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Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation

Petra Dersch,[1,2] Kerstin Schmidt[2] and Erhard Bremer[1*,2]

[1]Max-Planck-Institut für Terrestrische Mikrobiologie, Karl-von-Frisch Strasse, D-3550 Marburg, Germany.
[2]Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Germany.

Summary

Mutations in the structural gene (*hns*) for the *Escherichia coli* nucleoid-associated DNA-binding protein H-NS cause highly pleiotropic effects on gene expression, site-specific recombination, transposition of phage Mu, the stability of the genetic material and the topological state of the DNA. We have investigated the regulation of *hns* expression and found that *hns* transcription is subjected to stationary phase induction and negative autoregulation. A set of *hns–lacZ* protein and operon fusions was constructed *in vitro* and integrated in single copy into the *attB* site of the bacterial genome. Quantification of β-galactosidase activity along the bacterial growth curve showed that *hns* expression increases approximately 10-fold in stationary phase compared with exponentially growing cells. Immunological detection of the H-NS protein in growing and stationary phase cells supported the genetic data and showed that H-NS synthesis varies with growth phase. In addition, primer extension experiments demonstrated that the amount of *hns* mRNA is elevated in stationary phase cultures and that *hns* transcription is directed by a unique promoter functioning in both log and stationary phase. Disruption of the *hns* gene by an insertion mutation led to the derepression (approximately fourfold) of the expression of an *hns–lacZ* operon fusion integrated at the *attB* site, showing that *hns* transcription is subjected to negative regulation by its own gene product. Autoregulation of *hns* expression is particularly pronounced in log phase. Both stationary phase control and autoregulation of *hns* transcription are associated with a 130 bp fragment that contains the *hns* promoter. In order to study the interaction of H-NS with its own regulatory region, we developed an efficient overproduction procedure and a simple purification scheme for H-NS. DNA gel retardation assays showed that the H-NS protein can preferentially interact with a restriction fragment carrying the *hns* promoter. This restriction fragment showed features of curved DNA as judged by two-dimensional polyacrylamide gel electrophoresis performed at 4°C and 60°C.

Received 26 October, 1992; revised and accepted 15 February, 1993. *For correspondence (address no. 1). Tel. (6421) 286681; Fax (6421) 285833.

Introduction

The DNA of the *Escherichia coli* chromosome is more than 1000 times longer than the bacterial cell and must therefore be efficiently packaged and condensed into a chromatin-like structure, i.e. the bacterial nucleoid. The nucleoid must be a dynamic structure in order to allow timely DNA replication and selective gene expression in response to rapidly changing nutritional and environmental conditions. A small number of so-called histone-like DNA-binding proteins are associated with the bacterial DNA, and it is generally assumed that these proteins play an important role in the organization of the chromosomal DNA into a highly ordered structure (for overviews, see Drlica and Rouvière-Yaniv, 1987; Pettijohn, 1988; Schmid, 1990). One of the most abundant nucleoid-associated DNA-binding proteins is H-NS (H1a) whose amino acid sequence has been determined by Falconi *et al.* (1988). The H-NS protein is a non-basic polypeptide consisting of 137 amino acid residues, many of which are negatively or positively charged (pI = 5.6). It is heat stable and exists predominantly as a homodimer in solution because of strong hydrophobic interactions (Falconi *et al.*, 1988). H-NS has been shown by immunoelectron microscopy to exist primarily in the bacterial nucleoid, and its overproduction leads to a strong compaction of the chromosomal DNA *in vivo* (Spurio *et al.*, 1992). Such a compaction of DNA was also observed *in vitro* using purified H-NS protein (Rimsky and Spassky, 1986), suggesting that an important physiological function of H-NS is connected with the organization of the bacterial nucleoid. H-NS can bind to both linear and supercoiled DNA and has a very high affinity for double-stranded DNA, but it can also interact with single-stranded DNA and RNA (Rimsky and Spassky, 1986). Although H-NS binds DNA relatively non-specifically (Spassky *et al.*, 1984), it displays

a preference for curved DNA segments (Bracco *et al.*, 1989; Yamada *et al.*, 1990; Owen-Hughes *et al.*, 1992).

The structural gene (*hns*) for the H-NS protein has been genetically (Higgins *et al.*, 1988) and physically mapped to the 27.5 min region of the *E. coli* chromosome (May *et al.*, 1990; Göransson *et al.*, 1990; Hulton *et al.*, 1990). Mutations in this gene have been repeatedly recovered during searches for regulatory mutations affecting a large number of unrelated chromosomal and plasmid-encoded genes and have been designated as *bglY*, *pilG*, *virR*, *drdX* and *osmZ* (for an overview see Higgins *et al.*, 1990). Lesions in *hns* affect gene expression (Higgins *et al.*, 1988; Graeme-Cook *et al.*, 1989; May *et al.*, 1990; Göransson *et al.*, 1990; Dorman *et al.*, 1990; Lucht and Bremer, 1991; Gutierrez and Devedjian, 1991; Hromockyi *et al.*, 1992), site-specific inversion events (Spears *et al.*, 1986; Higgins *et al.*, 1988), transposition of bacteriophage Mu (Falconi *et al.*, 1991), the formation of chromosomal deletions (Lejeune and Danchin, 1989) and the motility of bacterial cells (Yamada *et al.*, 1991; Hinton *et al.*, 1992). In addition, *E. coli* and *Salmonella typhimurium* strains carrying certain *hns* alleles display alterations in the DNA supercoiling of reporter plasmids (Higgins *et al.*, 1988; Dorman *et al.*, 1990; Hinton *et al.*, 1992; Owen-Hughes *et al.*, 1992). Both increases and decreases in the synthesis of a sizeable number of specific *E. coli* proteins are observed in *hns* mutants; this altered pattern in protein composition is a reflection of the highly pleiotropic nature of *hns* mutations (Bertin *et al.*, 1990; Yamada *et al.*, 1991). The primary sequence of H-NS lacks typical DNA-binding motifs and it is obvious from the multitude of effects conferred by *hns* mutations that H-NS cannot be classified as a sequence-specific regulatory protein. The connecting theme underlying the diverse phenotypes of *hns* strains might lie in the participation of H-NS in structuring the dynamic bacterial nucleoid. Alterations in the fluidity of the genome, the topological state of the chromosomal DNA, the preferential binding of H-NS to curved DNA sequences or its interactions with sequence-specific regulatory proteins might explain its profound influence on a variety of important cellular functions (Higgins *et al.*, 1988; 1990; Lejeune and Danchin, 1989; May *et al.*, 1990; Göransson *et al.*, 1990; Hulton *et al.*, 1990; Yamada *et al.*, 1991; Falconi *et al.*, 1991; Owen-Hughes *et al.*, 1992).

Whatever the mechanism, it is clear that H-NS plays a very important physiological role for the bacterial cell. Therefore, one can expect that the amount of H-NS at any given time during the bacterial life cycle is critical. Overproduction of the H-NS protein from cloned copies of its structural gene has been shown to be highly detrimental to cell growth (May *et al.*, 1990; Hulton *et al.*, 1990; Spurio *et al.*, 1992). CspA, the major cold-shock protein of *E. coli*, exerts a positive control on expression of the *hns* structural gene, which is enhanced three- to fourfold after a shift from 37°C to 10°C (La Teana *et al.*, 1991). In addition, biochemical experiments have shown that the amount of H-NS (H1a) is correlated with the growth phase of the bacterial culture and that its concentration increases from approximately 2000 monomers during early exponential growth to approximately 18000 monomers per cell in stationary phase cells (Spassky *et al.*, 1984; Rimsky and Spassky, 1986). The precise mechanism by which this variation in H-NS content in the cell is achieved is unknown.

In order to gain a deeper understanding of the genetic control of *hns* expression, we have constructed a set of chromosomal *hns–lacZ* protein and operon fusions and have used these hybrid genes to study transcriptional control of *hns* in response to the growth phase and the synthesis of H-NS itself. In this paper, we report that *hns* transcription is enhanced in stationary phase and subjected to negative autoregulation by its own gene product. Both the stationary phase control and autoregulation of *hns* expression are associated with a 130 bp DNA fragment carrying the *hns* promoter and its transcription initiation site. We demonstrate that the purified H-NS protein binds preferentially to a DNA segment containing the *hns* regulatory region, and we provide evidence to show that this DNA segment displays features of curved DNA.

Results

Construction of hns–lacZ *protein and operon fusions*

A previous biochemical study has indicated that the amount of the H-NS protein increases in stationary phase (Spassky *et al.*, 1984). To facilitate the analysis of *hns* expression we constructed a set of *hns–lacZ* protein and operon fusions carried by low-copy-number plasmids. A 409 bp *Hin*cII–*Rsa*I restriction fragment containing the *hns* promoter (La Teana *et al.*, 1989; May *et al.*, 1990), the transcriptional initiation site and 12 codons of the *hns* coding region (Fig. 1A) was fused in frame to the *'lacZ* gene present on the pSC101-derived low-copy-number fusion vector pGP20 (Table 1). This *Hin*cII–*Rsa*I restriction fragment does not carry the promoter of the neighbouring *tdk* gene (Black and Hruby, 1991; Fig. 1B). Starting from the resulting *hns–lacZ* protein fusion plasmid pKS4, a derivative (pKS5) was constructed that contained only 22 bp of chromosomal DNA upstream of the *hns* −35 sequence (Fig. 1A). The same fragments were also cloned in front of a promoterless *lacZ* gene, yielding the low-copy-number *hns–lacZ* operon fusion plasmids pKS6 and pKS7 (Fig. 1B).

