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Thermoregulation of *Escherichia coli pap* transcription: H-NS is a temperature-dependent DNA methylation blocking factor

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Summary

The expression of Pap pili that facilitate the attachment of *Escherichia coli* to uroepithelial cells is shut off outside the host at temperatures below 26°C. Ribonuclease protection analysis showed that this thermoregulatory response was rapid as evidenced by the absence of *papBA* transcripts, coding for Pap pilin, after only one generation of growth at 23°C. The histone-like nucleoid structuring protein H-NS and DNA sequences within *papB* were required for thermoregulation, but the PapB and PapI regulatory proteins were not. *In vivo* analysis of *pap* DNA methylation patterns indicated that H-NS or a factor regulated by H-NS bound within the *pap* regulatory region at 23°C but not at 37°C, as evidenced by H-NS-dependent inhibition of methylation of the *pap* GATC sites designated GATC-I and GATC-II. These GATC sites lie upstream of the *papBAp* promoter and have been shown previously to play a role in controlling Pap pili expression by regulating the binding of Lrp, a global regulator that is essential for activating *papBAp* transcription. Competitive electrophoretic mobility shift analysis showed that H-NS bound specifically to a *pap* DNA fragment containing the GATC-I and GATC-II sites. Moreover, H-NS blocked methylation of these *pap* GATC sites *in vitro*: H-NS blocked *pap* GATC methylation at 1.4 μM but was unable to do so at higher concentrations at which non-specific binding occurred. Thus, non-specific binding of H-NS to *pap* DNA was not sufficient to inhibit methylation of the *pap* GATC sites. These results suggest that the ability of H-NS to act as a methylation blocking factor is dependent upon the formation of a specific complex of H-NS with *pap* regulatory DNA. We hypothesize that a function of H-NS such as oligomerization was altered at 23°C, which enabled H-NS to repress *pap* gene expression through the formation of a specific nucleoprotein complex.

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Introduction

Pap pili, encoded by the pyelonephritis-associated pili (*pap*) operon, are expressed by uropathogenic *Escherichia coli* and play a critical role in the colonization of host tissues in upper urinary tract infections (Hagberg *et al*., 1983; Lindberg *et al*., 1984; Roberts *et al*., 1989). At 37°C, the expression of Pap pili is subject to a phase variation control mechanism. Within a population of bacteria, some of the cells express pili (phase ON), while others cells do not (phase OFF) (Low *et al*., 1987; Blyn *et al*., 1989; van der Woude *et al*., 1996). At temperatures below 26°C, Pap pili expression is shut off (Göransson and Uhlin, 1984; White-Ziegler *et al*., 1990). This type of thermoregulatory response has been shown to control virulence factor expression in many bacterial genera including *Escherichia* (Roosendaal *et al*., 1986; Jordi *et al*., 1992), *Shigella* (Maurelli *et al*., 1984), *Bordetella* (Coote, 1991), *Yersinia* (Cornelis *et al*., 1989) and *Listeria* (Leimeister *et al*., 1992). Presumably, bacteria sense temperature and other environmental signals to determine whether they are inside or outside of a host. Low temperature may thus serve as an environmental cue to the bacterium that it is outside of a host. The subsequent shutoff of pili expression would allow the bacterium to conserve its resources by preventing the synthesis of protein structures that are needed only within a host and may be deleterious in non-host environments.

Thermoregulation of Pap pili expression occurs at the transcriptional level (Göransson and Uhlin, 1984; Blyn *et al*., 1989; White-Ziegler and Low, 1992) Using a single-copy *papBAp–lacZ* operon fusion in which the β-galactosidase gene is placed under the control of the *papBAp* promoter, we have shown that the expression of *papBA* mRNA, encoding the regulatory protein PapB and the main pilus structural protein PapA, is subject to both

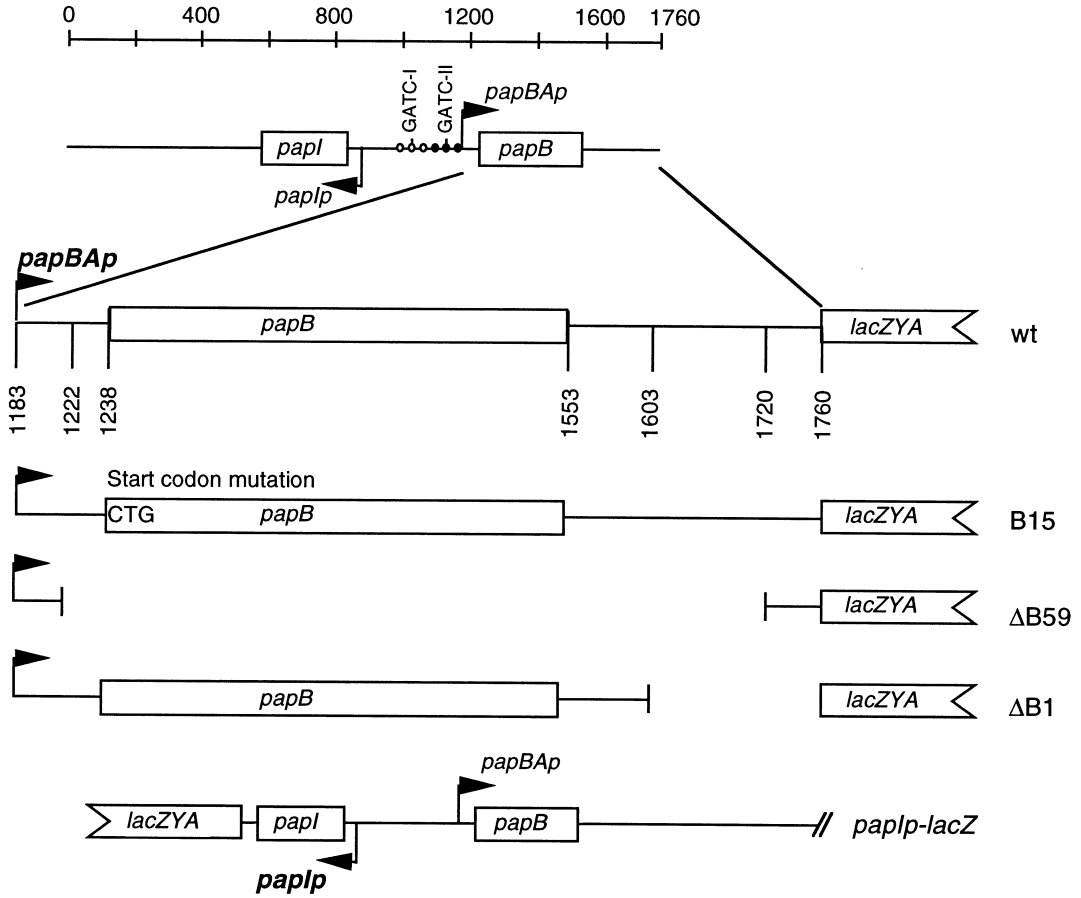

Fig. 1. The *pap* regulatory region and operon fusion constructs. The *papIBA* regulatory region is shown at the top with the basepair numbering system used in this study. The small open circles around GATC-I represent Lrp DNA binding sites 4, 5 and 6, whereas the small, closed circles around GATC-II represent Lrp binding sites 1, 2 and 3 (Nou *et al*., 1995). The wild-type *papBAp* fusion and *pap* mutant constructs are shown in the expanded scale below the *pap* regulatory region. The designations for each mutant construct are shown at the right (see Table 1). The *papIp* operon fusion is shown at the bottom.

phase variation and thermoregulatory control mechanisms (Blyn *et al*., 1989). At 37°C, both Lac^+ (phase ON) and Lac^- (phase OFF) colony phenotypes were observed as a result of phase variation. Lac^+ colonies arose from cells that were phase ON and expressed *papBA–lacZ* mRNA, while Lac^- colonies arose from cells that were phase OFF and did not express *papBA–lacZ* mRNA. In contrast, at 23°C, only Lac^- phase OFF colonies were observed, indicating that *papBA–lacZ* transcription was shut off at low temperature (Blyn *et al*., 1989; White-Ziegler and Low, 1992).

Although the mechanism by which thermoregulation of Pap pili expression occurs is not known, genetic analyses have indicated that the global regulator H-NS plays an essential role (Göransson *et al*., 1990). H-NS is a 15.5 kDa global regulatory protein that represses the transcription of many environmentally regulated operons in *E. coli* and other enteric bacteria (Ussery *et al*., 1994). The work described above with H-NS (Göransson *et al*., 1989a; 1990) was carried out using multicopy plasmid-encoded *pap* sequences, which abolish the Pap pili phase variation that is observed at 37°C in single-copy wild-type uropathogenic *E. coli* (Low *et al*., 1987; Blyn *et al*., 1989). Under multicopy *pap* conditions, the normal regulatory circuit involving leucine-responsive regulatory protein (Lrp), the *pap*-encoded co-regulatory protein PapI and deoxyadenosine methyltransferase (Dam) is disrupted (van der Woude *et al*., 1992). Pap phase variation is controlled by the differential methylation of two DNA GATC sites within the *pap* regulatory region (Braaten *et al*., 1994). The two GATC sites are targets for methylation by Dam (Blyn *et al*., 1990). The GATC-II site is located 53 bp upstream of the *papBA* transcription start site, whereas the GATC-I site is 102 bp upstream of GATC-II (Fig. 1). In phase OFF cells, the GATC-I site is methylated, and the GATC-II site is non-methylated, whereas the converse pattern is found in phase ON cells (Blyn *et al*., 1990). Lrp is a global regulator in *E. coli* that activates some genes and represses others (Calvo and Matthews, 1994), and Lrp is required to block Dam methylation of both *pap* GATC sites (Braaten

et al., 1991; Nou *et al*., 1993). In Pap phase OFF cells, Lrp binds co-operatively to three sites (designated as sites 1, 2 and 3) in the *pap* regulatory DNA and blocks methylation of the GATC-II site, which is located within Lrp site 2 (Fig. 1; Nou *et al*., 1995). Binding of Lrp at sites 1, 2 and 3 inhibits *pap* transcription, presumably because of steric hindrance of RNA polymerase to the *papBAp* promoter that lies between Lrp sites 2 and 3. The GATC-I site is methylated by Dam, as Lrp is not bound in this region in phase OFF cells (Braaten *et al*., 1994; Nou *et al*., 1995). Methylation of GATC-I reduces the affinity of Lrp for *pap* DNA sites 4 and 5 (Nou *et al*., 1993). As binding of Lrp to *pap* sites 4 and 5 is necessary for *papBA* transcription activation (Nou *et al*., 1995), we hypothesized that methylation of GATC-I locks cells in the phase OFF state until DNA replication occurs (Braaten *et al*., 1994).

