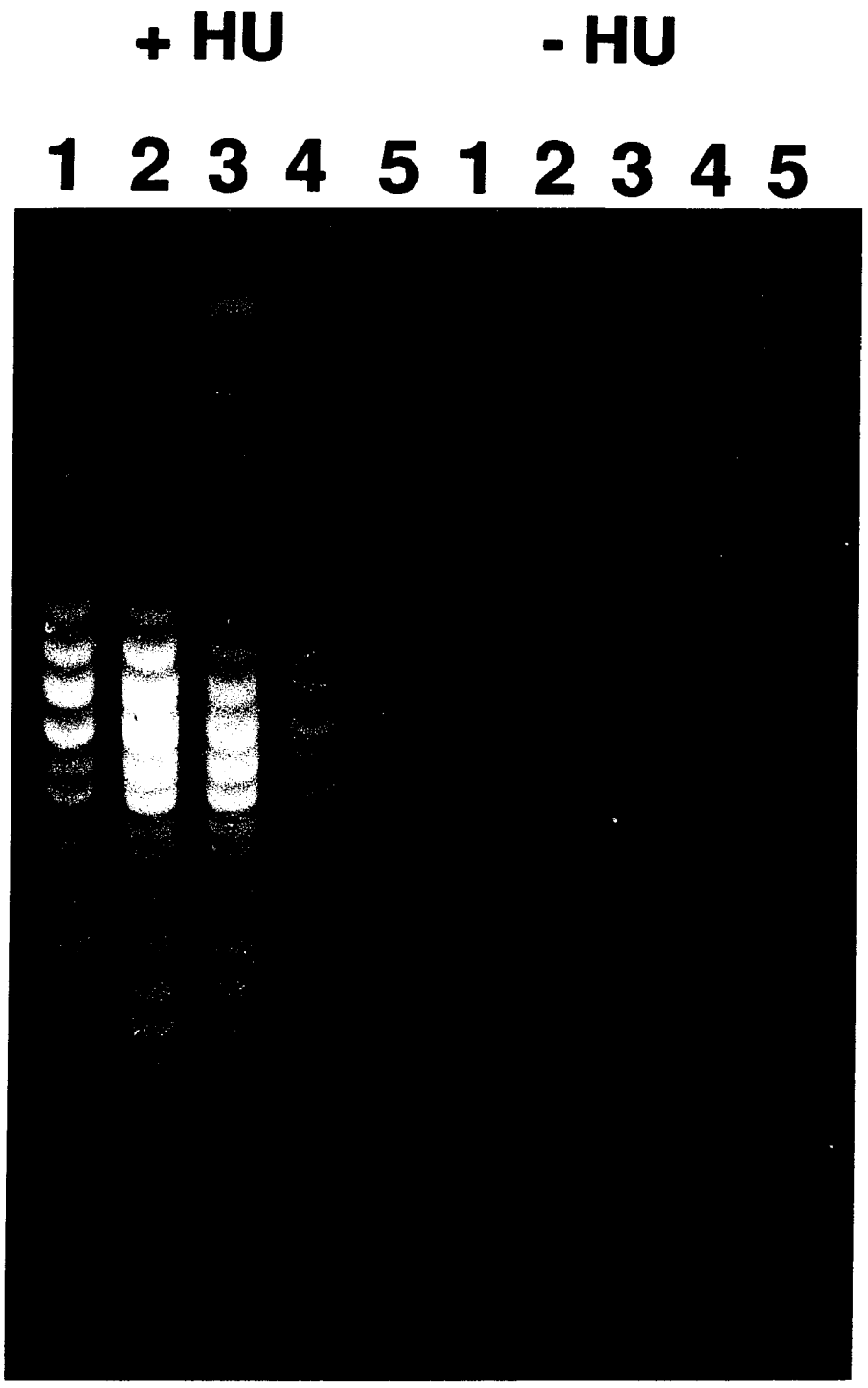
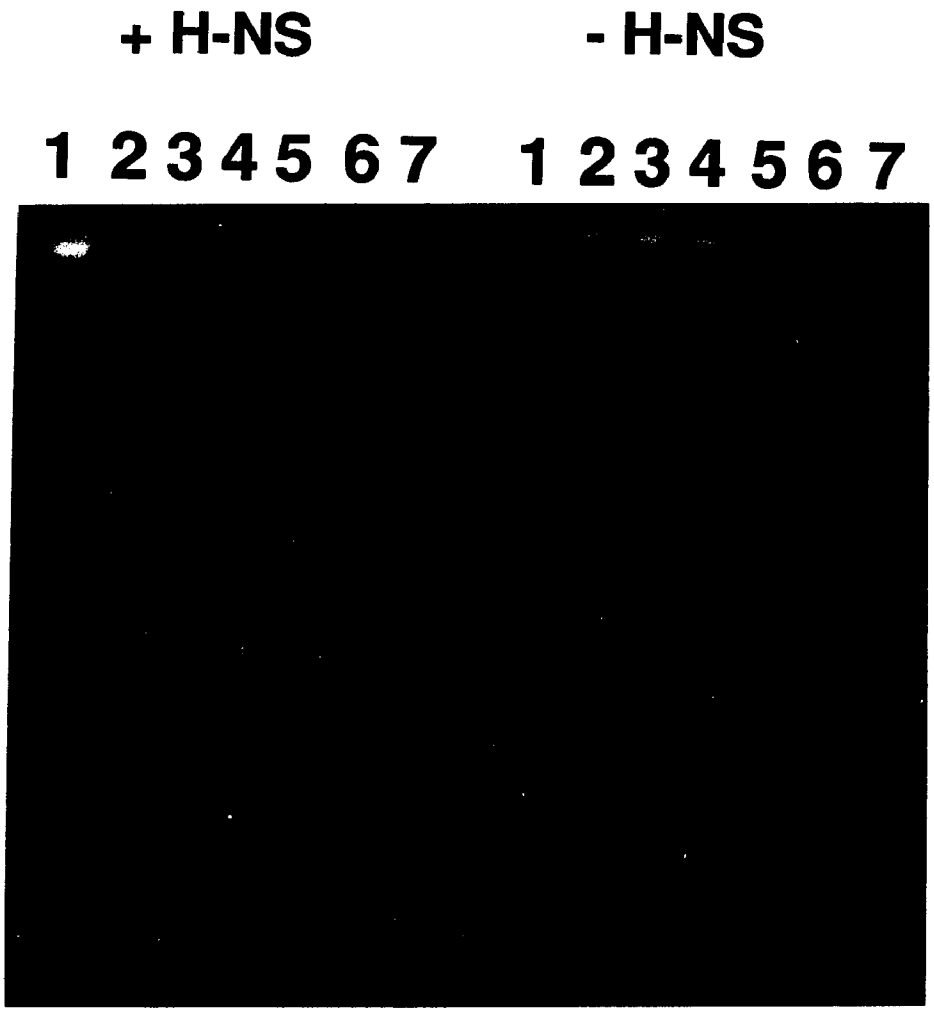