As a first step in the analysis of *hns* gene regulation, we used the strains carrying the various *hns–lacZ* fusion

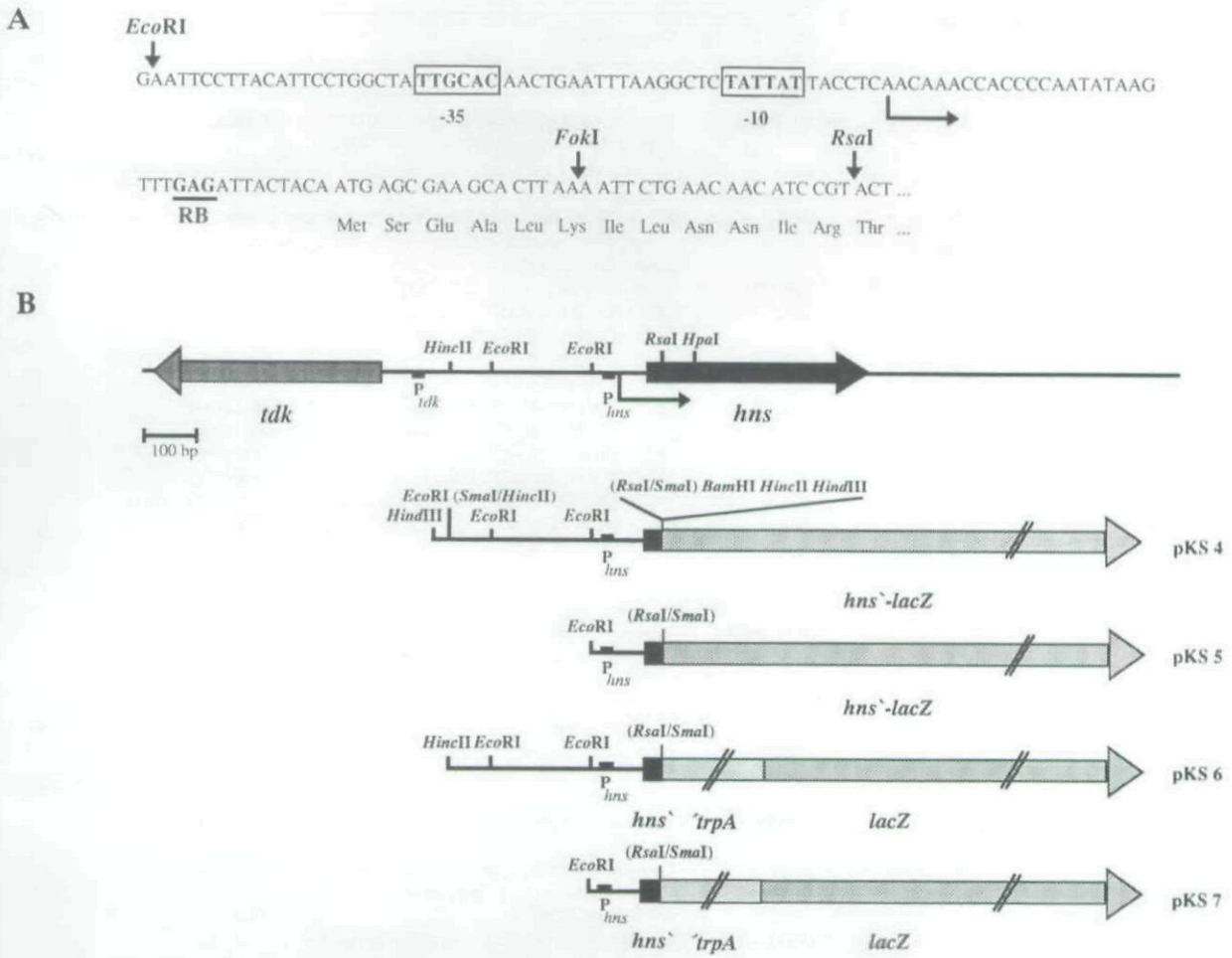

Fig. 1. A. DNA sequence of the *hns* promoter region.
B. Physical structure of the *hns–lacZ* protein and operon fusion plasmids. The genetic organization of the *tdk* (thymidine kinase; Black and Hruby, 1991) and *hns* regions of the *E. coli* chromosome (May *et al.*, 1990) is schematically shown. The positions of the promoters (P_{tdk}, P_{hns}) for the two genes and the transcription initiation site for the *hns* mRNA are indicated. Plasmids pKS4 and pKS5 carry *hns–lacZ* protein fusions, whereas plasmids pKS6 and pKS7 carry *hns–lacZ* operon fusions. The *lacZ* and *'trpA* material present in the hybrid genes is not drawn to scale. Genes marked with a prime are incomplete. Only the restriction sites relevant for this study are indicated and those destroyed during the cloning of *hns* restriction fragments are shown in brackets.

plasmids to monitor the synthesis of the H-NS–β-galactosidase hybrid protein and β-galactosidase in response to the growth phase of the cell cultures. Overnight cultures of the fusion strains were diluted 1000-fold into fresh Luria–Bertani (LB) medium, and cell growth was followed by measuring the optical density (OD_{578}) of these cultures. Log phase (OD_{578} = 0.6) and stationary phase (OD_{578} = 3.5) cultures of each *hns–lacZ* fusion strain were grown, adjusted to the same optical density and their total cellular proteins were then electrophoretically separated on a 7% SDS-polyacrylamide gel (Fig. 2). For both the protein (Fig. 2A) and operon fusions (Fig. 2B), we found that the products of these hybrid genes increased in the cultures grown to stationary phase. Thus, our results, in agreement with the data of Spassky *et al.* (1984) for H-NS (H1a) synthesis, demonstrate stationary phase induction of *hns* gene expression.

Growth-phase regulation of hns–lacZ *gene expression in protein and operon fusion strains*

To gain further insight into the stationary phase regulation of *hns* expression, we followed the expression of the *hns–lacZ* fusions along the bacterial growth curve. In order to avoid complications that might arise when cloned *hns–lacZ* fusions are used for such experiments, we transferred single copies of the *hns–lacZ* protein and operon fusions present on plasmids pKS4, pKS5 and pKS7 (Fig. 1B) into the *attB* site of the *E. coli* chromosome (see the *Experimental procedures*). The resulting strains

Table 1. Bacterial strains and plasmids.

Strain/Plasmid	Description	Source/Construction
Strain		
MC4100	F⁻, *araD139*, Δ(*argF–lac*)*U169*, *deoC1*, *flb5301*, *relA1*, *rpsL150*, *ptsF25*, *rbsR*	Casadaban (1976)
BL21	F⁻, *gal*, *met*, r⁻m⁻, *hsdS*, λ_{lys} p*lacUV5*-T7-gene 1, p*lacI*^{q}	Studier and Moffat (1986)
GP4	F⁻, *metB*, *ilv*, *rpsL*, Δ(*argF–lac*)*U169*, *deoR8*, *cytR9*, Φ(*tsx–lacZ*)1, (λp*lac*Mu55)	Gerlach *et al.* (1991)
RH90	MC4100 *rpos-359*::Tn*10*	Lange and Hengge-Aronis (1991)
BW10375	Δ(*gal–attB–bio*)*76*, *zbh-283*::Tn*10*	B. Wanner
GM230[a]	MC4100 Φ(*proU–lacZ*)hyb2, (λp*lac*Mu15), (Kan^{R}), *hns–205*::Tn*10*	Higgins *et al.* (1988)
BE1	W3110 *lrp-201*::Tn*10*	Ernsting *et al.* (1992)
JML87	MC4100 Φ(*proU–lacZ*)hyb7, (Kan^{R}), *polA1*	J. M. Lucht
KNS4[b]	MC4100 Φ(*hns–lacZ*)hyb2, (Kan^{R})	This study
KNS5[b]	MC4100 Φ(*hns–lacZ*)hyb2, (Kan^{R})	This study
PD32	MC4100 *hns-206*::Ap^{R}	This study
PD73[b]	MC4100 Φ(*hns–lacZ*)1, (Kan^{R})	This study
PD93	KNS4 *rpoS-359*::Tn*10*	This study
PD103	KNS4 *lrp-201*::Tn*10*	This study
PD110	PD73 *hns-206*::Ap^{R}	This study
Plasmid		
pTAC3422	Plasmid carrying the λ *int* gene (Cm^{R})	Atlung *et al.* (1991)
pTAC3591	Plasmid carrying an excisable *attP*-Kan^{R} cassette (Ap^{R})	Atlung *et al.* (1991)
pKT254 Ω-Ap	Plasmid carrying an ampicillin-resistance cassette (Ap^{R})	Fellay *et al.* (1987)
pHSG298	High-copy-number vector (Kan^{R})	Takeshita *et al.* (1987)
pGM7	Low-copy-number plasmid carrying *hns*⁺ (Cm^{R})	May *et al.* (1990)
pGP20	Low-copy-number *lacZ* protein fusion vector (Tet^{R})	P. Gerlach
pPD1	Low-copy-number over-expression vector carrying the ϕ 10 T7 promoter (Cm^{R})	P. Dersch
pPD3	pPD1, *hns*⁺ (Cm^{R})	This study
pPD9	Low-copy-number *lacZ* operon fusion vector (Ap^{R})	P. Dersch
pPD10	pGM7 carrying an ampicillin-resistance cassette cloned into the unique *Hpa*I site of the *hns* gene	This study
pPD17	pHSG298, *hns* carrying an ampicillin-resistance cassette cloned into the unique *Hpa*I site of (*hns-206*::Ap^{R})	This study
pKS4	pGP20; Φ(*hns–lacZ*)hyb2; see Fig. 1 (Tet^{R})	This study
pKS5	pGP20; Φ(*hns–lacZ*)hyb2; see Fig. 1 (Tet^{R})	This study
pKS6	pPD9; Φ(*hns–lacZ*)1, (Tet^{R})	This study
pKS7	pPD9; Φ(*hns–lacZ*)1, (Tet^{R})	This study