In phase ON cells, the GATC-I site is non-methylated, whereas the GATC-II site is methylated (Blyn *et al*., 1990). The PapI protein, along with Lrp, is necessary for blocking methylation of the GATC-I site (Braaten *et al*., 1991). PapI binds to Lrp and increases its affinity for *pap* Lrp binding sites 4 and 5 at the GATC-I region (Nou *et al*., 1995). This shift in Lrp binding protects the GATC-I site from methylation, freeing the GATC-II site to be methylated by Dam. By this mechanism, Lrp can act as both a repressor and an activator of *papBA* transcription (Nou *et al*., 1995; van der Woude *et al*., 1995). Lrp activates transcription when bound at the GATC-I site, whereas Lrp bound at the GATC-II site blocks transcription.

Additional regulatory proteins, including PapB and cAMP receptor protein (CRP), also play important roles in Pap phase variation. PapB activates *papI* transcription (Båga *et al*., 1985) by binding to the *pap* regulatory region upstream of the *papIp* promoter (Forsman *et al*., 1989). Pap phase variation occurs in *papB*$^-$ cells under conditions in which PapI is constitutively expressed (see *Results*), showing that PapB plays an indirect role in activating *pap* transcription. CRP binds 215.5 bp upstream of the *papBAp* promoter and is required for activation of both *papBAp* and *papIp* transcription (Göransson *et al*., 1989b) Recent data indicate that CRP is not required for translocation of Lrp to sites 4, 5 and 6 but plays a role at a later step (unpublished data).

In this study, we investigated the roles of PapI, PapB, H-NS and *pap* regulatory DNA sequences in Pap thermoregulation. These experiments were carried out under conditions in which a *papBAp–lacZ* fusion was present in single copy on the chromosome and Pap phase variation occurred (Low *et al*., 1987). Results showed that H-NS as well as DNA sequences within or near *papB* were required for thermoregulation of transcription at *papBAp*, but PapI and PapB were not. *In vivo* methylation analysis indicated that H-NS or an H-NS-regulated factor blocked methylation of the *pap* GATC sites at 23°C but not at 37°C. *In vitro* analyses at 23°C showed that H-NS bound specifically to the *pap* regulatory region and blocked methylation of the *pap* GATC-I and GATC-II sites. We hypothesize that an important step in the thermoregulation of Pap pili expression is the temperature-dependent formation of a specific H-NS–*pap* nucleoprotein complex.

Results

The relationship between PapI levels and thermoregulation

Previous results obtained using multicopy plasmid-encoded *pap* indicated that *papI* transcription as well as Pap pili expression was reduced at low temperature. Additionally, overproduction of PapI caused a loss of thermoregulation as evidenced by expression of Pap pili at 26°C (Göransson *et al*., 1989a). From these observations, it was hypothesized that thermoregulation of *papBA* transcription was an indirect effect caused by limiting amounts of PapI at low temperature. To determine if these observations were also true under conditions in which phase variation of Pap pili expression occurred, we carried out experiments using a single-copy, chromosomal *papIp–lacZ* transcriptional fusion (Fig. 1), which placed *lacZ* under the control of the *papIp* promoter (*E. coli* DL2838, Table 1). This *E. coli* isolate showed phase variation at 37°C with switch rates similar to those measured previously for the *papBAp* promoter (Table 2). At 23°C, all *E. coli* DL2838 colonies had a Lac$^-$ colony phenotype. We measured a 14-fold reduction in β-galactosidase activity at 23°C (15 Miller units) compared with 37°C (213 Miller units), demonstrating that *papI* transcription was thermoregulated.

To assess the relationship between PapI levels and thermoregulation of *papBA* transcription quantitatively, we analysed *E. coli* containing a single-copy *papBAp–lacZ* fusion (DL1504, Table 1). This *E. coli* isolate switched reversibly between phase OFF and ON states at 37°C (Table 2). At 23°C, all colonies of strain DL1504 displayed a phase OFF Lac$^-$ phenotype resulting from the loss of transcription of the *papBA* genes at low temperature (Table 3). Analysis of β-galactosidase levels showed that *papBA* expression was reduced 52-fold at 23°C compared with expression at 37°C (Table 3).

PapI levels in *E. coli* DL1504 were modulated by the introduction of plasmid pDAL262, which contains the *papI* gene under the control of the *lac* promoter (Table 1), and plasmid pPY1025 expressing the LacI^q repressor (Braaten *et al*., 1994). Regulation of PapI levels in the resulting DL2223 transformant could then be modulated by the addition of IPTG. Even in the absence of IPTG, PapI was overexpressed in DL2223 based on immunoblot analysis (data not shown) and an increase in the β-galactosidase level at 37°C (2250 units, Fig. 2A) relative to the DL1504 parent strain lacking pDAL262 (1082 units, Table 3). Unlike

Table 1. Bacterial strains, bacteriophages and plasmids used in this study.

Strain, plasmid or phage	Description (relevant genotype)	Reference or source
E. coli		
XL-2 Blue	*recA1 endA1* [F′ *lacIq ZΔM15 Tn*10 (Tet^r) Amy Cam^r]	Stratagene
DL379	MC4100 λ246 lysogen	Blyn *et al*. (1989)
DL1504	MC4100 λ354 lysogen	Braaten *et al*. (1994)
DL1505	MC4100 λ354-1 lysogen	Braaten *et al*. (1994)
DL1742	MC4100 λ354-1 lysogen *ΔpapI*	This study
DL1784	MC4100 *Δlrp*	vanderWoude *et al*. (1995)
DL1910	DL1504 *Δlrp*	vanderWoude *et al*. (1995)
DL1947	DL1504 *hns651*	vanderWoude *et al*. (1995)
DL1948	DL1910 *hns651*	vanderWoude *et al*. (1995)
DL1965	DL1505 *hns651*	This study
DL1976	MC4100 *hns651*	This study
DL2208	MC4100 λ354-15 lysogen	This study
DL2223	DL1504 containing pDAL262, pPY1025	This study
DL2754	UT5600 containing pDAL481	This study
DL2755	MC4100 λ354-59 lysogen	This study
DL2771	DL1504 containing pDAL468	This study
DL2773	DL2208 containing pDAL468	This study
DL2774	DL2755 containing pDAL468	This study
DL2838	MC4100 λ491 lysogen	This study
DL3052	MC4100 λ354-73 lysogen	This study
DL3056	MC4100 λ354-73 lysogen containing pDAL468	This study
DL3317	XL-2 Blue containing pDAL569	This study
MC4100	F$^-$ *araD139* D(*lacIPOZYA-argF*)*U169 rpsL thi-1*	Casadaban (1976)
NH757	B178*hns651tyrT*β::*Tn*10	Falconi *et al*. (1991)
UT5600	*ΔlacZ lacI^q ompT Δleu trpE thi-1 ΔfepA-ompT*	Rupprecht *et al*. (1983)
Bacteriophages		
Phage λRS45	*lacZYA imm^{21}*	Simons *et al*. (1987)
Phage λ246	λRS45-pDAL379 recombinant phage	Blyn *et al*. (1989)
Phage λ354	λRS45-pDAL354 recombinant phage	Braaten *et al*. (1994)
Phage λ354-1	λRS45-pDAL354-1 recombinant phage	Braaten *et al*. (1994)
Phage λ354-15	λRS45-pDAL354-15 recombinant phage	This study
Phage λ354-59	λRS45-pDAL354-59 recombinant phage	This study
Phage λ354-73	λRS45-pDAL354-73 recombinant phage	This study
Phage λ491	λRS45-pDAL491 recombinant phage	This study
Phage P1L4	Virulent phage P1	L. Caro
Plasmids		
pUHE2 PzI-2	ColE1 replicon containing the $P_{lac/ara-1}$ promoter)	Lutz and Bujard (1997
pACYC184	*cam-tet* p15A replicon	Chang (1978)
pDAL262	pTZ18R containing a 271 bp *papI* DNA sequence	Nou *et al*. (1993)
pDAL246	pRS551 containing 1.6 kb *papIB* DNA sequence (ΔB1))	Blyn *et al*. (1989
pDAL337	pTZ19U containing a 1.76 kb *papIB* regulatory sequence	Braaten *et al*. (1994)
pDAL354	pRS550 containing 1.76 kb *papIB* DNA sequence (*papBAp–lacZ*)	Braaten *et al*., (1994)
pDAL354-1	pDAL354 with GATC^{1028} site changed to GCTC	Braaten *et al*. (1994)
pDAL354-15	pDAL354 with ATG start codon of *papB* changed to CTG (B15)	This study
pDAL354-59	pDAL354 with a 503 bp *papB*/intergenic DNA deletion (ΔB59)	This study
pDAL354-73	pDAL354 containing a *papI* frameshift mutation	This study
pDAL468	pACYC184 containing a 271 bp *papI* DNA sequence	This study
pDAL481	pGEX-2T containing a 413 bp *hns* DNA sequence	This study
pDAL485	pRS551 with multiple cloning site of *Eco*RI–*Sph*I–*Bam*HI	This study
pDAL491	pRS551 containing 1176 bp *papIB* DNA sequence (*papIp-lacZ*)	This study
pDAL569	pUHE2 PzI-2 containing *hns* under $P_{lac/ara-1}$ promoter control	This study
pGEX-2T	plasmid containing multiple cloning site and GST DNA sequences	Pharmacia Biotech
pRS550	*amp-kan-lacZYA* pMB1 replicon	Simons *et al*. (1987)
pRS551	*amp-kan-lacZYA* pMB1 replicon	Simons *et al*. (1987)
pPY1025	pGB2 containing *lacI^q*	P. Youderian

Table 2. Pap phase switch frequencies for *papIp* and *papBAp* operon fusions.

Strain	*pap* operon fusion	Mutation	*papI* plasmid	37°C Switch frequency[a] (events/cell/generation) ON to OFF	OFF to ON
DL2838	*papIp–lac*	wt	–	1.79×10^{-2}	4.85×10^{-4}
DL1504[b]	*papBAp–lac*	wt	–	2.35×10^{-2}	5.54×10^{-4}
DL3052	*papBAp–lac*	*papI* frameshift	–	NA[c]	NA
DL3056	*papBAp–lac*	*papI* frameshift	pDAL468	1.28×10^{-2}	3.87×10^{-3}
DL2208	*papBAp–lac*	*papB* CTG start codon	–	NA	NA
DL2773	*papBAp–lac*	*papB* CTG start codon	pDAL468	1.56×10^{-2}	3.79×10^{-3}
DL1947	*papBAp–lac*	*hns651*	–	3.65×10^{-2}	1.66×10^{-4}

a. Pap switch frequencies were measured as described in *Experimental procedures*.
b. Nou *et al*. (1995).
c. NA, not applicable.