Figure 9. Topology of plasmid DNA in cells overproducing HNS. DNA was extracted from 1., NH1177 or 2., NH1205 at times 1. before heat induction; 2, 15 minutes post induction; 3, 30 minutes postinduction; 4. 60 minutes postinduction and 5, 3 hours post induction.

A



(Figure 8). When HNS was overexpressed, a dramatic relaxation in the topoisomer population of both pACYC184 and pPLc11 or pPLc2833, was observed (Figure 9). The reporter plasmid was relaxed by approximately 8 topoisomers, or about 25% of the supercoils found during rapid growth of the same cells. A supercoiling change of similar magnitude was observed in cells in which transcription had been poisoned with rifampicin (Wu, 1988). Thus, HU and HNS differ in their effect on the transcriptional and supercoiling state of the cell.

DISCUSSION

One of the problems that any organism must solve in order to survive is that of expressing certain classes of genes only at given times. The problem can be dealt with at the DNA level, one gene or operon at a time, using strategies of repression and activation. Coordinate regulation of groups of genes, using these strategies, allows the cell to react efficiently to changes in its environment. Eukaryotes, too, use coordinate regulation. But eukaryotes have access to another regulatory strategy at the DNA level, that of packaging genes into chromatin. The opening and closing of chromatin domains appears to be a higher-order form of organization which controls the access of regulatory factors to DNA (Freeman, 1992).