a. We have used the designation *hns* for mutations originally described as *osmZ* (Higgins *et al.*, 1988) but have retained the allele numbers originally assigned to the *osmZ* mutations to avoid confusion. The *hns-205*::Tn*10* allele is therefore identical to the previous described *osmZ-205*::Tn*10* mutation.
b. The *hns–lacZ* fusions carried by these strains are integrated as a single copy at the *attB* site in the chromosome.

were grown overnight in LB medium to stationary phase and diluted 1000-fold into fresh LB medium. Growth and β-galactosidase activity of the cultures were then continuously monitored (Fig. 3). The initial β-galactosidase activity measured in the freshly diluted cultures was high. Subsequently, the enzyme activity decreased rapidly when the cultures began to grow logarithmically and strongly increased again as soon as they entered stationary phase (Fig. 3). Thus, *hns–lacZ* expression is low in exponentially growing cells and is stimulated approximately 10-fold when cell growth slows down and the cultures enter stationary phase. This is a specific effect since the β-galactosidase activity of a *tsx–lacZ* operon fusion strain did not show a variation of enzyme activity in response to growth phase (Fig. 3A). Since both the *hns–lacZ* protein and operon fusion strains show the same pattern of *hns* expression, the data presented in Fig. 2 and Fig. 3 strongly suggest that synthesis of the H-NS protein

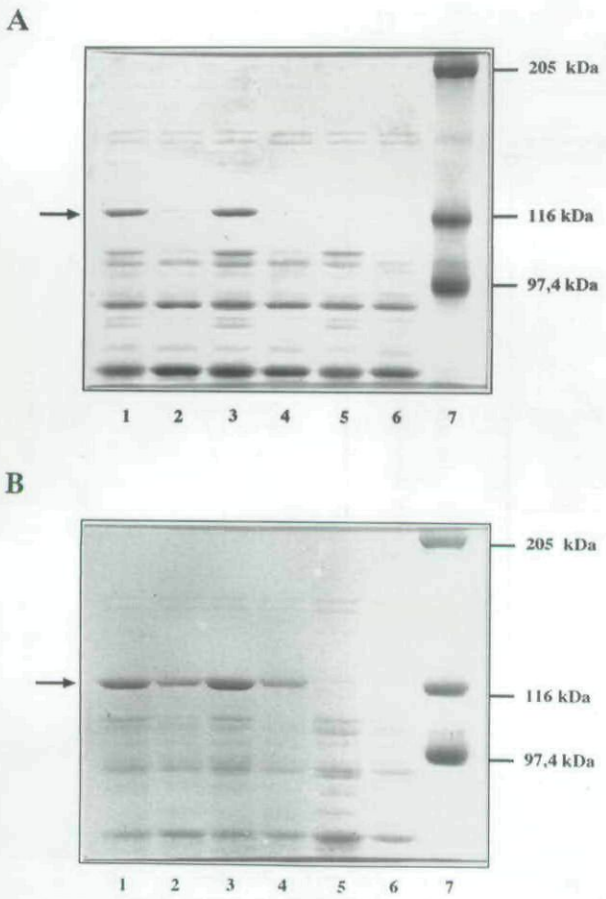

Fig. 2. Stationary phase-induced expression of the plasmid-encoded *hns–lacZ* protein and operon fusion plasmids. Total cell extracts were prepared from log- and stationary phase LB cultures of strain MC4100 carrying the various protein (A) and operon (B) fusion plasmids, and the proteins were electrophoretically separated on a 7% SDS–polyacrylamide gel. The gels were stained with Coomassie brilliant blue. The arrows indicate the position of the H-NS-β-galactosidase hybrid protein synthesized in the *hns–lacZ* protein fusion strains (A) and the β-galactosidase produced in the *hns–lacZ* operon fusion strains (B). In (A) and (B), lanes, 1, 3 and 5 show proteins synthesized in stationary phase cultures, whereas the polypeptides produced in log-phase cultures are shown in lanes 2, 4 and 6. A molecular mass standard (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa) is shown in lane 7.
A. MC4100(pKS4), lanes 1 and 2; MC4100(pKS5), lanes 3 and 4; MC4100(pGP20, *lacZ* protein fusion vector), lanes 5 and 6.
B. MC4100(pKS6), lanes 1 and 2; MC4100(pKS7), lanes 3 and 4; MC4100(pD9, *lacZ* operon fusion vector), lanes 5 and 6.

is regulated in response to growth phase at the level of *hns* transcription.

Synthesis of the H-NS protein in response to growth phase and mapping of the transcriptional initiation site

To further confirm the induction of H-NS synthesis in stationary phase, we visualized the amount of the *E. coli* H-NS in log- and stationary phase cultures using a polyclonal serum raised against the purified H-NS protein. Consistent with the results obtained with the *hns–lacZ* fusions, the amount of H-NS was found to be significantly greater in cells grown to stationary phase than in log-phase cells (Fig. 4A, lanes 3 and 4). The antiserum used for the Western blot experiment is highly specific for the H-NS protein. No H-NS protein can be detected in cell extracts of stationary phase cells of strain PD32 (*hns-206*::Ap^R) (Fig. 4A, lane 5), which carries a chromosomal insertion of an ampicillin-resistance cassette cloned into the unique *Hpa*I site (codon 37) of the *hns* gene (Fig. 1B). Western blotting of total cell proteins prepared from log-phase and stationary phase cultures of *S. typhimurium* LT2 (Fig. 4A, lanes 1 and 2) indicates that the amount of H-NS varies in response to growth phase in this organism as well.

The transcription initiation site for the *hns* gene in log-phase cells was previously mapped by primer extension, and a unique start site for the *hns* mRNA was found (La Teana *et al.*, 1989). To test whether the same promoter directs transcription of the *hns* mRNA in both log-phase and stationary phase cells, we mapped the *hns* transcription initiation site in cells carrying the low-copy-number *hns–lacZ* protein fusion plasmid pKS4 (Fig. 1B). Total mRNA was prepared from cells grown to log and stationary phase, a 17 bp primer complementary to the *lacZ* portion of the *hns–lacZ* transcript was hybridized to the mRNA and extended with reverse transcriptase in the presence of [α-^{35}S]-dATP, and the reaction products were electrophoresed on a 4% DNA sequencing gel. Using the same primer, DNA sequence reactions of double-stranded pKS4 DNA were prepared and run next to the primer extension products to allow the unambiguous assignment of the transcription initiation site(s) (Fig. 4B). In both the log- and stationary phase cells of strain MC4100 (pKS4), the same transcript was found, but the amount of the mRNA was greater in stationary phase cells than in log-phase cells (Fig. 4B). The transcription initiation site inferred from the primer extension product of the *hns–lacZ* protein fusion is identical to that described by La Teana *et al.* (1989) for the *hns* gene from *E. coli* grown to log phase. We did not detect additional transcripts initiating upstream of the *hns* −35 region (data not shown). Thus, our data show that a unique promoter is directing *hns* mRNA synthesis in growing and stationary cells and that the activity of this promoter is subjected to growth phase control.