DL1504, which was transcriptionally inactive at 23°C (21 units, Table 3), plating of isolate DL2223 at 23°C showed that phase ON colonies were present (data not shown) and β-galactosidase levels were higher than those of DL1504 (between 500 and 1000 Miller units depending on IPTG concentration, Fig. 2A). The ratio of *papBA* expression at 37°C compared with 23°C for strain DL2223 was decreased as PapI expression was induced via the addition of IPTG. At 10 μM IPTG, the 37°C/23°C ratio was less than one, indicating that the thermoregulatory response was blocked by PapI addition.

PapI and PapB are not required for Pap thermoregulation

The results above and previous data (Göransson *et al*., 1989a) indicated that *papI* transcription was thermoregulated and that overexpression of PapI disrupted thermoregulation of the *papBAp* promoter. To explore the relationship between PapI levels and thermoregulation further, we constructed a low-copy plasmid (pDAL468) that constitutively expressed PapI under the control of a vector promoter. Immunoblot analysis showed that the amount of PapI in *E. coli* DL1504 containing pDAL468 (DL2771) was significantly higher than that of PapI expressed from *E. coli* DL1504 lacking this plasmid, and the PapI level of cells containing pDAL468 grown at 23°C was higher than that of cells grown at 37°C (Fig. 2B, inset). This higher level of PapI expressed by plasmid pDAL468 resulted in a sevenfold increase in the OFF to ON switch frequency and a twofold increase in *papBA* transcription (compare *E. coli* DL3056 with DL1504, Table 2 and Fig. 2B). The *papI* frameshift mutation in *E. coli* DL3052 was complemented at 37°C when transformed with plasmid pDAL468, constructing isolate DL3056 (Fig. 2B). However, at 23°C, transcription from the *papBAp* promoter was still thermoregulated as evidenced by a sixfold reduction in *papBA* transcription at 23°C compared with 37°C (DL3056, Fig. 2B). Similar 23°C results (eightfold reduction) were obtained after the introduction of pDAL468 into *E. coli* isolate DL1504, the wild-type *papBAp–lacZ* fusion, constructing isolate DL2771 (Fig. 2B). These results showed that the Pap thermoregulatory response did not correlate with the amount of PapI present, as *papBA* transcription was reduced at 23°C compared with 37°C under conditions in which the amount of PapI was higher at 23°C than it was at 37°C.

In a second approach to determine the relationship between PapI and *papBA* thermoregulation, we analysed

Table 3. H-NS is required for thermoregulation of *papBA* transcription.

Strain	Genotype	CAA added (0.2%)	β-Galactosidase activity[a] 37°C	23°C	37°C/23°C β-galactosidase ratio
DL1504	Wild type	–	1082 ± 241	21 ± 6	52
DL1947	*hns651*	–	184 ± 7	178 ± 15	1
DL1504	Wild type	+	76 ± 33	5 ± 1	15
DL1947	*hns651*	+	130 ± 9	174 ± 21	1
DL1505	GCTC-I	+	1989 ± 293	47 ± 4	42
DL1965	GCTC-I *hns*651	+	1290 ± 365	768 ± 221	2
DL1910	Δ*lrp*	+	4 ± 2	3 ± 2	1
DL1948	Δ*lrp hns 651*	+	645 ± 49	694 ± 116	1

a. β-galactosidase activity is expressed as Miller units (Miller, 1972) and was measured as described in *Experimental procedures*. Analyses were at either 37°C or at 23°C in M9 glycerol medium with 0.2% casamino acids added where indicated. Error is expressed as ± 1 standard deviation from the mean.

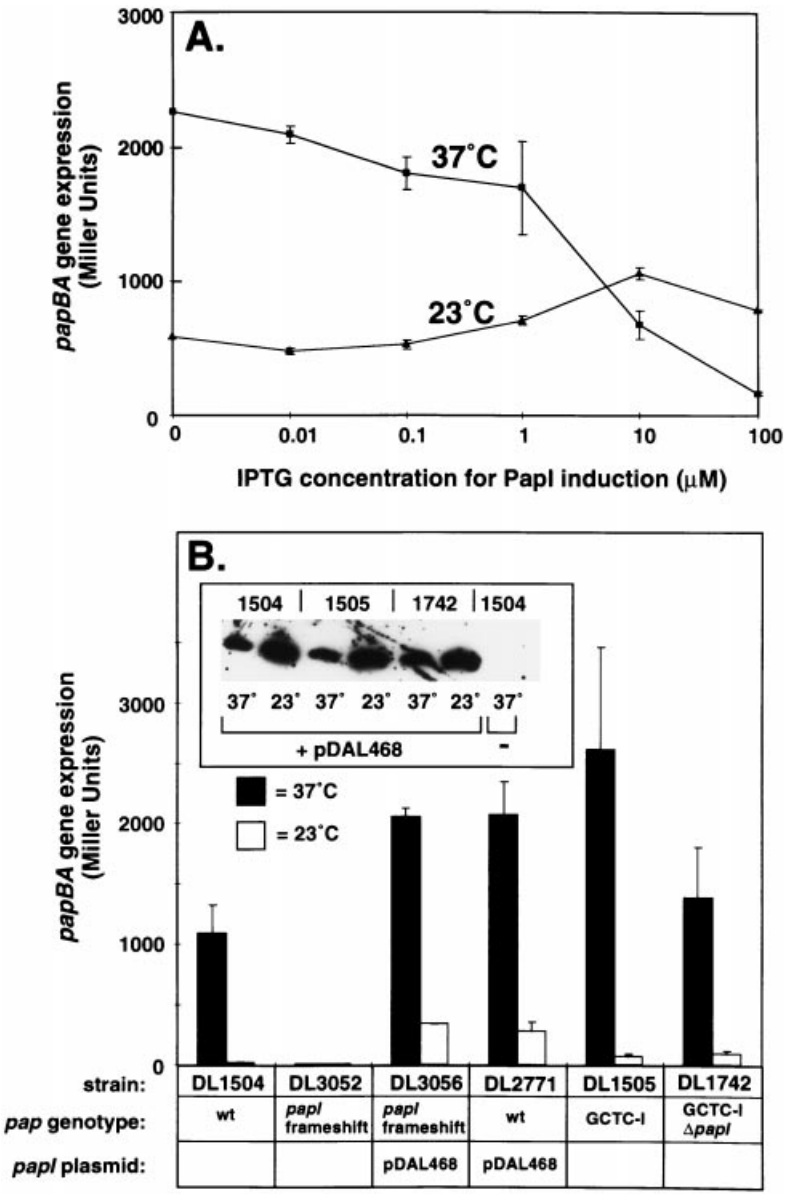

Fig. 2. Quantitation of the effects of PapI on *pap* thermoregulation.
A. The effects of increasing levels of PapI on *papBA* transcription at 23°C and 37°C. For this study, *E. coli* isolate DL2223 containing a wild-type *papBAp–lacZ* fusion, high plasmid pDAL262 (*lacZp–papI*) and plasmid pPY1025 ($lacI^q$) was used. The levels of IPTG shown were added to cultures to induce expression of PapI, and the expression of β-galactosidase from the *papBAP–lacZ* fusion was measured (Miller units) (Miller, 1972).
B. The analysis of *papBAp* expression at 23°C and 37°C from both wild-type and *pap* mutant constructs (see *Experimental procedures*). The error bars represent one standard deviation from the mean. The inset shows PapI levels from *E. coli* isolates containing low-copy plasmid pDAL468, detected by immunoblotting (*Experimental procedures*). When *papI* was in single copy, PapI protein was undetectable (*E. coli* DL1504).

E. coli DL1505, which displayed a PapI-independent locked ON phenotype at 37°C as a result of a mutation in the GATC-I site (GCTC-I) that precludes methylation by Dam (Braaten *et al*., 1994). Transcription from the *papBAp* promoter was still thermoregulated in *E. coli* DL1505 to a level similar to wild-type isolate DL1504 (37-fold decrease at 23°C compared with 37°C, Fig. 2B). Deletion of the *papI* gene reduced *papBA* transcription twofold at 37°C but did not significantly affect the LacZ expression level measured at 23°C. Thus, PapI was not required for the repression of *papBA* transcription at low temperature. Taken together, these results showed that, although very high levels of PapI resulted in the expression of *papBA* transcripts at 23°C, the intracellular level of PapI did not correlate with the level of thermoregulation, and PapI was not required for thermoregulation of *papBAp* transcription.

To examine the role of the PapB protein in the thermoregulation of *papBA* transcription, *papBAp–lacZ* fusions containing mutations in the *papB* gene and DNA regions downstream of *papB* were analysed for transcription at 37°C and 23°C. Strain DL2208 contains a *papBAp–lacZ* fusion in which the ATG start codon of the *papB* gene was altered to CTG (designated as the B15 mutation). Transcription of *papBA* in DL2208 was blocked at both 23°C and 37°C (Fig. 3) owing to the loss of translation of PapB, which was necessary for the activation of transcription of *papI* (Båga *et al*., 1985; Forsman *et al*., 1989) PapI, in turn, was required for *papBA* transcription. To restore

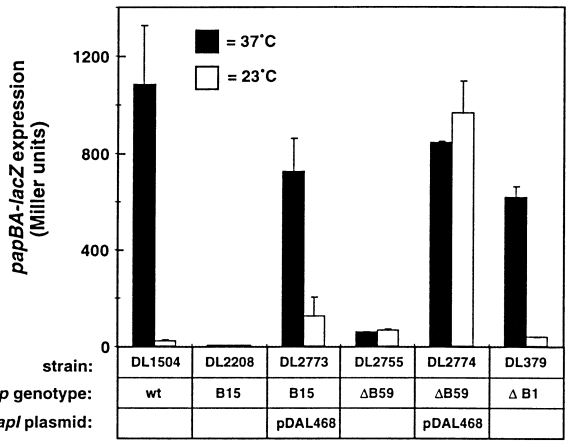

Fig. 3. Quantitation of the effects of *papB* on *pap* thermoregulation. The effects of mutations within the *papB* DNA region on transcription from *papBAp–lacZ* operon fusions were measured by quantitation of β-galactosidase activity (*Experimental procedures*). The *pap* mutations shown at the bottom are depicted in Fig. 1. The error bars represent one standard deviation from the mean.

papBA transcription in mutant isolate DL2208, the low-copy plasmid pDAL468 expressing PapI was introduced to construct isolate DL2773 (Table 1). This resulted in the restoration of *papBA* transcription and phase switching at 37°C (Table 2), supporting the conclusion that PapB activates *papBA* transcription indirectly by activating *papI* transcription and PapI expression. At 23°C, transcription of the *papBA* genes was reduced, as demonstrated by a uniform Lac$^-$ colony phenotype. Measurement of β-galactosidase activities showed that the transcription of *papBA* was reduced sixfold at 23°C compared with 37°C in DL2773 (Fig. 3). These results showed that thermoregulation occurred in the absence of the PapB protein and, thus, PapB was not required for the thermoregulatory response.