The nucleoid of *E. coli*, its folded chromosomal mass, is bound by a number of factors, including RNA and DNA polymerases, specific activators and repressors, and the

nucleoid associated proteins HU, IHF, FIS and HNS. The interaction of the supercoiled chromosome with factors that bind it leads to constraint of about half the chromosome's supercoils.

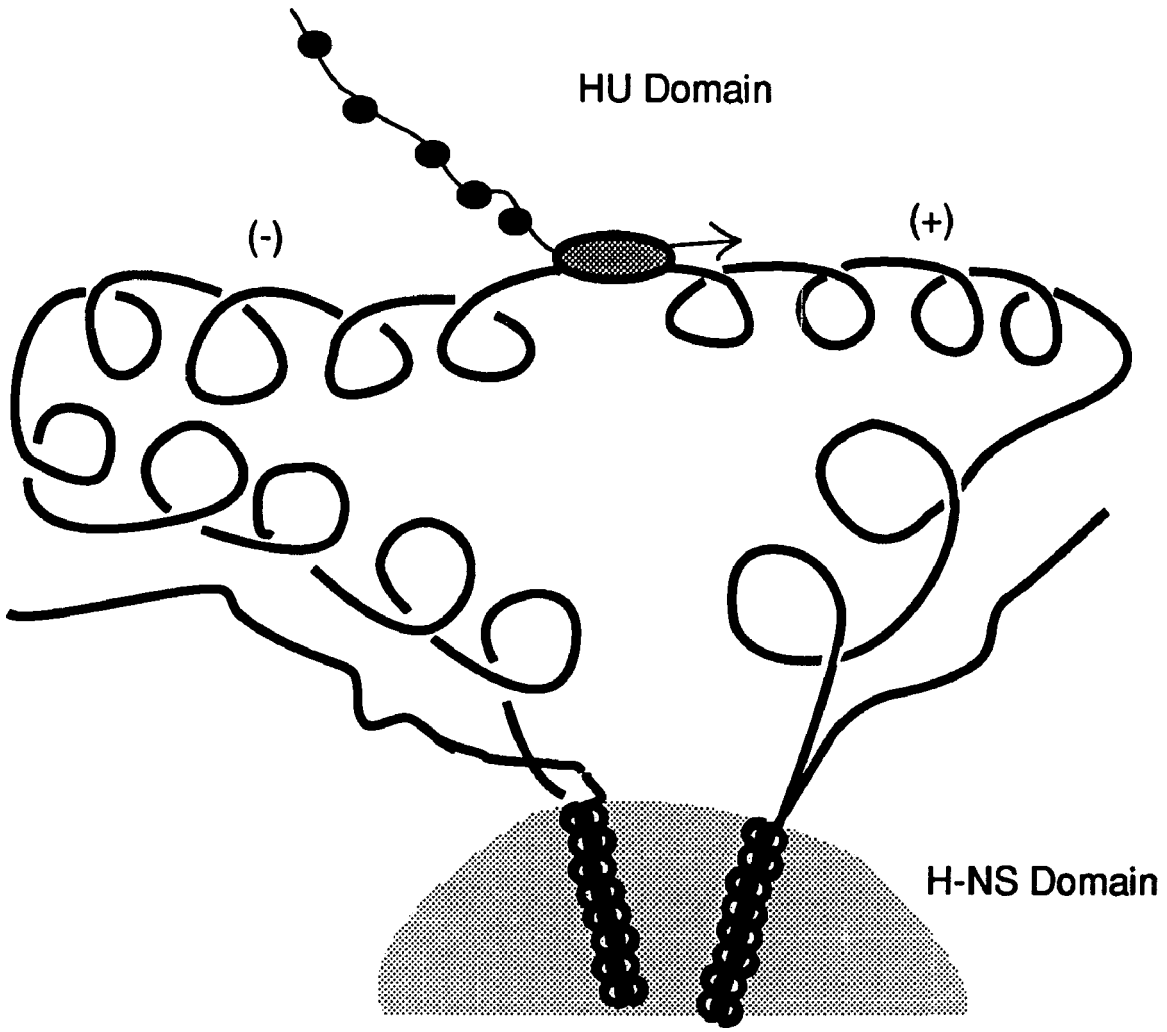
HNS was discovered in a search for the factors that influence the stability of bacteriophage Mu in the *E. coli* chromosome. Mu became more transpositionally active in an HNS mutant. Recently we proposed a model for the interaction of HNS with a transcriptional factor important in stabilizing Mu lysogens.