The RpoS and Lrp regulatory protein are not involved in hns *expression*

A number of stationary-phase-induced genes of *E. coli* are regulated by RpoS (KatF), a putative alternative sigma factor (Lange and Hengge-Aronis, 1991; Siegele and Kolter, 1992). This information prompted us to test whether the expression of *hns* was controlled by RpoS as well. The *rpoS-359*::Tn*10* allele was introduced by P1*vir*-mediated transduction into strain KNS4 carrying a

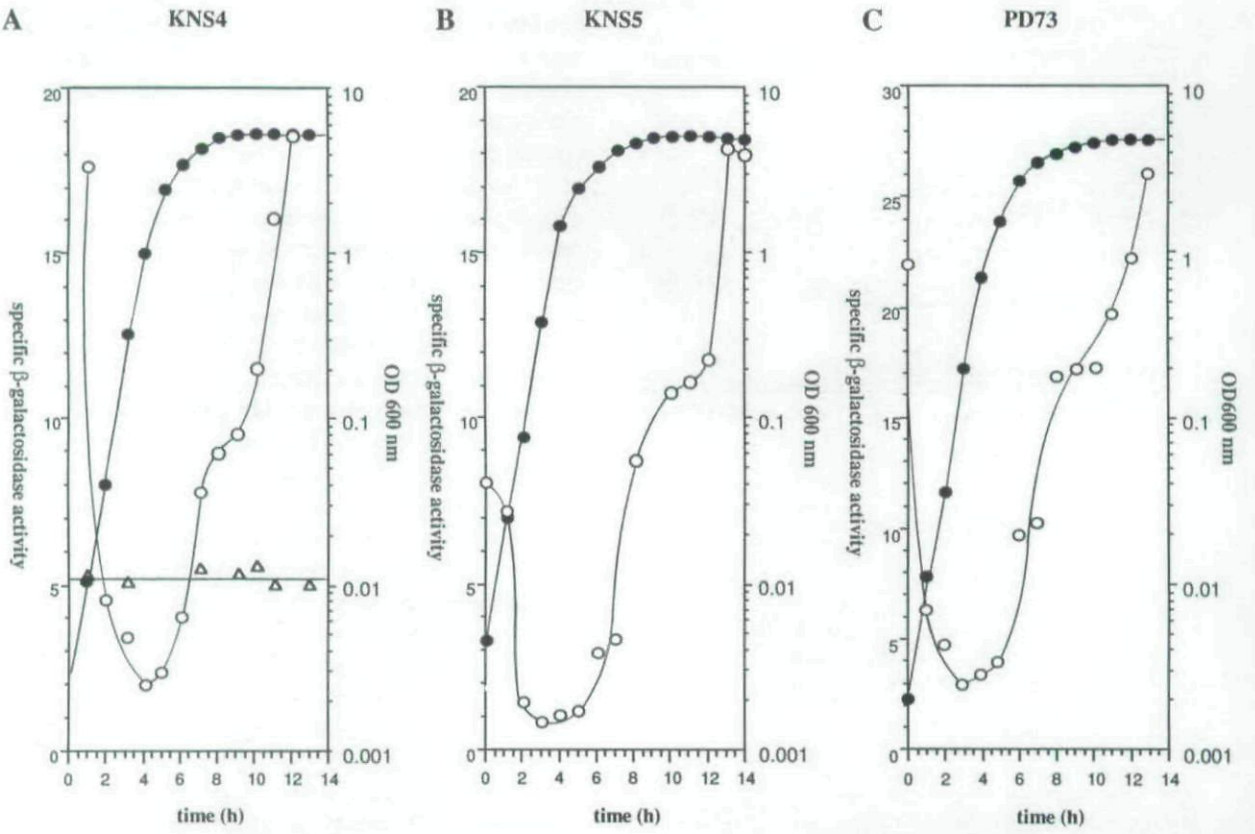

Fig. 3. Stationary phase-induced expression of chromosome-encoded *hns–lacZ* protein and operon fusions. The *hns–lacZ* protein fusions from plasmids pKS4 and pKS5 and the *hns–lacZ* operon fusion from plasmid pKS7 (Fig. 1B) were transferred as a single copy into the *attB* site of the *E. coli* chromosome. The fusion strains were grown overnight in LB medium at 37°C and diluted 1000-fold in fresh LB medium. The growth of the cultures (closed symbols) was spectrophotometrically monitored at OD_{600}, and the β-galactosidase activity (open symbols) of the cultures was determined.
A, B. *hns–lacZ* protein fusion strains KNS4 and KNS5, respectively.
C. *hns–lacZ* operon fusion strain PD73.
The β-galactosidase synthesized in the *tsx–lacZ* operon fusion strain GP4 (A, open triangles) was measured as a control.

chromosomal *hns–lacZ* protein fusion integrated at *attB*. Beta-galactosidase activity was then measured in an isogenic pair of *rpoS*$^+$ and *rpoS*$^-$ strains grown to log and stationary phase. No significant differences in the level of *hns* expression were found (Table 2). Hence, synthesis of H-NS is not dependent on the RpoS (KatF) regulatory protein.

Expression of *hns* is enhanced by cold shock (La Teana *et al.*, 1991). The involvement of the leucine-responsive regulatory protein (Lrp), a global regulator for the expression of at least 30 genes in *E. coli*, in the adaptation to a cold environment has recently been discussed (Ernsting *et al.*, 1992). We therefore tested the effect of the *lrp-201*::Tn*10* insertion on *hns–lacZ* expression and found no indication for the involvement of the Lrp protein in the regulation of the *hns* structural gene (Table 2).

Expression of the hns *gene is autoregulated*

Mutations in *hns* are highly pleiotropic and result in both increased and decreased synthesis of many *E. coli* proteins (Bertin *et al.*, 1990; Higgins *et al.*, 1990). We investigated a possible function of H-NS in the regulation of the expression of its own structural gene. For these experiments an ampicillin-resistance cassette was engineered into the cloned *hns* gene after codon 37 (*hns-206*::Ap^R; see the *Experimental procedures*) and the mutant *hns* allele was recombined into the bacterial chromosome. No H-NS-related polypeptide that cross-reacted with an anti-H-NS serum was detected in Western blots of whole-cell extracts of strains carrying the *hns-206*::Ap^R mutation (Fig. 4A, lane 3). We introduced the *hns-206*::Ap^R mutation into the *hns–lacZ* operon fusion strain PD73 (the *lac* fusion is integrated at *attB*) and monitored β-galactosidase activity in the resulting strain PD110 (*hns-206*::Ap^R). The presence of the *hns-206*::Ap^R allele resulted in an approximately fourfold derepression of *hns–lacZ* expression which was particularly evident during log phase of the cultures (Fig. 5). From these genetic data we conclude that the DNA-binding protein

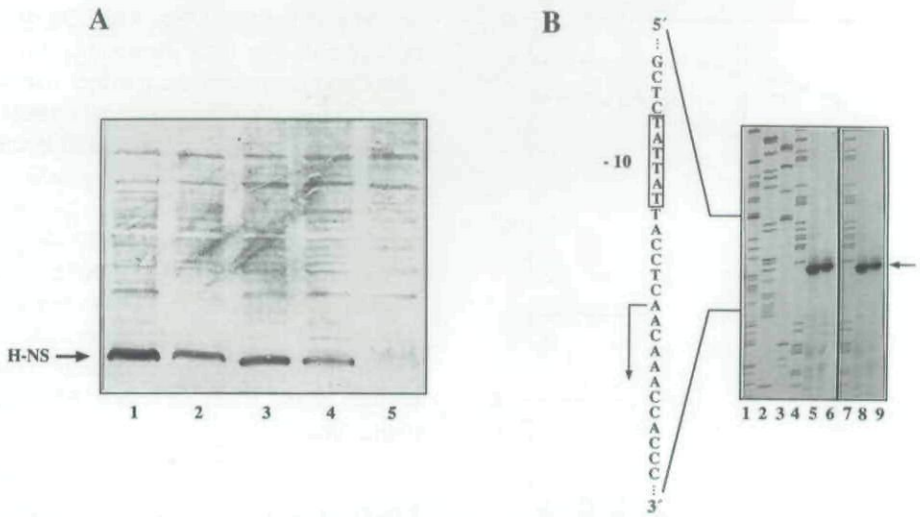

Fig. 4. Stationary phase-induced synthesis of H-NS and mapping of the *hns* transcription initiation site.
A. Whole-cell proteins were prepared from cultures of *S. typhimurium* LT2 (lanes 1 and 2), the *E. coli* K-12 strain MC4100 (*hns*$^+$) (lanes 3 and 4) and its *hns-206*::Ap^R derivative, strain PD32 (lane 5). The cultures were either grown to stationary phase (OD_{578} = 3.5; lanes 1, 3 and 5) or to log phase (OD_{578} = 0.6; lanes 2 and 4). The proteins were electrophoretically separated on a 15% SDS–polyacrylamide gel, transferred to a sheet of Immobilon blotting membrane and the bound proteins were then reacted with an antiserum raised against the *E. coli* H-NS protein. Prior to the preparation of the whole cell protein extracts, the optical density of the cultures was adjusted to an OD_{578} of 0.7. Coomassie brilliant blue-stained SDS–polyacrylamide gels were run to ensure that the same amount of total-cellular protein was present in each protein sample used for the Western blot.
B. Total RNA was prepared from log-phase (OD_{578} = 0.6) and stationary phase cells, (OD_{578} = 3.5) of strain MC4100 carrying the low-copy-number *hns–lacZ* protein fusion plasmid pKS4 (see Fig. 1). A synthetic primer was hybridized to the *lacZ* portion of the *hns–lacZ* transcript and extended with reverse transcriptase in the presence of a radiolabelled deoxyribonucleotide. The reaction products (lane 5, stationary phase cells; lane 6, log-phase cells) were electrophoretically separated on a DNA-sequencing gel along with a DNA sequencing reaction (lanes 1–4) of plasmid pKS4. The −10 region of the *hns* promoter and the *hns* transcription initiation site are indicated on the left. Lanes 7, 8 and 9 show a lower exposure of lanes 4, 5 and 6.