DNA sequences within papB *are required for Pap thermoregulation*

The results above strongly indicated that PapI and PapB were not required for *pap* thermoregulation. To determine if DNA sequences within the *pap* regulatory region played a role in thermoregulation, we constructed *pap* deletions downstream of the *papBAp* promoter (Fig. 1). Deletion of the entire *papB* coding sequence and the downstream region to *pap* bp 1720 (Fig. 3, ΔB59) greatly reduced *pap* transcription at both 23°C and 37°C as expected, as PapB activates *papBA* transcription indirectly as discussed above. Complementation with low-copy plasmid pDAL468 (PapI$^+$) resulted in a level of *papBA* transcription at 37°C near to that of wild-type *pap*, but no decrease in transcription was observed at 23°C (Fig. 3, DL2774). In contrast, deletion of *pap* bp 1603–1760 (ΔB1) did not affect the thermoregulatory response significantly, indicating that this downstream intergenic sequence was not necessary for thermoregulation (Fig. 3, DL379). Together, these results showed that DNA sequences within the *papB* region were necessary for *papBA* thermoregulation.

H-NS is required for Pap thermoregulation

Previous work demonstrated that a mutation in *hns* designated *drdX* disrupted temperature-dependent repression of *pap* gene expression from multicopy plasmids (Göransson *et al*., 1990). We determined the effect of H-NS on *papBA* transcription from the single-copy chromosomal *pap* operon by transduction of the *hns651* insertion mutation (Falconi *et al*., 1991) into *E. coli* isolate DL1504 to construct DL1947 (Table 1). Thermoregulation of *pap* transcription in the *hns*$^+$ control isolate DL1504 (a 37°C/23°C ratio of 52) was lost in the absence of H-NS (37°C/23°C ratio of 1; DL1947, Table 3). Similar results were obtained in medium containing casamino acids, which increased the growth rate of cells lacking H-NS by about twofold. Although casamino acids reduced the LacZ level of DL1504, thermoregulation of *pap* transcription still occurred (Table 3). These results indicated that H-NS was required for thermoregulation of *papBA* transcription.

The β-galactosidase expression measured for DL1947 in M9 glycerol medium at 37°C was significantly lower than that observed for the wild-type strain DL1504 grown at 37°C (Table 3). This decrease in β-galactosidase expression at 37°C was caused by a low percentage of phase ON bacteria in the DL1947 cultures (4–8%). Based on this observation, phase variation rates were measured for the *hns651* mutant strain DL1947 at 37°C. The phase ON to phase OFF switch frequency was 1.5-fold higher than the wild-type strain DL1504, and the phase OFF to phase ON switch frequency was threefold lower in the *hns651* mutant (Table 2). These data indicated that H-NS was not only required for thermoregulation of *papBA* transcription at 23°C, but also influenced the rate at which bacteria switched between phase states at 37°C.

Because only a small fraction of *E. coli* lacking H-NS were in the *pap* phase ON transcription state, we analysed *E. coli* DL1505 containing the GCTC-I mutation, which was phase-locked ON at 37°C. These mutant cells showed a 42-fold thermoregulatory response at 23°C similar to wild-type *E. coli* (Table 3). Introduction of the *hns651* mutation (DL1965) almost completely inhibited the thermoregulatory response, supporting the conclusion that H-NS was required for the thermoregulation of *papBA* transcription.

H-NS is a temperature-dependent DNA methylation blocking factor

In vivo *methylation protection analysis*. The data presented above showing that H-NS as well as DNA

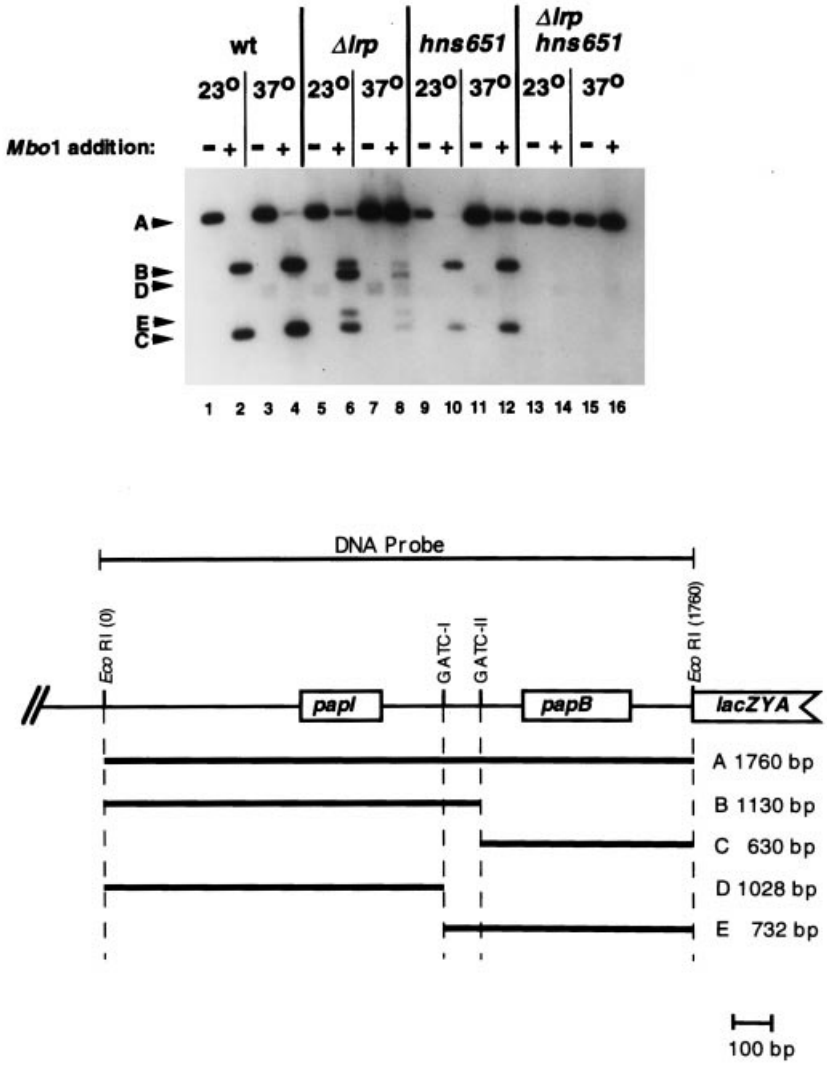

Fig. 4. Analysis of the temperature dependence of *pap* DNA methylation patterns from wild-type and mutant *E. coli* strains. The *E. coli* strains analysed are shown at the top: DL1504 (wt), DL1910 (Δ*lrp*), DL1947 (*hns651*) and DL1948 (Δ*lrp*, *hns651*) (Table 1). The restriction enzyme *Mbo*I, which digests only fully non-methylated GATC sites, was added where indicated. Restriction enzyme *Eco*R1, which cuts at two sites flanking the entire *pap* regulatory region, was added to all samples. DNAs for analysis were isolated from bacteria grown at 23°C or 37°C, as indicated. The figure at the top is a Southern blot of the restriction digests using the 1.76 kb *pap* DNA probe shown. Each band in the Southern blot shown has been given a letter designation that corresponds to the diagram below. Bands 'B' and 'C' represent a non-methylated GATC-II site, whereas bands 'D' and 'E' represent a non-methylated GATC-I site.

sequences within or near *papB* were required for Pap thermoregulation suggested the possibility that H-NS (or an H-NS-regulated factor) might bind in a temperature-dependent manner to *pap* regulatory DNA. This hypothesis was tested by monitoring the methylation patterns of the *pap* GATC-I and GATC-II sites using Southern blot analysis. As Lrp protects these *pap* GATC sites from Dam methylation (see *Introduction*), we reasoned that binding of H-NS might have a similar effect. DNAs isolated from cells grown at 23°C and 37°C were digested with the restriction endonucleases *Eco*RI, which cuts at the borders of the *pap* regulatory DNA region, and *Mbo*I, which cuts only non-methylated GATC sites (Braaten *et al*., 1994).

The wild-type strain DL1504 grown at either 37°C or 23°C predominantly displayed a phase OFF DNA methylation pattern based on the presence of DNA fragments 'B' and 'C', which result from *Mbo*I digestion at non-methylated GATC-II sites (Fig. 4). This high fraction of DNA with a phase OFF methylation pattern correlated well with the low amount of transcription observed in casamino acids at both temperatures tested (DL1504, Table 3). To determine the effect of H-NS on methylation protection of the *pap* GATC sites, an Lrp$^-$ *E. coli* isolate (DL1910) was analysed specifically to distinguish H-NS-mediated methylation protection. At 37°C, very little protection of either *pap* GATC site was observed in the absence of Lrp (Fig. 4, lane 8), consistent with previous data showing that, at 37°C, Lrp is the major methylation blocking factor for *pap* regulatory DNA (Braaten *et al*., 1991). Transcription of *papBA* was shut off in isolate DL1910, as Lrp is essential for *pap* transcription (Table 3). In contrast, at 23°C, methylation protection of both the GATC-I and the GATC-II sites was observed (Fig. 4, lane 6) as evidenced by the presence of DNA fragment pairs 'D/E' and 'B/C' respectively. These results indicated that another protein besides Lrp protected the GATC sites from methylation by Dam at 23°C but not at 37°C.

To determine if the temperature-dependent protection of

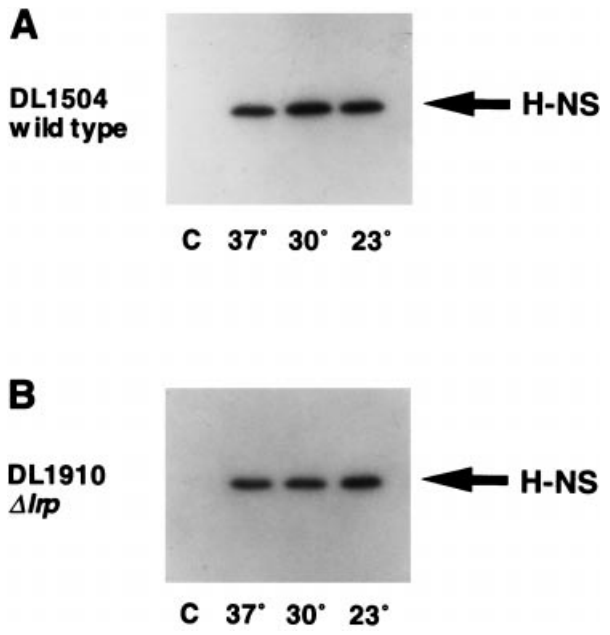

Fig. 5. Quantitation of H-NS levels as a function of temperature. Immunoblot analysis using antiserum raised against H-NS was carried out as described in *Experimental procedures*. Equal amounts (20 μg) of total cellular protein were loaded in each lane. The 'C' lane contained cell extract from *E. coli* DL1947 *hns651* grown at 37°C (Table 1).

the *pap* GATC sites observed required H-NS, we measured the effect of the *hns651* mutation on methylation protection of the *pap* GATC sites in *lrp*$^+$ and *lrp*$^-$ backgrounds. *E. coli* (*lrp*$^+$) containing the *hns651* mutation (DL1947) showed a predominantly phase OFF DNA methylation pattern at both 23°C and 37°C (Fig. 4, lanes 10 and 12) owing to a low level of phase ON cells in medium containing casamino acids, as discussed above. Introduction of *hns651* into Lrp$^-$ isolate DL1910 (DL1948) showed that neither *pap* GATC site was protected from DNA methylation at 23°C or 37°C (Fig. 4, lanes 14 and 16). These results indicated that H-NS or an H-NS-regulated protein was required for temperature-dependent protection of the *pap* GATC sites from Dam methylation. The basal *papBA* transcription observed in the absence of Lrp and H-NS in DL1948, which is independent of the upstream Lrp binding sites (van der Woude *et al*., 1995), was not thermoregulated (Table 3). This result was consistent with the data above and previous results (Göransson *et al*., 1990) indicating that H-NS was required for *pap* thermoregulation.