In vitro HNS and HU can constrain supercoils. Surprisingly, the two proteins interfere with eachother in this activity, so that each is able to inhibit supercoiling by the other (submitted).

Here we have shown that the effects of HU and HNS in the cell are quite different. While HU does not interfere with global transcription, HNS stops it. The effect of HNS is rapid and dramatic, reducing transcription to background levels within minutes of induction and inhibiting transcription for hours after the cells are returned to the non-inducing growth temperature. The cells are found to be static, not growing, rather than no longer capable of growth, since they display little loss of viability when plated after the induction of HNS.

Why has the cell maintained two supercoil constraining proteins? The answer may lie in a difference between the DNA structures that they create (Figure 10). DNA bound to

Figure 10. One model for the differential roles of two nucleoid proteins in organizing the bacterial chromosome.


HU Domain
(-)
(+)
H-NS Domain

HU is amenable to the passage of the transcriptional machinery, while DNA bound to HNS is not. We suggest that the two proteins define transcriptionally active and inactive domains of the chromosome. At low concentrations, HNS can help specific repressors silence transcription. Higher levels of HNS may allow the effect to propagate, causing larger regions of the chromosome to become transcriptionally occluded. HNS is most abundant during stationary phase, when transcription is especially quiet (Seigele, 1992), and when our model would predict a great deal of a silencing factor would be needed.

The antagonism between HU and HNS *in vitro* provides a way for domains to switch between an inactive HNS dominated condition and an HU dominated active state: HNS and HU can disrupt each other's DNA complexes. This may allow active and inactive domains to invade one another, allowing silenced regions of the chromosome to become more easily activated when needed or preventing inopportune expression of genes that are not useful in the cell's growth state or environment. As a regulatory strategy, this scheme would have a parallel in higher organisms, where DNA can be organized into closed and open chromatin.

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SUMMARY

Bacteria, like eukaryotes, keep some genes silent. Bacteria doubling rapidly in rich media will not express the genes that enable them to utilize poor carbon sources (16). Cells growing in media of low osmolarity will not express the genes that allow them to synthesize and import osmoprotectants (3,12,14). Bacteriophages lysogenizing host cells will repress the genes for factors that allow them to enter a lytic pathway (8,28). The ability to repress and activate genes gives bacteria an important level of control over the balance of resources in the cell.

The nucleoid associated protein HNS plays a role in the regulation of several apparently unrelated genetic systems (17). The first section of this dissertation identifies the bacteriophage Mu early operator region, the site of the genetic switch that determines whether Mu lysogenizes or lyses cells, as one genetic region with which HNS interacts. In a search for genetic factors whose phenotype is stimulation of Mu transposition (smt), mutations in the gene *hns* increased the frequency of Mu transposition, showing that in the cell HNS helps to stabilize Mu lysogens. Biochemical studies indicated that low concentrations of HNS increased the binding of purified repressor to its operator site, suggesting that HNS stabilizes Mu lysogens by decreasing the concentration of repressor needed to keep

lytic transcription from occuring. In the presence of HNS, repressor protected its operator sites about twice as well as it can alone, while cells with a disrupted *hns* maintained lysogeny only half as well as cells with wild type HNS present. We concluded that HNS helps maintain repression of the Mu early operator region by helping repressor silence transcription from the promoters that control Mu's lysis/lysogeny switch. HNS may enhance transcriptional repression of other operons by stabilizing other repressors.