H-NS negatively modulates the expression of its own structural gene.

Overproduction and purification of the H-NS protein

In order to provide physical evidence for the interaction of the H-NS protein with the *hns* regulatory region, highly purified H-NS was needed, and therefore we developed a simple and efficient overproduction and purification scheme for this non-specific DNA-binding protein. Overproduction of the H-NS protein is highly detrimental to cell growth (May *et al.*, 1990; Hulton *et al.*, 1990) and results in a strong compaction of the bacterial nucleoid (Spurio *et al.*, 1992). To allow the efficient overproduction of H-NS, we cloned the intact *hns* gene into a newly constructed low-copy-number plasmid, pPD1, that contains the inducible phage T7ϕ10 promoter. Induction of *hns* expression under the control of the T7 promoter/RNA polymerase system led to the strong production of a 16kDa protein (Fig. 6). The molecular weight of this polypeptide is

Table 2. Effects of *rpoS* and *lrp* mutations on *hns–lacZ* expression.

	Genotype		OD_{578}		Beta-galactosidase Activity	
Strain	*rpoS*	*lrp*	logarithmic	stationary	logarithmic	stationary
KNS4	+	+	0.6	4.8	2.5	16.3
PD93	−	+	0.53	4.15	2.4	16.3
PD103	+	−	0.65	4.55	2.8	20.7

Cultures were grown in LB medium overnight at 37°C and the β-galactosidase activity was determined (stationary phase cultures). Aliquots were diluted 1000-fold into fresh LB medium and grown for approximately 3h (logarithmic cultures). Specific β-galactosidase activity is expressed as μmol substrate (ONPG) cleaved per min per mg protein. The symbol + indicates the presence of chromosome-encoded *rpoS*$^+$ or *lrp*$^+$ genes; the symbol − indicates the presence of the *rpoS-359*::Tn*10* or *lrp-201*::Tn*10* mutation. All strains carry the same Φ(*hns–lacZ*)hyb2 protein fusion integrated as a single copy into the *attB* site; the strains are *hns*$^+$.

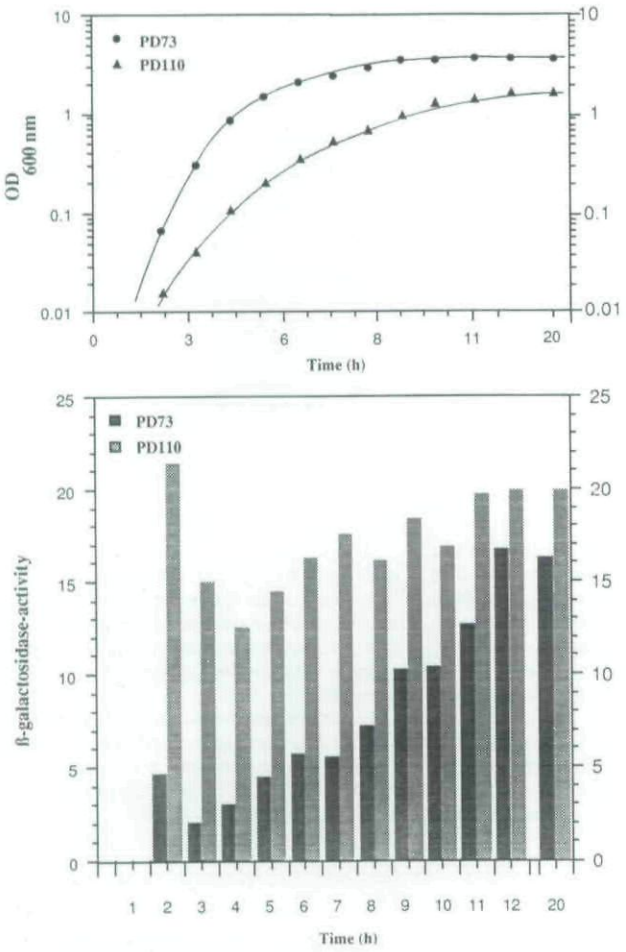

Fig. 5. Autoregulation of *hns* expression. The strains used for the experiment carry the same *hns–lacZ* operon fusion from plasmid pKS7 (see Fig. 1B) integrated as a single copy into the *E. coli* chromosome at *attB*. They differ in their *hns* alleles: PD73 (*hns*$^+$); PD110 (*hns-206*::Ap^R). The cells were grown in LB medium at 30°C overnight and diluted into fresh LB medium. Both their growth (OD_{600}) (A) and their β-galactosidase activity (B) were monitored.

consistent with the molecular weight of H-NS (M_r = 15402) calculated from its known amino acid sequence (Falconi *et al.*, 1988). Densitometer scanning of the SDS gel shown in Fig. 6 revealed that the 16kDa protein comprised approximately 14% of total cellular proteins (data not shown).

For the purification of the H-NS protein we took advantage of its high affinity to double-stranded DNA (Rimsky and Spassky, 1986). The overproduced H-NS protein was precipitated with ammonium sulphate from crude cell lysates, dialysed extensively, loaded on to a double-stranded DNA cellulose affinity column and eluted under high salt (500mM NaCl) conditions. This chromatographic step was sufficient to obtain large quantities (1mg H-NS/litre culture) of highly purified H-NS protein. The H-NS preparation was free of other contaminating proteins as judged by staining SDS gels with Coomassie brilliant blue (Fig. 6) or silver nitrate (data not shown). The purity and identify of the purified 16kDa protein with the authentic H-NS (Falconi *et al.*, 1988) was proven by automated Edman degradation of the first 12 *N*-terminal amino acids. The experimentally determined sequence of the *N*-terminus was identical to the amino acid sequence published by Falconi *et al.* (1988). As previously reported by Laine *et al.* (1984) for the H-NS protein expressed from a chromosomal gene, two amino acids (Met and Ser) were detected in the first Edman degradation cycle showing that the *N*-terminal Met residue is removed from only a fraction of the H-NS protein (I. Rasched, personal communication).

Interaction of the H-NS protein with its own regulatory region

To study the interaction of H-NS with the regulatory region of its own structural gene, we performed protein–DNA-binding assays. Restriction enzyme digestion of the *hns–lacZ* protein fusion plasmid pKS4 (Fig. 1B) with both *Hin*dIII and *Hin*cII cleaves the plasmid into six fragments (Fig. 7; the 620bp band is actually composed of two restriction fragments). The *hns* promoter is present on the 460bp fragment (Fig. 7, lane 1). Restriction digests of pKS4 were incubated with increasing concentrations

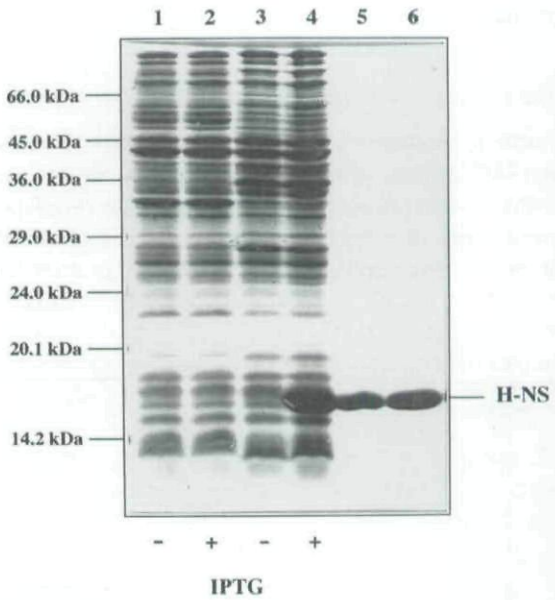

Fig. 6. Overproduction and purification of the H-NS protein. Cultures of strain BL21 (pPD1, vector plasmid; lanes 1 and 2) and strain BL21 (pPD3, *hns*$^+$; lanes 3 and 4) were grown in the absence (−) or presence (+) of IPTG. Total cell extracts were prepared, and the proteins were electrophoretically separated on a 15% SDS–polyacrylamide gel. Lanes 5 and 6: purified H-NS protein (4μg in lane 5 and 8μg in lane 6).