The H-NS-dependent methylation protection of the *pap* GATC-I and GATC-II sites observed at 23°C could be the result of a temperature-dependent increase in H-NS level. However, immunoblot analysis of *E. coli* DL1504 demonstrated that there was only a 1.5-fold increase in H-NS at 23°C compared with 37°C (Fig. 5A). Moreover, there was no increase in the H-NS level at 23°C compared with 30°C, and yet Pap phase variation occurred at 30°C with switch frequencies similar to those measured at 37°C (Blyn *et al*., 1989). Similar results were obtained with the Lrp$^-$ isolate DL1910 used to analyse the role of H-NS in *pap* GATC site methylation protection (Fig. 5B). These results indicated that the H-NS-dependent methylation protection of *pap* GATC sites was not the result of an increase in the H-NS level at 23°C.

In vitro *DNA binding and methylation protection analyses*. The results shown in Fig. 4 indicated that H-NS or a factor regulated by H-NS blocked methylation of the GATC-I and GATC-II sites in the *pap* regulatory region at 23°C. By analogy with Lrp, which binds to and is required to block methylation of these *pap* GATC sites (Braaten *et al*., 1994; Nou *et al*., 1995), we determined if H-NS bound specifically to the *pap* regulatory region containing the GATC-I and GATC-II sites. This was carried out at 23°C with a competitive electrophoretic mobility shift assay (EMSA), which has been used previously to show that H-NS bound specifically to bent DNA upstream of the *proU* promoter (Lucht *et al*., 1994). Plasmid pDAL337 containing a 1.76 kb *pap* regulatory DNA fragment was digested with restriction endonucleases as shown in Fig. 6A to yield different-sized vector and *pap*-specific DNA fragments. These DNA fragments were incubated with purified H-NS and analysed by EMSA. As shown in Fig. 6B, H-NS had the highest affinity for the 363 bp *pap* DNA fragment containing the GATC-I and GATC-II sites (see arrow), with complete shift occurring at 0.7 μM H-NS. This affinity of H-NS for the upstream regulatory region of the *papBAp* promoter was similar to that measured for the *proU* promoter (Lucht *et al*., 1994). In contrast, complete shift of the *papI* (854 bp) and *papB* (543 bp) containing DNA fragments as well as vector DNAs (146, 1268 and 1449 bp) occurred at higher H-NS concentrations (Fig. 6B). These results indicated that H-NS specifically bound to the *pap* regulatory region containing GATC-I and GATC-II.

To determine if methylation of the *pap* regulatory DNA by Dam was blocked by H-NS, an *in vitro* methylation protection assay was carried out. A 327 bp non-methylated *pap* regulatory DNA fragment (Fig. 6A) was radiolabelled at the GATC-I proximal end and incubated with H-NS, followed by the addition of Dam. To determine the methylation state of the *pap* GATC sites, DNAs were phenol extracted to remove H-NS and incubated with *Mbo*I, which digests only at non-methylated GATC sites. As shown in Fig. 7, digestion of the non-methylated 327 bp *pap* DNA (designated 'uncut') with *Mbo*I yielded a 125 bp radiolabelled fragment, representing digestion at GATC-I (Fig. 7A, lanes 1 and 2). Treatment of the 327 bp *pap* DNA with Dam inhibited *Mbo*I digestion (Fig. 7A, lane 3), indicating that, under these conditions, the *pap* GATC sites were 100% methylated. To determine if H-NS could

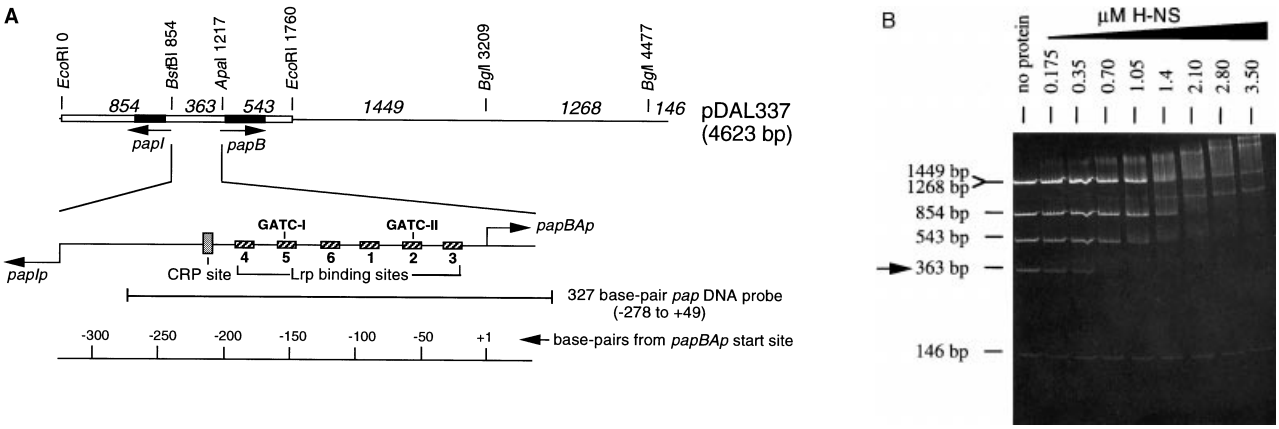

Fig. 6. Competitive electrophoretic mobility shift assay.
A. A restriction map of plasmid pDAL337 is shown at the top. The basepair locations of each restriction site are shown relative to the single *Eco*RI site, and the sizes of each DNA restriction fragment are shown. The bottom portion of the figure shows an expanded view of the 363 bp *pap* regulatory region including locations of the divergent *papIp* and *papBAp* promoters, the six Lrp binding sites that include the GATC-I and GATC-II sites (Nou *et al*., 1995) and the CRP site. The 327 bp DNA fragment used as a probe in Fig. 7 is also shown. The scale shown at the bottom is relative to the *papBA* transcription start site.
B. Electrophoretic mobility shift analysis (EMSA) was carried out as described in *Experimental procedures* using 0.5 μg of plasmid pDAL337 digested with *Eco*RI, *Bst*BI, *Apa*I and *Bgl*II (see A). The sizes of DNA restriction fragments are shown at the left with the arrow indicating DNA fragments containing the *pap* regulatory region. Purified H-NS was added at the concentrations shown at the top.

block methylation of the *pap* GATC sites by Dam, H-NS was added to the 327 bp *pap* DNA before incubation with Dam (Fig. 7A, lanes 4–8). Results showed that H-NS blocked methylation of both the *pap* GATC-I and GATC-II sites as evidenced by the presence of 125 bp and 223 bp DNA fragments respectively (Fig. 7A). A parallel mobility shift analysis showed that, although all of the *pap* probe was in complex with H-NS at 0.7 μM H-NS, no methylation protection occurred (Fig. 7B). Doubling the H-NS level resulted in a slower moving protein–DNA complex, and maximal methylation protection was observed. As the H-NS level was increased, the mobility of H-NS–*pap* DNA complexes appeared to be slightly reduced, and methylation of the *pap* GATC sites was not inhibited (Fig. 7). These results showed that H-NS blocked methylation of the *pap* GATC sites *in vitro* and suggested that this activity was caused by specific binding of H-NS, as methylation protection was observed at low levels of H-NS at which binding was specific but not at higher H-NS levels at which non-specific binding occurred (Fig. 6B).

Temperature-dependent repression of pap *transcription occurs within one cell generation*

To understand further the mechanism by which *pap* thermoregulation occurred, *papB* mRNA levels, β-galactosidase levels and the percentage of bacteria remaining in the phase ON DNA methylation state were measured in wild-type strain DL1504 after a temperature shift from 37°C to 23°C. During the first generation of growth at 23°C, *papB* mRNA levels initially increased fourfold compared with the amount of mRNA present in the initial 37°C culture (Fig. 8A). Measurement of the half-life ($t_{1/2}$) of *papB* mRNA at 23°C after rifampicin addition demonstrated that the $t_{1/2}$ was increased fivefold at 23°C ($t_{1/2} = 4$ min) compared with 37°C ($t_{1/2} = 45$ s) (Fig. 9), which could account for the initial increase in *papB* mRNA observed at 23°C, assuming a constant mRNA synthesis rate. By the end of the first generation of growth at 23°C, *papB* mRNA was not detectable, indicating that transcription initiated at the *papBAp* promoter was totally shut off (Fig. 8A).

Concurrent with the measurement of *papB* mRNA, β-galactosidase activity was also monitored at each time point following the temperature shift to 23°C. Initially, the β-galactosidase activity increased about twofold, then gradually decreased from the first generation through succeeding generations (Fig. 8B). By 10 generations of growth, the β-galactosidase level reflected the low level of *papB* mRNA present at 23°C (Fig. 8B). Notably, the rapid repression of *papB* transcription following a 37°C to 23°C shift, measured using an RNase protection assay (Fig. 8A), was not observed using β-galactosidase activity as a measure of *papBA* transcription (Fig. 8B). Presumably, this discrepancy in results was caused by a higher stability of β-galactosidase than *papB* mRNA at 23°C.