We also observed that the effect of HNS depended on the growth history of the cells. Cells grown in different media showed different amounts of transposition, and western blots of the proteins extracted from these cells showed HNS migrating at different positions in acrylamide gels, perhaps corresponding to different migration positions of HNS variants previously reported (25). This suggests that HNS is differentially modified depending on the culture conditions of the cells. This differential modification may be a clue to how the HNS stabilization of repressor binding could be released: HNS modification may occur as a response to environmental changes, just as phosphorylation of other proteins is used as a signal (4,19,27). It will be interesting to purify the different modified variants of HNS and determine their biochemical properties.

In the second segment of this research, we looked at the biochemical effects of HNS and were surprised to find that it can stabilize supercoils. This activity, which had

been miised in earlier studies, is an important one. Ever since the supercoiling of the bacterial chromosome was determined to be half constrained, identifying the factors involved in this constraint has been a puzzle. HU can account for a major fraction of the constrained supercoils, as can RNA polymerase. The identification of a second supercoiling protein allows assignment of a source of constraint for much of the remaining stable supercoiling.

Testing whether the supercoil stabilizing effects of HU and HNS were synergistic, we got another surprise: HU and HNS are antagonistic in their interaction with DNA. HU destabilizes supercoils introduced by HNS and vice versa. This antagonism suggests that there is a difference between the way that HU and HNS stabilize supercoils on DNA. We suggest that they could use two different types of binding: perhaps HU stabilizes supercoils by wrapping DNA around a protein core, as nucleosomes do, while HNS stabilizes interwound forms of DNA. Alternatively, one of the proteins might underwind the DNA when it binds, and the underwound DNA form might destabilize interaction with the other protein.

Footprinting experiments might help determine the mode of HNS binding and crosslinking experiments with both proteins might give some indication of whether they can come into contact on plasmids.

Even though the mechanism of the antagonism between HU and HNS has yet to be worked out, this antagonism suggests

that HU and HNS may stabilize supercoils differently in the cell, and that each protein can "invade" the other's supercoiled DNA so that DNA regions constrained by HNS might be released from that constraint by HU, and vice versa.

We embarked on the third part of these studies wondering what the effect of these supercoil-constraining proteins in the cell is. If their roles in the cell are similar, overexpressing them might give similar results. Using overexpressing plasmids to produce a ~40-fold excess of either protein in the cell, we looked at the effects of excess HU and HNS on growth, transcription and DNA topology. The effects of the two proteins were dramatically different: while overexpression of HU had little effect on growth, overexpression of HNS stopped cell growth within ten minutes of induction. Cells overexpressing HNS remained alive but did not grow for a period of six to eight hours. After that period, the cells resumed growth at the original doubling rate. Non-growing cells plated during the six to eight hour lag period grew into colonies that looked like the parent colonies and broth cultures grown from these colonies showed the same induction profile again if HNS was induced a second time.

Transcription in cells overexpressing HNS was also very different from in cells overexpressing HU. Cells in which HU was overproduced showed little difference in global transcription, as measured by uridine incorporation, from cells with normal HU levels. Overexpression of HNS,

however, stopped transcription within minutes of induction, and transcription was at the assay's background level during the long period in which the cells did not grow. Just before cell growth resumed, the cells began transcribing again.

Examining proteins isolated from the cells showed that HU and HNS increased to approximately the same fold overproduction. HU overproducing cells grown continuously in the presence of the inducer IPTG did not show any change in transcription, though the level of HU in the cell remained elevated. In the HNS overproducing cells, the signal for induction, high temperature, was removed after fifteen minutes of induction. Protein profiles showed that the level of HNS remained elevated during the period when growth and transcription stopped, and that the levels of HNS had begun dropping back toward normal when transcription, then growth, resumed.