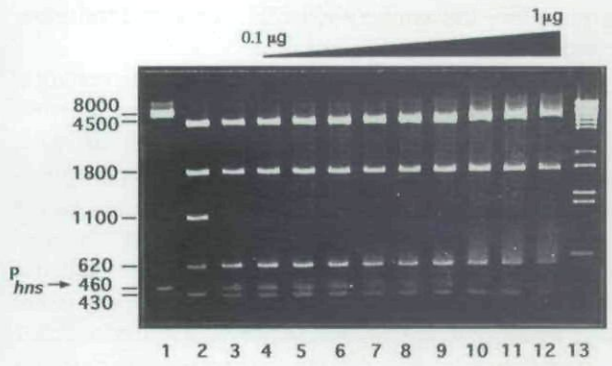

Fig. 7. Interaction of the purified H-NS protein with its own promoter region. DNA from the *hns–lacZ* protein fusion plasmid pKS4 (Fig. 1B) was digested with *Hin*dIII (lane 1), *Hin*cII (lane 2) and *Hin*dIII plus *Hin*cII (lanes 3–12). The restriction fragments originating from the *Hin*dIII plus *Hin*cII digestion were incubated with increasing concentrations of the purified H-NS protein (lane 4, 0.1 µg; lane 5, 0.14 µg; lane 6, 0.19 µg; lane 7, 0.25 µg; lane 8, 0.33 µg; lane 9, 0.45 µg; lane 10, 0.6 µg; lane 11, 0.8 µg; lane 12, 1.0 µg) and electrophoretically separated on a 1% agarose gel. A molecular weight standard (λ DNA cut with *Bst*EII) was run in lane 13. The position of the 460 bp *Hin*dIII fragment carrying the *hns* promoter (P_{hns}) is indicated by an arrow.

(0.1–1 µg) of purified H-NS, the formed protein–DNA complexes were then separated by electrophoresis on a 1% agarose gel and the DNA was visualized by staining with ethidium bromide. The use of defined restriction fragments for the H-NS–DNA-binding assay allowed us to assess readily whether the H-NS protein exhibits a preferential affinity for a DNA segment that contained the *hns* promoter region. The addition of small amounts of H-NS to the reaction mixture was sufficient to cause the preferential disappearance of the DNA restriction fragment containing the *hns* promoter (Fig. 7, lanes 6 and 7), and this phenomenon was dependent on the concentration of the H-NS protein in the DNA-binding assay (Fig. 7). Thus, in agreement with the negative effect of H-NS on the expression of its own structural gene (Fig. 5), we found that the H-NS protein at low concentrations can interact efficiently with DNA segments encoding the *hns* regulatory region. We did not observe a defined H-NS–DNA complex over the concentration range of H-NS used in the experiment documented in Fig. 7. However, an increase in the H-NS concentration above 1 µg in the gel retardation assay first led to a 'smearing' of all six restriction fragments of plasmid pKS4 and subsequently to the appearance of higher-molecular-weight DNA–protein complexes of each restriction fragment (data not shown).

The promoter region of hns *shows features of curved DNA*

Because a preferential interaction of the H-NS protein with curved DNA sequences has been reported (Bracco *et al.*, 1989; Yamada *et al.*, 1990), we investigated whether the *hns* promoter region displayed features of curved DNA. Non-curved DNA sequences migrate on two-dimensional polyacrylamide gels run at two different temperatures (4°C and 60°C) in a straight diagonal line, whereas curved fragments show anomalously slow electrophoretic mobility at 4°C, but not at 60°C. Consequently their position deviates from the diagonal line when the second dimension of the gel is run at 60°C (Yamada *et al.*, 1990). The *hns–lacZ* protein fusion plasmid pKS4 (Fig. 1B) was separately cleaved with *Hin*dIII and *Hin*cII (Fig. 7, lanes 1 and 2), the digests were mixed together, and run in the first dimension at 4°C and in the second dimension at 60°C on an 8% polyacrylamide gel (Fig. 8). The restriction digests of pKS4 produced seven restriction fragments, two of which, the 460 bp and the 1100 bp fragments, contain the *hns* promoter region. The 460 bp restriction fragment clearly exhibited anomalous electrophoretic mobility (Fig. 8). Such altered electrophoretic mobility was also detected for the 1100 bp fragment (Fig. 8); however, this effect was not very pronounced, probably because of the larger size of the restriction fragment and the relative position of the *hns* promoter region within the DNA fragment.

Discussion

Recent studies have made it increasingly clear that the nucleoid-associated DNA-binding protein H-NS plays an important role in the ability of *E. coli* and *S. typhimurium* to adapt and respond to changing nutritional and environmental growth conditions. Gene transcription, site-specific recombination, deletion formation and phage Mu

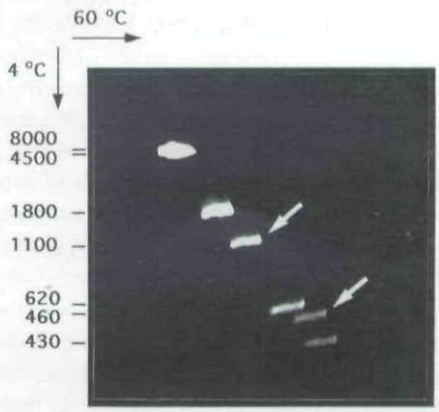

Fig. 8. The *hns* promoter fragment exhibits features of curved DNA. DNA from *hns–lacZ* protein fusion plasmid pKS4 (Fig. 1B) was separately cleaved with *Hin*dIII and *Hin*cII and the restriction digests were mixed. The resulting restriction fragments were then electrophoretically separated on an 8% polyacrylamide gel at 4°C in the first dimension and the subsequently electrophoresed at 60°C in the second dimension. Restriction fragments carrying the *hns* promoter are indicated by arrows.

transposition are affected by the H-NS protein, but the mechanism by which it influences these diverse cellular processes is not yet completely understood (Higgins *et al.*, 1990). In the present study we focused on the genetic control of the structural gene for H-NS and show that *hns* expression, in addition to its already known cold-shock regulation (La Teana *et al.*, 1991), is growth phase controlled and negatively autoregulated.

A previous study by Spassky *et al.* (1984) has provided biochemical evidence to show that the amount of H-NS (H1a) increases in stationary phase cells. Our data support this report and demonstrate that the increased content of H-NS in stationary phase cultures is largely caused by the genetic regulation of *hns* transcription. Three lines of evidence demonstrate that *hns* expression is induced in stationary phase. Strains carrying single-copy *hns–lacZ* fusions integrated into the *attB* site show approximately 10-fold increased β-galactosidase activity when the cultures enter stationary phase. Both *hns–lacZ* protein and operon fusion strains exhibit the same pattern of induction, strongly suggesting that growth-phase control of *hns* expression is achieved at the transcriptional level. Consistent with this conclusion is our finding that in primer extension experiments higher amounts of *hns–lacZ* transcripts can be detected in cultures grown to stationary phase. In addition, immunological methods allowed us to demonstrate directly the difference in H-NS content in log-phase and stationary phase cultures of a strain expressing a chromosomal *hns* wild-type gene. Taken together, three different experimental approaches have provided compelling evidence for stationary phase induction of *hns* expression in *E. coli*. We visualized the amount of H-NS in *S. typhimurium* LT2 using the *E. coli* H-NS antiserum and found that the production of the H-NS protein in this organism varies with growth phase as well. Recently, Hinton *et al.* (1992) used a multicopy *hns–lux* operon fusion and Western analysis of H-NS content in a wild-type *S. typhimurium* strain to investigate the growth-phase control of H-NS synthesis. These authors found no indication for stationary phase induction of *hns–lux* expression and a variation in the level of H-NS when cells enter stationary phase. The basis for the discrepancies between the gene fusion data is not clear. However, the use of different reporter genes (*lacZ* and *lux*) (Owen-Hughes *et al.*, 1992) and the single-copy and multicopy nature of the *hns* gene fusions might contribute to the different results reported by Hinton *et al.* (1992) and by us. Our data on the stationary phase induction of *hns* expression in *E. coli* do not agree with a report by Tanaka *et al.* (1991), who detected no variation in the amount of H-NS during cell growth by immunoblot analysis. We found that deviation from the optimal antigen–antibody concentration in the Western blot experiment readily yields non-quantitative data (our unpublished results) and this might explain the difference between our data and the study cited above.

We found, in addition to the stationary phase control of *hns* transcription, that the expression of the *hns* structural gene is subjected to negative control by its own gene product. Autoregulation of *hns* expression is particularly pronounced during log phase (fourfold) and it subsides largely during stationary phase (Fig. 5). This is a somewhat surprising finding since one would expect that the increased H-NS content in stationary phase cells would lead to stronger autoregulation of *hns* during this growth phase. Thus it appears that the ability of H-NS to modulate expression of its own structural gene is reduced in stationary phase cells. The mechanism by which this might be achieved is unknown but could possibly be correlated with the variation in the relative abundance of H-NS isoforms (H1a, H1b, H1c) during cell growth (Spassky *et al.*, 1984). Since the H-NS protein lacks typical DNA-binding motifs it is not yet clear how it recognizes and interacts with DNA. The diverse cellular functions influenced by H-NS strongly suggests that it is not a typical sequence-specific regulatory protein. We consider it unlikely that autoregulation of *hns* expression simply functions according to a classical repressor and operator type mechanism of gene regulation.