At 37°C, the fraction of cells containing a non-methylated *pap* GATC-I site is a direct measure of the fraction of cells in the phase ON transcription state. Conversely, the fraction of cells with a non-methylated *pap* GATC-II

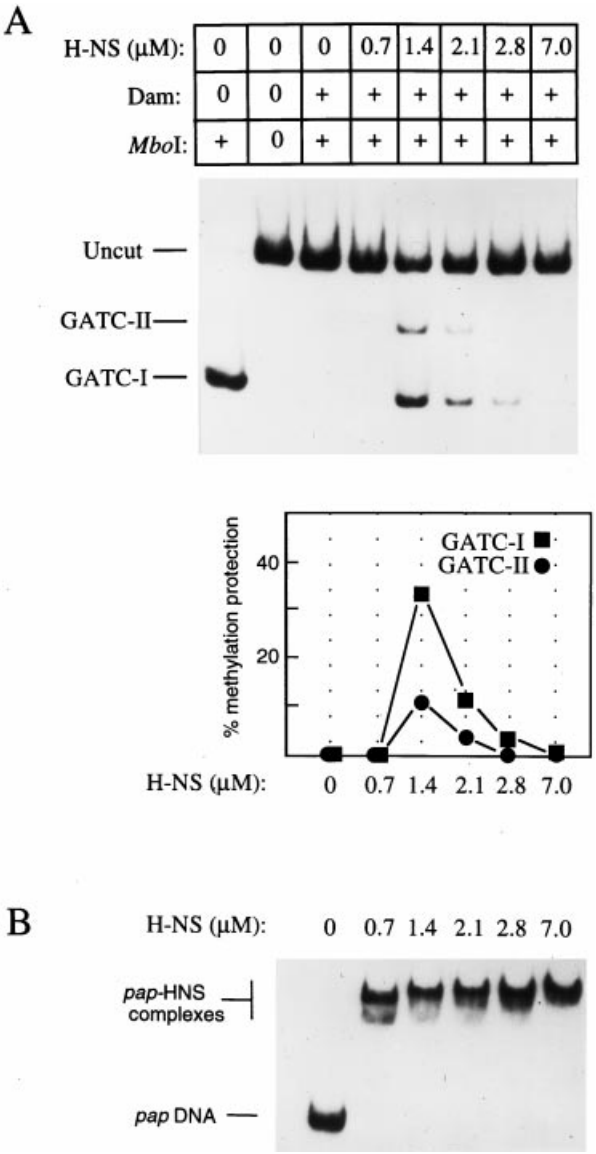

Fig. 7. *In vitro* DNA methylation protection analysis.
A. H-NS at the concentrations indicated was incubated with ^{32}P-labelled *pap* regulatory DNA (0.49 nM of the 327 bp DNA fragment shown in Fig. 6A) for 20 min at 23°C. Dam (0.8 units) was added (+) or not added (0) for 30 min, and samples were prepared for restriction digestion with *Mbo*I and analysed on a 4% acrylamide gel as described in *Experimental procedures*. The locations of the 327 bp ('uncut') *pap* regulatory DNA and *pap* DNA fragments resulting from *Mbo*I digestion at GATC-II and GATC-I are shown on the left. Quantitation of each lane of the acrylamide gel is shown lined up directly below and is expressed as the percentage of *pap* GATC sites that were protected from Dam methylation by H-NS.
B. Electrophoretic mobility shift analysis was performed in parallel with the methylation protection analysis shown in (A) directly above as described in *Experimental procedures*.

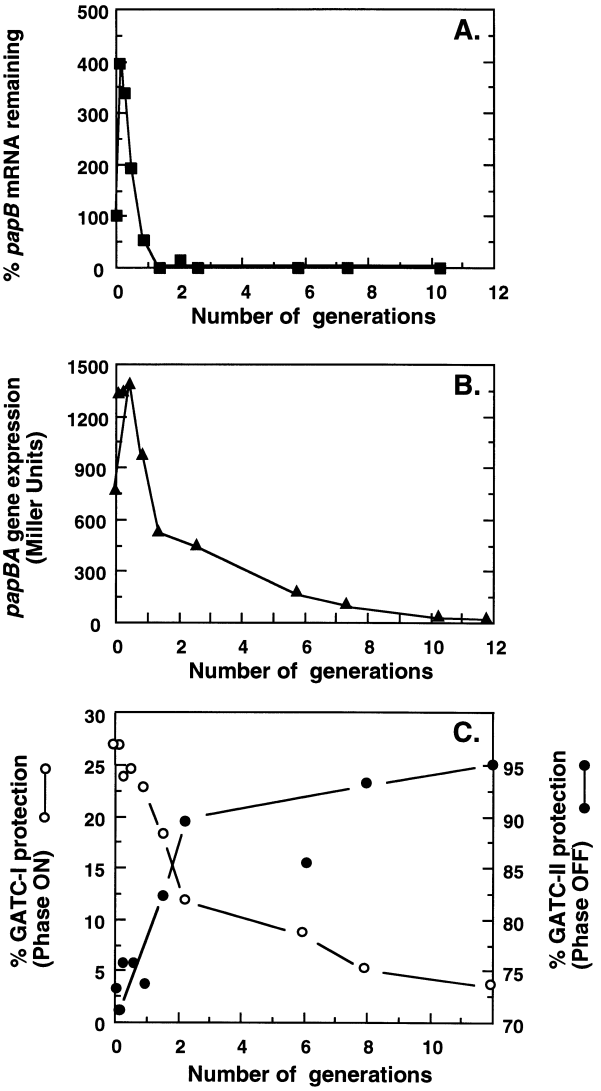

Fig. 8. Temporal analysis of *papBA* mRNA levels and *pap* GATC methylation states after a 37°C to 23°C temperature downshift. Phase ON (Lac$^+$) *E. coli* isolate DL1504 was grown in M9 glycerol medium at 37°C. Different dilutions were shifted to 23°C, and each diluted culture was grown to exponential phase (A_{600} = 0.6), at which time DNA and RNA were isolated, and β-galactosidase expression was measured from the sample. The generation time at 23°C was approximately 4 h.
A. *papB* mRNA was quantitated using an RNase I protection assay as described in *Experimental procedures*. The ordinate shows the percentage of *papB* mRNA present relative to the level measured at '0' time.
B. The level of β-galactosidase activity expressed from the *papBAp–lacZ* fusion was quantitated at different times.
C. The percentage of *pap* DNAs containing a non-methylated *pap* GATC-I site (ON methylation state, open circles) or a non-methylated GATC-II site (OFF methylation state, closed circles) is shown. DNA methylation analyses were carried out by digestion with *Mbo*I and Southern blotting (*Experimental procedures*).

site is a measure of the fraction of cells in the phase OFF transcription state (Nou *et al*., 1995). Therefore, to determine the effect of the 37°C to 23°C temperature shift on *pap* phase variation, we measured the fraction of cellular DNA containing non-methylated GATC-I (ON state) and GATC-II sites (OFF state) by Southern blot analyses of *Mbo*I-digested *pap* DNAs. We observed a rapid decrease in methylation protection of the GATC-I site after a shift to

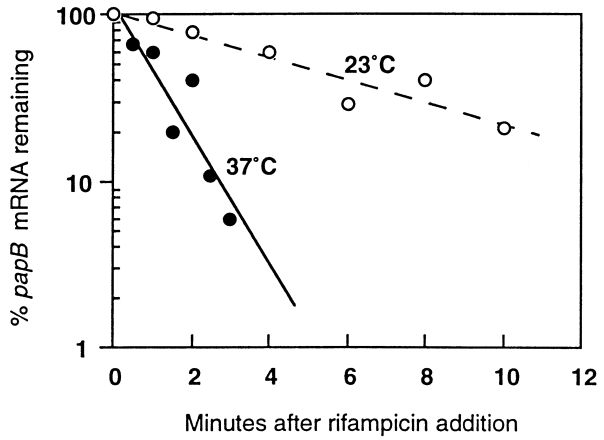

Fig. 9. Determination of *papB* mRNA half-lives at 23°C and 37°C. *E. coli* DL1504 was grown in M9 glycerol medium at 37°C to exponential phase, and one half of the culture was shifted to 23°C. After 20 min, rifampicin was added to each culture aliquot and, at different times, samples were taken for quantitation of *papB* mRNA as described in *Experimental procedures*.

23°C (Fig. 8C). This was probably caused by dissociation of Lrp from sites 4 and 5 in the GATC-I region (Braaten *et al*., 1991). The loss of methylation protection at GATC-I after a shift to 23°C was accompanied by a reciprocal increase in methylation protection at GATC-II (Fig. 8C). These results suggested that, after dissociation of Lrp from GATC-I, Lrp reassociated at GATC-II. Alternatively, it is possible that H-NS might also have bound at GATC-II, as H-NS blocked methylation of GATC-II at 23°C in the absence of Lrp (Fig. 4). In any case, a comparison of Fig. 8A and C shows that temperature-dependent repression of *papBA* transcription at 23°C involved more than just the transition of cells from the phase ON to phase OFF states. After one generation of growth at 23°C, *papBA* transcription was shut off (Fig. 8A), even though over 20% of cells remained in the phase ON state (Fig. 8C) Thus, transcription in these phase ON cells was shut off rapidly at 23°C preceding the transition to the phase OFF methylation state.

Discussion

The results presented here provide a framework for understanding the mechanism by which thermoregulation of *pap* gene expression occurs. Our data indicate that H-NS, which is required for Pap thermoregulation (Table 3; Göransson *et al*., 1990), is a temperature-dependent DNA methylation blocking factor *in vivo*, inhibiting methylation of the *pap* regulatory DNA GATC sites at 23°C but not 37°C (Fig. 4). H-NS could block the methylation of the *pap* GATC sites directly by binding to *pap* regulatory DNA or indirectly via the regulation of another DNA binding protein. We favour the former hypothesis, as our results showed that H-NS bound specifically to *pap* regulatory DNA containing the GATC-I and GATC-II sites (Fig. 6B) and blocked methylation of these sites by Dam *in vitro* (Fig. 7). These data raise the possibility that H-NS blocked *pap* transcription by competing with and/or altering the binding of Lrp with *pap* DNA, which includes the GATC-I and GATC-II sites. H-NS could act as a transcriptional repressor by inhibiting *papBAp* activation that results from binding of Lrp to sites 4 and 5 (Braaten *et al*., 1994; Nou *et al*., 1995). H-NS might also stabilize the phase OFF transcription state by interacting with Lrp bound at sites 1, 2 and 3 (van der Woude *et al*., 1996; see Fig. 6A). Alternatively H-NS could alter the interaction of RNA polymerase with the *papBAp* promoter as was recently suggested for *bgl* repression (Mizuno *et al*., 1996).