We suggest that the difference between the consequences of overexpression of the proteins reflects a difference in how they interact with DNA. Immuno-electron microscopy has placed HU at the periphery of the cell (7) where transcription occurs (22). Similar experiments have not yet been done to locate HNS. Still, it has been observed that cells entering stationary phase compact their DNA into the central region of the cell, apparently drawing it away from the periphery (7). HNS has been shown to silence transcription with *E. coli* RNA polymerase from plasmids *in*

vitro, (V.McGovern, unpublished observations) and to prevent open complex formation by RNA polymerase prebound to promoters (26). HNS may deform DNA on binding so that RNA polymerase may not pass (21). No similar effects have been shown for HU.

Interestingly, *E. coli* naturally express HNS at a high level during stationary phase, when they silence much of their transcription. It is tempting to speculate that the long growth lag produced by HNS overexpression is an artificial stationary phase. During true stationary phase a few specific genes are transcribed (2,13) and experiments looking for transcription of those genes during HNS overexpression may be valuable.

A model for the role of HNS in the chromosome has been proposed in which the cell uses its chromosome as a "pleiotropic physiological barometer" (5) that can sense environmental changes and act as a global regulatory signal, alerting genes to bring about physiological changes. This model suggests that HNS plays an active role in determining the superhelicity of the chromosome, acting to establish different set points of chromosomal supercoiling at which different groups of genes will be expressed (10).

While it is clear that some promoters are able to sense DNA topology (11,23,24,18), whether the cell uses this potential source of information in a global control system is an open question.

The work in this thesis suggests a different model for the role of HNS in cells. We suggest that the pleiotropic phenotypes of HNS mutants are not a consequence of changes in a global regulatory system but rather reflect a loss of repression of a diverse group of normally silent genes. Our results lead us to believe that the role of HNS in cells is not to set levels of supercoiling but to prevent inappropriate transcription of silenced genes, and that the changes in supercoiling levels observed by workers in some genetic systems are a consequence of changes in transcription, not changes in global superhelicity.

The Liu and Wang model (15) and later experiments (29,31) demonstrate that transcription and supercoiling are inexorably connected, so it is difficult to design experiments that look at one without the other. Still, the cell appears to have balanced gyrase and topoisomerase against each other to maintain a single supercoiling set point (20,18,30), and the average superhelicity of plasmids rapidly returns to a fairly constant level after an environmental shift (1), suggesting that the cell tries to maintain a single supercoiling set point. DNA isolated from hns^- cells sometimes has a topology different from that of wild type cells, but these changes in topology have been variable, and changes have occurred that both increase and decrease plasmid supercoiling (6,9,10), so no prediction about the direction of change in supercoiling mediated by HNS is possible.

A model in which HNS alters transcription by silencing genes provides a simpler explanation for the pleiotropic phenotype of *hns* mutants. If HNS is not a repressor but a protein that helps keep transcription from occuring in silenced genes, the changes in transcription and in supercoiling will depend on the conditions under which the mutant is being observed: HNS mutants in aerated L broth culture may be prone to derepression of a different set of genes than those observed on minimal plates.

The effects of HNS on transcription, the biochemical antagonism between HU and HNS, and the different effects of the two proteins in the cell lead us to propose a role for HNS in the organization of the bacterial chromosome. We suggest that HNS organizes the regions of the chromosome that are transcriptionally silenced into inactive domains, complexing with these genes in a conformation that is inaccessible to the transcription machinery. This inaccessible form may be built upon HNS that is interacting with specific repressor-DNA complexes during rapid cell growth, but we propose that when the cellular concentration of HNS increases, during stationary phase or during overexpression, the inactive domain may also be propagated over larger regions of the chromosome.

HU, which does not interfere with the passage of the transcription machinery, may organize DNA in an active domain (or a set of active domains) keeping the fraction of the chromosome that is being transcribed organized and

compacted as the genes are expressed. It is possible that electron micrographs have already revealed the organizational scheme drawn by this model: the transcriptionally active HU-associated region might correspond to the HU-associated peripheral DNA seen in electron micrographs, while the transcriptionally inactive HNS domain DNA might be related to the compact central region of DNA observed in the same pictures.

The organizational strategy that we propose might be a bacterial parallel of the organization of eukaryotic chromatin: in eukaryotes, genes transcriptionally silenced in closed regions can only be expressed when the array of proteins around them changes, turning silent heterochromatin into active euchromatin.

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