Both the stationary phase control and the autoregulation of *hns* transcription are associated with a small 130 bp *Eco*RI–*Rsa*I DNA fragment. In addition to the −10 and −35 regions, this DNA segment carries only 22 bp of upstream sequences, the transcription initiation site and a small part (12 codons) of the *hns* coding region (Fig. 1A). It is remarkable that an almost identical fragment (*Eco*RI–*Fok*I; see Fig. 1A) confers the CspA-mediated increase in *hns* transcription during severe cold shock (La Teana *et al.*, 1991). The *hns* −10 and −35 sequences (Fig. 1A) exhibit significant homologies to the consensus sequence of σ^{70}-dependent promoters and have a typical 17 bp spacer sequence (Harley and Reynolds, 1987). Our mapping of the *hns* mRNA initiation site used in stationary and log-phase cells provides conclusive evidence that a unique promoter directs *hns* transcription under both circumstances. Thus, the stationary phase control of *hns* expression does not appear to be mediated by an alternative sigma factor that specifically directs *hns* mRNA synthesis under stationary phase conditions. We have clearly ruled out the involvement of RpoS (KatF), a protein showing significant homologies to sigma factors and which is required for the stationary phase-induced expression of many *E. coli* genes (Lange and Hengge-Aronis, 1991; Siegele and Kolter, 1992), in the transcriptional regulation of *hns*. DNA binding studies with purified H-NS protein have shown that it interacts co-operatively with double-stranded DNA (Rimsky and Spassky, 1990) and that H-NS exhibits a higher affinity for curved DNA

segments than for non-curved sequences (Bracco *et al.*, 1989; Yamada *et al.*, 1990; Owen-Hughes *et al.*, 1992). The electrophoretic properties of a restriction fragment carrying the *hns* regulatory region point to the presence of a curved DNA segment in the vicinity of the *hns* promoter, and we found that this curved restriction fragment is preferentially recognized by H-NS *in vitro*. This finding, therefore, provides additional evidence for the negative autoregulation of *hns* transcription observed in *hns–lacZ* fusion strains *in vivo*.

Besides H-NS, a number of DNA-binding proteins (HU, IHF and Fis) are associated with the bacterial nucleoid and their structure and function have already been studied in considerable detail (Drlica and Rouvière-Yaniv, 1987; Pettijohn, 1988; Schmid, 1990). We draw attention to the fact that the negative feedback control of *hns* described here is shared by the structural genes for the HU, IHF and Fis proteins (Miller, 1981; Kohno *et al.*, 1990; Ninnemann *et al.*, 1992). Likewise, expression of the *fis* gene is subjected to growth-phase control but, in contrast to the stationary phase induction of *hns* transcription, Fis synthesis is enhanced during log phase and nutritional upshifts (Ninnemann *et al.*, 1992). Since the H-NS, HU, IHF and Fis proteins participate in a wide variety of cellular processes, careful control of their intracellular amount at a given time during the bacterial growth cycle is obviously of great importance for cell physiology.

The data presented in this report add another level of complexity to the known regulatory features of *hns* expression. Cold-shock regulation (La Teana *et al.*, 1991), stationary phase control and autoregulation (this study) are all associated with a very small DNA segment encompassing the *hns* promoter. These different regulatory circuits provide the cell with a mechanism for the fine tuning of the intracellular H-NS content, thus allowing timely and efficient adjustment of the dynamic nucleoid to the requirements imposed by changing nutritional and environmental conditions. Genetic analysis and DNA-footprinting studies with H-NS and the cold-shock regulatory protein CspA are now required to dissect the structurally and functionally important regulatory elements within the *hns* promoter region.

Experimental procedures

Growth conditions, chemicals, bacterial strains and plasmids

The *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. For some experiments cultures of *S. typhimurium* LT2 (laboratory collection) were used. Rich (LB), MMA and M9 minimal media supplemented with 0.4% glucose as a carbon source and 1% casamino acids were prepared as described (Miller, 1972; Silhavy *et al.*, 1984). Cells were grown, unless indicated otherwise, at 37°C with vigorous aeration. Lactose–MacConkey agar plates and BS indicator plates containing bromothymol blue and salicin were used to score the Lac- and Bgl-phenotypes of bacterial strains (Miller, 1972; Schnetz *et al.*, 1987). Agar plates containing 0.1 ml of a 10 mg ml^{-1} solution of X-gal in dimethylformamide were used to distinguish between $LacZ^+$ and $LacZ^-$ phenotypes of bacterial strains. Antibiotics were used at the following concentrations: kanamycin (Kan), 50 mg l^{-1}; ampicillin (Ap), 50 mg l^{-1}; tetracycline (Tet), 5 mg l^{-1}; chloramphenicol (Cm), 30 mg l^{-1} and rifampicin 100 mg l^{-1}.

Genetic procedures and construction of bacterial strains

Standard techniques were used for the growth of bacteria and generalized transduction with bacteriophage P1*vir* (Miller, 1972; Silhavy *et al.*, 1984). To obtain an *E. coli* strain lacking the H-NS protein, we constructed *in vitro* an insertion of an ampicillin-resistance cassette into the unique *Hpa*I site (Fig. 1B) within the *hns* gene (the restriction enzyme cuts the *hns* gene after codon 37; May *et al.*, 1990) and transferred this *hns* allele by *in vivo* homologous recombination into the bacterial chromosome. The low-copy-number hns^+ plasmid pGM7 (May *et al.*, 1990) was linearized with *Hpa*I, and a 2.7 kb *Sma*I restriction fragment from plasmid pKT254 Ω-Ap (Fellay *et al.*, 1987) carrying an ampicillin-resistance gene was ligated into the *Hpa*I site present in the *hns* gene (*hns-206*::Ap^R). From the resulting low-copy-number plasmid, pPD10, a 4.7 kb *Sal*I restriction fragment carrying the disrupted *hns* gene was isolated and cloned into the unique *Sal*I site of the high-copy-number plasmid pHSG298 (kanamycin resistance, Kan^R) (Takeshita *et al.*, 1987), resulting in plasmid pPD17. Plasmid pPD17 was then transformed into the *polA1* strain JML87 that carries the osmoregulated Φ(*proV–lacZ*)hyb7 protein fusion (Table 1) by selecting for colonies resistant to low concentrations of ampicillin (50 μg ml^{-1}). pBR322-derived plasmids cannot replicate in such a *polA* strain (Saarilahti and Palva, 1985) but can integrate into the chromosomal *hns* gene via homologous recombination yielding an hns^+/*hns-206*::Ap^R merodiploid. Because of the intact copy of the *hns* gene, the Φ(*proV–lacZ*)hyb7 fusion is expressed at very low levels, resulting in a Lac^- phenotype on lactose–MacConkey indicator plates. Homologous recombination between the duplicated *hns* sequences can yield derivatives of the original strain that have lost both the pHSG298 vector sequences (kanamycin sensitive, Kan^S) and the *hns* wild-type gene, leaving the mutant *hns-206*::Ap^R copy in the bacterial chromosome. Disruption of the integrity of *hns* will lead to derepression of the expression the Φ(*proV–lacZ*)hyb7 fusion, resulting in a Lac^+ phenotype on lactose–MacConkey plates (Higgins *et al.*, 1988; May *et al.*, 1990; Lucht and Bremer, 1991). A P1*vir* lysate was prepared on such an Lac^+ Ap^R Kan^R strain and used to transduce the hns^+ strain MC4100 (Table 1) to Ap^R. The resulting transductants showed a strong Bgl^+ phenotype, characteristic of many *hns* mutants (Higgins *et al.*, 1988; Lucht and Bremer, 1991). In addition, the *hns-206*::Ap^R insertion present in one of these transductants, strain PD32 (Table 1), was mapped by phage P1*vir* transduction of the *hns-205*::Tn*10* insertion from strain GM230 (Table 1) into strain PD32 by selecting for tetracycline-resistant (Tet^R) colonies. All 50 Tet^R transductants tested were found to be sensitive to ampicillin, attesting to the expected tight genetic linkage between the *hns-206*::Ap^R and *hns-205*::Tn*10* mutations. Western blot experiments with an H-NS–antiserum confirmed the absence of the H-NS protein in strain PD32 (Fig. 4A). To transfer the various

hns–lacZ fusion constructed *in vitro* (see below) as a single copy into the bacterial chromosome, we used the system described by Atlung *et al.* (1991). A restriction fragment carrying the phage λ *attP* site and a kanamycin-resistance (Kan^R) cassette but no origin of replication was isolated from preparative digests of plasmid pTAC3591 (Table 1) and ligated with restriction fragments carrying the various *hns–lacZ* protein and operon fusions. The ligation products were transformed into a derivative of strain MC4100 carrying the λ *int*$^+$ plasmid pTAC3422 (chloramphenicol resistance, Cm^R) (Table 1). Synthesis of the Int protein allows the site-specific integration of the ligated fragments into the *attB* site in the *E. coli* chromosome, and strains carrying such insertions can be selected as Kan^R colonies. P1 *vir* lysates were prepared on such strains and used to transduce strain MC4100 to Kan^R. The absence of plasmid pTAC3422 (Cm^R) in the transductants was tested by streaking colonies on LB plates containing chloramphenicol. The integration of the *hns–lacZ* fusion into the *attB* site was genetically mapped in representative $LacZ^+$ Kan^R Cm^S transductants by transducing these strains with a P1 *vir* lysate prepared on strain BW10375 carrying a Tn*10* insertion (*zbh-283*::Tn*10*) tightly linked to the Δ(*gal–attB–bio*)*76* deletion. The Tet^R transductants yielded in each case predominantly $LacZ^-$ Kan^S colonies, proving the integration of the *hns–lacZ* fusions into the *attB* site.