Based on the results shown here, it is likely that a key step in repressing *pap* transcription in response to a temperature downshift is the temperature-dependent alteration of binding of H-NS to *pap* regulatory DNA, observed as an increase in methylation protection of the *pap* GATC-I and GATC-II sites (Fig. 4). Previous data indicated that H-NS levels were transiently increased about fourfold in response to a temperature downshift from 37°C to 10°C (LaTeana *et al*., 1991). However, our results showed that H-NS levels were similar at 23°C and 30°C (Fig. 5) and thus could not account for the alteration in H-NS binding observed. It is also possible that H-NS-mediated methylation protection requires a co-factor that is active only at 23°C. However, this does not seems likely, as purified H-NS protected methylation of the GATC sites in the absence of other factors (Fig. 6B). A third possibility that we favour is that the conformation of H-NS is altered by temperature. This could occur directly as an intrinsic property of H-NS or indirectly by a temperature-dependent modification of H-NS. Previous results using isoelectric focusing showed that a number of isoforms were present (Spassky *et al*., 1984; Ussery *et al*., 1994), although the role(s) of these isoforms in H-NS function is not known. These possibilities can be addressed by determining if H-NS blocks methylation of the *pap* GATC sites at 37°C *in vitro* (the results shown in Fig. 6 were obtained at 23°C) and if the relative levels of different H-NS isoforms are altered at 23°C compared with 37°C.

Although H-NS blocked methylation of the *pap* GATC sites *in vitro* at 23°C, it did so only at low concentrations at which specific binding was observed but not at higher concentrations at which H-NS bound non-specifically (Figs 6 and 7). These results are consistent with previous observations indicating that the complexes formed between H-NS and specific DNA sites are distinguishable from complexes formed between H-NS and non-specific sites (Tippner and Wagner, 1995). Recently, it has been shown that oligomerization of H-NS is required for binding to

specific but not non-specific DNA binding sites and for bending of non-curved DNA (Spurio *et al*., 1997). Binding of H-NS to the AT-rich DNA sequences that adjoin the *pap* GATC-I (GATCTTTTAT) and GATC-II sites (TTAAAA-GATC) may aid the formation of multimeric protein–DNA complexes that sequester the GATC sites and prevent Dam methylation. Higher levels of H-NS that result in occupancy of non-specific *pap* DNA binding sites might further alter DNA conformation and disrupt H-NS interactions, allowing Dam access to the GATC sites. A prediction of this model is that H-NS lacking the ability to oligomerize (but retaining DNA binding activity) would be unable to block *pap* GATC methylation.

Temporal analysis of *papB* mRNA levels following a 37°C to 23°C downshift showed that *pap* transcription was shut off within one cell generation (Fig. 8A). However, after one cell generation, a significant fraction of cells (about 20%) remained in the phase ON methylation state (Fig. 8C). Thus, although H-NS increased the phase ON to phase OFF rate at 23°C, it must also have shut off transcription from the *papBAp* promoter in the phase ON cells remaining in the first generation following a shiftdown from 37°C to 23°C. Previously, it was hypothesized that thermoregulation of *papBA* transcription was caused by limiting amounts of PapI at low temperature. Based on Northern blot analysis, it was suggested PapI would be limiting because *papI* transcription was thermoregulated (Göransson *et al*., 1989a). As PapI is required to activate transcription from the *papBAp* promoter, a lack of PapI would lead to a loss of *papBA* transcription. Thus, the thermoregulation of *papBA* would simply be an indirect effect of the thermoregulation of *papI* transcription. This theory was supported by the observation that overproduction of PapI at low temperature caused a loss of thermoregulation (Göransson *et al*., 1989a). The data presented in this paper contradict this hypothesis. Using a constitutively expressed plasmid-encoded *papI* gene, we have shown that *papBA* transcription was reduced six- to eightfold at 23°C (Fig. 2B, DL3056 and DL2771) in the presence of a higher level of PapI than was present in cells in which *papBA* transcription occurred at 37°C (Fig. 2B, DL1504). Thus, the level of PapI did not correlate with thermoregulation of *papBA* transcription. Moreover, our data showed that *papBA* thermoregulation occurred in the absence of PapI (Fig. 2B, DL1742). Together, these results strongly indicated that *papI* does not play a direct role in the thermoregulation of *papBA* transcription.

Why is transcription from the *papIp* promoter thermoregulated? First, a reduction in transcription of *papBAp* would reduce *papIp* transcription indirectly via a decrease in the level of PapB, which is an activator of *papI* transcription (Forsman *et al*., 1989). Secondly, our recent data indicate that transcription from the *papIp* and divergent *papBAp* promoters is dependent upon the same nucleoprotein complex organized by Lrp and CRP. Mutations that block binding of Lrp to the GATC-I region shut off both *papBAp* and *papIp* transcription (unpublished data). Thus, the temperature-dependent dissociation of Lrp from the GATC-I region observed by DNA methylation pattern analysis (Fig. 8C) would be expected to block both *papBAp* and *papIp* transcription.

Previous results have shown that mutations in Lrp binding site 3, adjacent to GATC-II, blocked *papBA* thermoregulation. These *pap* mutations resulted in a PapI-independent, phase-locked ON phenotype at both 37°C and 23°C (Nou *et al*., 1995). In contrast, a mutation in GATC-I (GCTC-I) was also locked ON and PapI-independent (DL1505, Fig. 2B) but could still thermoregulate (phase-locked ON at 37°C and transcriptionally inactive at 23°C). Why do these two *pap* mutations result in different thermoregulatory phenotypes? One possibility is that specific binding of H-NS to *pap* DNA at 23°C might stabilize the phase OFF state by interaction with Lrp bound at sites 1, 2 and 3 around GATC-II (discussed above). Mutation of site 3 would counteract this stabilization by reducing Lrp affinity for *pap* sites 1, 2 and 3, which are highly co-operative (Nou *et al*., 1995), allowing formation of the phase ON state in which Lrp binds to sites 4, 5 and 6. An alternative hypothesis raised by the results presented here is that DNA sequences within Lrp site 3 (CATAAAAAAC) may also serve as an H-NS binding site critical for H-NS-mediated repression. According to either of these hypotheses, the GCTC-I mutant would maintain its thermoregulatory response because Lrp and H-NS could still interact near the unaltered GATC-II region to shut off *pap* transcription. These hypotheses are being tested.

In *Salmonella typhimurium*, the *proU* operon, like the *pap* operon, is controlled by H-NS. H-NS represses transcription of the *proU* operon under conditions of low osmolarity (Higgins *et al*., 1988; Dattananda *et al*., 1991; Lucht and Bremer, 1991). A DNA region involved in the repression of *proU* transcription was identified downstream of the *proU* promoter at nucleotide positions +73 to +274 within the coding region of the first gene of the operon (Fletcher and Czonka, 1995). H-NS has been shown to bind to the AT-rich DNA flanking the *E. coli proU* promoter, with a strong binding site between positions +60 and +110 (Lucht *et al*., 1994), in good agreement with the DNA region involved in *proU* repression localized in *S. typhimurium* (Fletcher and Czonka, 1995). These data are consistent with the hypothesis that H-NS binds to a region within the *proU* operon to repress transcription from the *proU* promoter. The results presented here show that a DNA sequence required for temperature-dependent repression of *papBA* transcription is located within and/or adjacent to the *papB* coding sequence, although the PapB protein was not required for *papBA* thermoregulation (Figs 1 and 3). The role of this *cis*-acting *papB* DNA in

thermoregulation is not clear. Results in Fig. 6B indicated that the affinity of H-NS for *papB* DNA (543 bp DNA fragment) was lower than for *pap* regulatory DNA (363 bp DNA fragment). However, it is still possible that binding of H-NS to a region of this DNA (defined by deletion ΔB59, Fig. 1) is required for thermoregulation. Further mutational analyses will need to be carried out to determine the exact *pap* DNA sequence required for thermoregulation and its function.

Experimental procedures

Bacterial strains, plasmids, bacteriophage and media

The strains, plasmids and bacteriophage used in this study are shown in Table 1. Luria–Bertani (LB) broth, LB agar, M9 minimal (M9) broth and M9 agar were prepared according to Miller (1972). The carbon source used in M9 media was glycerol at a final concentration of 0.2%. Casamino acids, when used, were at a final concentration of 0.2%. Antibiotics, when used, were at the following final concentrations: kanamycin, 25 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; tetracycline, 12.5 μg ml^{-1}; and chloramphenicol, 34 μg ml^{-1}. The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal; Gold Biotechnology) was used at a final concentration of 40 μg ml^{-1} of M9 agar medium.

Plasmid pDAL468 contains a 271 bp fragment encoding the *papI* gene cloned into the vector pACYC184 and constitutively expresses PapI. To construct plasmid pDAL468, plasmid pDAL262 was cut with *Taq*1, end filled with Klenow enzyme and subsequently digested with the restriction enzyme *Sph*I. The 271 bp fragment containing *papI* was isolated and ligated into *Eco*RV–*Sph*I-digested pACYC184. The resulting plasmid pDAL468 is chloramphenicol resistant.

Construction of pap *mutant derivatives*

Each of the following *pap* derivatives was constructed using the plasmids designated below. Each *pap* DNA sequence was recombined onto phage λRS45, integrated into the *E. coli* chromosome, and single-copy lysogens were used for analysis as described previously (Simons *et al*., 1987; Braaten *et al*., 1994).

Plasmid pDAL491, containing a *papIp–lacZ* transcription fusion, was constructed by introducing a *Sph*I site into the multiple cloning site of plasmid pRS551 (Simons *et al*., 1987) to construct pDAL485. A 1176 bp *Eco*RI–*Sph*I *pap* DNA fragment containing the *papI* and *papB* genes was ligated into *Eco*RI–*Sph*I-digested pDAL485 to construct pDAL491. On this plasmid, the *papI* rho-dependent terminator site has been removed, allowing the *papIp* promoter to drive *lacZ* transcription. Plasmid pDAL354 contains a 1.76 kb *papIB* regulatory sequence with the *papBAp* promoter controlling *lacZ* transcription (Nou *et al*., 1995). Plasmid pDAL354-15 has an A to C transversion mutation within the start codon of *papB*, changing ATG to CTG. Plasmid pDAL354-59 contains a 503 bp deletion of the *pap* sequence from bp 1222 to bp 1720 (see Fig. 1). Plasmid pDAL354-73 contains a 2 bp insertion frameshift mutation, 5′-TG-3′, between *pap* base 806 and 807 of *papI*, resulting in a nearby translational stop site. The Δ*papI* mutation in *E. coli* DL1742 was constructed as described previously (Nou *et al*., 1995).

Calculation of switch frequencies

Pap phase switch frequencies were calculated as described previously (Blyn *et al*., 1989) and are based on data from two to three separate colonies.

Measurement of β-galactosidase activity

Each bacterial strain was inoculated from a frozen −70°C stock onto M9 agar containing glycerol, incubated at 37°C and passed once on M9 agar. After growth for 36 h, a Lac$^+$ colony (a Lac$^-$ colony from those strains that did not express any Lac$^+$ colonies) was isolated and resuspended in 1 ml of M9 salts. Two flasks containing 15 ml of prewarmed M9 broth with glycerol were inoculated with 100 μl of the colony suspension and incubated at either 37°C or 23°C in a shaking water bath. The cultures were grown to log phase (OD_{600} = 0.25–0.9), and β-galactosidase activities were measured as described previously (Miller, 1972). This inoculation method ensured that all strains had grown for 9–11 generations at either 37°C or 23°C before β-galactosidase measurements. All β-galactosidase activities were an average from two or more separate cultures grown under identical conditions.

Bacterial chromosomal DNA isolation and Southern analysis

Bacteria were collected from cultures grown as described under the measurement of β-galactosidase activities (see above) with the exception of the temperature downshift experiment (see below). Chromosomal DNAs were isolated as described previously (Ausubel *et al*., 1987), digested with the appropriate restriction enzymes and analysed on a 1.2% agarose gel. The separated DNA fragments were subsequently transferred and cross-linked to Hybond-N membrane (Amersham) using Stratagene's Posiblot system and the UV Stratalinker 1800.

The Southern blot analysis shown in Fig. 4 was performed using a 1.76 kb *Eco*RI ^{32}P-labelled *pap* DNA fragment (see Fig. 1, bp 1–1760). The temperature downshift experiment (Fig. 8C) was carried out using an 80 bp ^{32}P-labelled DNA fragment derived from the inter-GATC-I/GATC-II *pap* DNA region (Fig. 1). Quantitation of the fraction of *pap* DNAs in the phase ON or phase OFF states was carried out by phosphorimager analysis (Bio-Rad Phosphoranalyst) or film densitometry.

Antibody production to GST–H-NS fusion protein

The *hns* gene was cloned into plasmid pGEX-2T (Pharmacia Biotech). Plasmid pGEX-2T contains coding sequences for glutathione-*S*-transferase (GST) and a thrombin cleavage site. For protein expression, the plasmid construct containing *hns-gst* (pDAL481) was transformed into the bacterial strain UT5600 constructing DL2754 (Table 1).

Expression of recombinant GST–H-NS protein was carried out by inducing cells in log phase with IPTG (0.5 mM). Bacteria

were harvested by centrifugation, washed once in phosphate-buffered saline, pH 7.4 (PBS), and resuspended in 5 ml of PBS containing protease inhibitors (pepstatin A, 1 μg ml^{-1}; antipain, 1 μg ml^{-1}; leupeptin, 1 μg ml^{-1}; chymostatin, 1 μg ml^{-1}; and phenylmethylsulphonyl fluoride, 10 μg ml^{-1}). The bacterial cells were broken by sonication, Triton X-100 was added to 1% final concentration, and the extract was clarified by centrifugation. Glycerol was added to the supernatant to a final concentration of 10%, and the extract was frozen at −70°C.

The GST–H-NS fusion protein was isolated using glutathione–agarose beads (Sigma). Elution was carried out using a reduced glutathione buffer (10 mM in 50 mM Tris, pH 8.0). Immunoblot analysis showed that the GST–H-NS fusion protein was of the predicted size of 42 kDa (26 kDa GST plus 16 kDa H-NS). Antiserum against the GST–H-NS fusion protein was raised in rabbits (Cocalico Biologicals).

Immunoblotting

The immunoblot analyses shown in Figs 2 and 5 were performed as described previously (Harlow and Lane, 1988). Briefly, equal amounts of total cellular protein were analysed using SDS–PAGE. Proteins were transferred to nitrocellulose (Costar) using a transblot electroblotter (Bio-Rad). Polyclonal antisera to H-NS and PapI were adsorbed against total *E. coli* proteins bound to sepharose beads (Sambrook *et al*., 1989). The H-NS antiserum was purified using beads coated with proteins from the *hns651* strain DL1976 (van der Woude *et al*., 1995). Detection of H-NS and PapI was carried out using [^{125}I]-protein A (Dupont NEN). Preflashed radiographic film was exposed to membranes containing radiolabelled proteins, and individual bands were quantitated using film densitometry (Bio-Rad).

H-NS purification

H-NS was purified from *E. coli* DL3317 (Table 1) containing plasmid pDAL569, which has *hns* under the control of the $P_{lac/ara-1}$ promoter from plasmid pUHE2 PzI-2 (Lutz and Bujard, 1997). Plasmid pDAL569 was constructed as follows. A 411 bp DNA fragment encompassing the entire H-NS coding sequence was amplified from *E. coli* chromosomal DNA using an H-NS PCR primer with a *Kpn*I site adjacent to the *hns* start codon (5′-CAACAAGGTACCATGAGCGAAGCACTTAAAATTC-3′) and a PCR primer with an *Xba*I site adjacent to the H-NS stop codon (5′-CAACAATCTAGATTATTGCTTGATCAGGAAATCG-3′). The *hns* PCR product DNA and vector pUHE2 PzI-2 were digested with *Kpn*I and *Xba*I. The appropriate DNA fragments were isolated from agarose gels, ligated and transformed into *E. coli* XL-2 Blue (Stratagene). DNA sequence analysis of the resulting plasmid pDAL569 showed that the *hns* DNA sequence was error-free. Two litres of DL3317 were grown at 37°C to an $A_{600} = 0.8$, and H-NS overexpression was induced by the addition of IPTG and arabinose (1 mM and 0.05% final concentrations respectively). After a 2 h induction, cells were harvested by centrifugation and resuspended in 30 ml of PG buffer [50 mM sodium phosphate buffer, pH 7.4 containing EGTA (1 mM), β-mercaptoethanol (10 mM), phenylmethylsulphonyl fluoride (PMSF; 1 mM) and glycerol (2%)]. This and all subsequent steps were carried out at 4°C. Bacteria were broken by passage through a French pressure cell at 18 000 p.s.i., and cell debris was separated by centrifugation at 22 000 × *g* for 1 h. After bringing the volume to 60 ml by the addition of PG buffer, cell lysate was loaded onto a phosphocellulose P-11 column (30 ml bed volume) and eluted with a 0–1.0 M NaCl linear gradient (300 ml). Fractions eluting at 0.35–0.45 M NaCl contained H-NS and were dialysed against TN buffer (10 mM Tris HCl, pH 8.0, 0.1 M NaCl, 1 mM β-mercaptoethanol) using 3 kDa cut-off dialysis membranes (Spectrapor). Final purification was carried out by FPLC on a mono-Q 5/5 column (Pharmacia LKB) equilibrated in TN buffer with elution by a 0.1–1.0 M NaCl linear gradient (5 ml). H-NS eluted as a sharp peak at 0.5 M NaCl and was judged to be 98% pure based on SDS–PAGE analysis.

Competitive electrophoretic mobility shift assay (EMSA)

Purified H-NS (see above) was incubated with restriction-digested *pap* plasmid pDAL337 (0.5 μg) in binding buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA, 80 mM NaCl, 10 mM β-mercaptoethanol, 4% glycerol, 50 μg ml^{-1} bovine serum albumin). After a 20 min incubation, protein–DNA complexes were separated on 4% acrylamide gels in 0.5 × TBE buffer (45 mM Tris-borate, 1 mM EDTA). DNA was visualized by ethidium bromide staining.

In vitro *methylation protection assay*

The ability of H-NS to protect the *pap* GATC-I and GATC-II sites from methylation by Dam was determined using a 327 bp *pap* DNA probe containing Lrp binding sites 1–6 spanning bp −278 to +49 (see Fig. 6A). This 327 bp *pap* DNA was amplified by polymerase chain reaction (PCR) using ^{32}P-end-labelled oligonucleotide 5′-CTCTATGTTTGCTTTATTTGTTC-3′ and non-labelled oligonucleotide 5′-CCCTTCTGTCGGGCCCC-3′ with plasmid pDAL337 DNA template. H-NS was incubated with radiolabelled *pap* DNA (0.49 nM) in binding buffer (see above) containing 80 μM S-adenosyl methionine (SAM) for 20 min at 23°C. Dam (0.8 units) was then added for 30 min followed by a 10 min incubation at 65°C to inactivate Dam. H-NS, which is heat stable, was removed by phenol extraction. Residual phenol was removed by centrifugation using Micro Biospin-30 columns (Bio-Rad), and DNAs were digested with restriction endonuclease *Mbo*I, which cuts at non-methylated GATC sites. DNA analysis was carried out using 4% acrylamide gels in 0.5 × TBE buffer (above) followed by autoradiography (see Fig. 7A). A parallel EMSA experiment was carried out by incubating H-NS with radiolabelled 327 bp *pap* DNA for 20 min as described above for methylation protection and analysis on 4% acrylamide/TBE gels (see Fig. 7B). Quantitation of the fraction of DNAs protected from methylation was performed using phosphorimager analysis (Bio-Rad).

Ribonuclease protection assay

The RNA probes used for ribonuclease protection were synthesized *in vitro* using DNA templates constructed by PCR. The 5′ end of the *papB* gene was amplified by PCR from chromosomal

DNA of strain DL1504 (Table 1) using oligonucleotide primer 5′-CGGAATTCGCCATGATGTTTTTATCTGAGTACC-3′ and oligonucleotide5′-CTGGATCCTAATACGACTCACTATAGG-GAGGCTCCTTACGGGAGTGCC-3′, which contains a phage T7 RNA polymerase promoter at the 3′ end to allow production of the antisense RNA by phage T7 RNA polymerase. Similarly, the 5′ end of the *murF* gene was amplified by PCR using oligonucleotide 5′-CGGAATTCATGATTAGCGTAACCCTT-A-3′ and oligonucleotide 5′-CTGGATCCTAATACGACTCA-CTATAGGGAGGGGGTGATATCTGCACCTT-3′ containing a T7 RNA polymerase promoter at the 3′ end. Antisense RNA probes were labelled with [α-^{32}P]-UTP by *in vitro* transcription using T7 RNA polymerase (Ambion). The *murF* RNA probe was used as an internal control, as similar levels of transcript are expressed at both 37°C and 23°C (data not shown). Full-length RNA probes from *in vitro* transcription reactions were isolated by electrophoresis in a 6% acrylamide gel.

Ribonuclease protection assays were performed using the RPA II kit (Ambion). For each time point, 1.25 μg of total cellular RNA, isolated as described previously (Hagen and Young, 1978), was used, and both the radiolabelled *papB* and *murF* probes were added to each RNA sample in molar excess. RNA samples were separated by electrophoresis (8% acrylamide), preflashed radiographic film was exposed to the gel and bands were quantitated by densitometry. The *papB* mRNA levels were quantitated and corrected for differences in sample load using the *murF* as an internal control.

Measurement of papB *mRNA half-lives*

To determine the half-lives of the 5′ end of the *papB* mRNA at 23°C and 37°C, bacterial cultures were grown at 37°C to mid-log phase and divided into two flasks. One flask was incubated at 37°C and the other at 23°C. Rifampicin (20 mg ml^{-1} in methanol) was added to a final concentration of 200 μg ml^{-1} to both flasks after a 20 min preincubation. Aliquots of 5 ml were removed at various time points for RNA isolation. Levels of *papB* RNA were quantitated as described above using the ribonuclease protection assay.

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