Methods used with nucleic acid construction of plasmids

Routine manipulations of nucleic acids were all as described previously (Sambrook *et al.*, 1989). DNA sequencing of the fusion junction in the *hns–lacZ* protein fusion present in plasmid pKS4 (Fig. 1) was performed with the dideoxy chain termination method (Sanger *et al.*, 1977) modified for double-stranded DNA templates with the Sequenase 2.0 kit using the conditions recommended by the supplier (United States Biochemical Corp.). A synthetic oligonucleotide primer (5′-GTTTTCCCAGTCACGAC-3′) that hybridized to the *lacZ* material of the *hns–lacZ* hybrid gene was used for the sequencing reaction. The same primer was used for the mapping of the transcription initiation site of the *hns–lacZ* protein fusion from plasmid pKS4. Total RNA was prepared by hot acid phenol extraction from cultures of strain MC4100(pKS4) grown in MMA with 0.2% glucose as the carbon source to log phase (OD_{578} = 0.6) and to stationary phase (OD_{578} = 3.5). The total amount of RNA was spectrophotometrically (OD_{260}) determined as described by Sambrook *et al.* (1989) and the purity of the RNA was checked by determining the OD_{260}/OD_{280} ratio. An OD_{260} of 1 corresponds to approximately 40 μg ml^{-1} of RNA (Sambrook *et al.*, 1989). For the primer extension reaction, the synthetic primer was hybridized with 50 μg of RNA and extended with avian-myeloblastosis virus (AMV) reverse transcriptase in the presence of radiolabelled [α-^{35}S]–dATP. The size of the reaction product was determined on a 4% DNA-sequencing gel under denaturing conditions.

For the construction of an *hns–lacZ* protein fusion, a 409 bp *Hin*cII–*Rsa*I restriction fragment from pGM7 (May *et al.*, 1990) was cloned into the unique *Sma*I site within the polylinker region of the low-copy-number fusion vector pGP20 (Tet^R) (Table 1). Those plasmids into which the *Hin*cII–*Rsa*I fragment had inserted in such a way that the *hns′* and *′lacZ* coding sequences were properly aligned were identified by their $LacZ^+$ phenotype after transformation into strain MC4100 (Table 1). DNA sequencing of the junction between the two genes confirmed the in-frame fusion of the *hns′* segment to the *lacZ* indicator gene. One of the resulting plasmids was designated pKS4 (Fig. 1B). Chromosomal material upstream of the *hns* promoter was deleted from pKS4 by cleaving the plasmid DNA with *Eco*RI and religation, resulting in plasmid pKS5 (Fig. 1B). For the construction of an *hns–lacZ* operon fusion a 700 bp *Bam*HI fragment was isolated from pKS4 and inserted into a low-copy-number operon fusion vector pPD9 (ampicillin resistance, Ap^R) (Table 1). This manipulation removed the Ap^R cassette from pPD9, restored the integrity of the *′tet* gene carried by pPD9 and aligned the desired orientation of the *hns* promoter with the promoterless *lacZ* indicator gene. One of the plasmids obtained was pKS6 (Fig. 1B) and it was used to construct a derivative, pKS7, from which a chromosomal *Eco*RI fragment had been removed (Fig. 1B). For the overexpression of the H-NS protein, we cloned the *hns*$^+$ gene into low-copy-number overexpression vector pPD1 (P. Dersch *et al.*, in preparation) carrying the T7φ10 promoter (Tabor and Richardson, 1985). A 2.7 kb *Sal*I restriction fragment carrying *hns* was excised from plasmid pGM7 (May *et al.*, 1990) and ligated into the unique *Sal*I site of pPD1; the resulting plasmid was designated pPD3.

Overproduction and purification of the H-NS protein and preparation of an H-NS antiserum

Plasmid pPD3 (T7φ10 promoter, *hns*$^+$) (Table 1) was transformed into strain BL21, which carries a chromosomal copy of the structural gene for the T7 RNA polymerase under the control of the *lacPO* regulatory sequences (Studier and Moffat, 1986). The H-NS protein was purified from 5 l of strain BL21(pPD3); for its overproduction, one-litre portions were employed to maximize the efficiency of H-NS synthesis. One litre of M9 minimal medium supplemented with 0.4% glucose and 1% casamino acids was inoculated with 10 ml of an overnight culture of strain BL21(pPD3) and grown at 37°C to an OD_{578} of 0.7. Expression of the T7 RNA polymerase structural gene was induced by adding IPTG to a final concentration of 2.5 mM, and the culture was then grown for an additional 30 min. The *E. coli* RNA polymerase was subsequently inhibited by adding rifampicin to a final concentration of 100 μg ml^{-1}. After growth of the culture for an additional hour, the cells were harvested by centrifugation, and the combined cell pellets from the five 1 l cultures were resuspended in 50 ml of TED buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT). The cells were disrupted by passing them three times through a French-Press cell at 103 500 kPa and the cell lysate was centrifuged in a Beckman L7-65 ultracentrifuge at 100 000 × *g* for 4 h to obtain a clear supernatant fraction. Solid ammonium sulphate was added to 40% saturation and the precipitated proteins were discarded. Additional ammonium sulphate was added to the cleared cell lysate to 60% saturation, and the protein precipitate was recovered by centrifugation, dissolved in 20 ml of buffer A (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 5% glycerol and 150 mM NaCl) and dialysed overnight against 4 l of buffer A. The sample was applied to a double-stranded DNA cellulose column (Pharmacia; 1 cm × 10 cm) previously equilibrated with buffer A, and the column was then washed with 30 ml of buffer A containing 250 mM NaCl. The H-NS protein was eluted from the double-stranded DNA cellulose column in a single step by applying 30 ml of buffer A containing 500 mM NaCl. Fractions (2 ml) of the eluate were collected and their protein content was analysed on 15% SDS–polyacrylamide gels stained with Coomassie brilliant blue. Those fractions that contained only H-NS were pooled, dialysed

against buffer A, and stored in 100 μl aliquots at −20°C. The protein concentration of the purified H-NS polypeptide was determined by the BioRad Protein Assay (BioRad) using bovine serum albumin as a standard.

To obtain an H-NS antiserum, a rabbit was injected with 1 ml of an H-NS solution (250 μg protein resuspended in buffer A) and 1 ml of complete Freund's adjuvant. After 14 d, the rabbit was given a booster injection of the same dose of H-NS protein resuspended in buffer A; 14 d later, the blood of the animal was collected and the production of H-NS antibodies was tested by Western blotting using $H\text{-}NS^+$ and $H\text{-}NS^-$ cell extracts.

Gel retardation assay

The binding of H-NS to defined restriction fragments was carried out in 20 μl reaction mixtures containing increasing concentrations of purified H-NS protein (0.1–1 μg) and 1 μg DNA of plasmid pKS4 (Fig. 1B) cut with *Hin*dIII and *Hin*cII. The reaction buffer contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 80 mM NaCl, 5 mM DTT and 5% glycerol. The reaction mixtures were incubated for 20 min at room temperature, and subsequently loaded on to a 1% agarose gel. The gel was run in 0.5 × TBE buffer (Sambrook *et al.*, 1989) and stained with ethidium bromide.

Gel electrophoresis, preparation of protein samples, immunological detection of H-NS, and β-galactosidase assays

For the immunological detection of the H-NS protein, total cellular extracts of *E. coli* strains were electrophoretically separated on 15% SDS–polyacrylamide gels (Laemmli, 1990) and transferred (Towbin *et al.*, 1979) to a sheet of Immobilon (Millipore). The bound proteins were then probed with the rabbit H-NS antiserum. The antigen–antibody complexes were visualized with a second goat anti-rabbit immunoglobulin G alkaline-phosphatase-conjugated antibody (Sigma) using 5-bromo-4-chloro-3-indolylphosphate and nitroblue-tetrazolium chloride (Boehringer) as substrates. Specific β-galactosidase activity, expressed as micromoles of substrate (2-nitrophenyl-β-D-galactoside; ONPG) cleaved per min per mg of protein, was assayed as described by May *et al.* (1986).

Notes added in proof

Recently Ueguchi *et al.* (1993 *Mol Gen Genet* **236:** 171–178) published a report on the regulation of the *E. coli hns* gene. In agreement with the data reported here, these authors show that *hns* expression is influenced by growth phase and subjected to autoregulation. They also state that they previously (Tanaka *et al.* (1991) *Agric Biol Chem* **55:** 3139–3141) failed to notice the accumulation of H-NS in stationary phase because of the use of large amounts of cellular proteins for the immunoblotting analysis of H-NS content, conditions which preclude accurate quantification of the reactive protein.

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