Molecular Cell, Vol. 12, 947–957, October, 2003, Copyright ©2003 by Cell Press

The Mechanism by which DNA Adenine Methylase and PapI Activate the Pap Epigenetic Switch

Aaron D. Hernday,[1] Bruce A. Braaten,[2] and David A. Low[1,2,*]
[1]Biomolecular Sciences and Engineering
[2]Department of Molecular, Cellular, and Developmental Biology
University of California, Santa Barbara
Santa Barbara, California 93106

Summary

The expression of pyelonephritis-associated pili (Pap) in uropathogenic *Escherichia coli* is epigenetically controlled by a reversible OFF to ON switch. In phase OFF cells, the global regulator Lrp is bound to *pap* sites proximal to the pilin promoter, whereas in phase ON cells, Lrp is bound to promoter distal sites. We have found that the local regulator PapI increases the affinity of Lrp for the sequence "ACGATC," which contains the target "GATC" site for DNA adenine methylase (Dam) and is present in both promoter proximal and distal sites. Mutational analyses show that methylation of the promoter proximal GATC^{prox} site by Dam is required for transition to the phase ON state by specifically blocking PapI-dependent binding of Lrp to promoter proximal sites. Furthermore, our data support the hypothesis that PapI-dependent binding of Lrp to a hemimethylated GATC^{dist} site generated by DNA replication is a critical component of the switch mechanism.

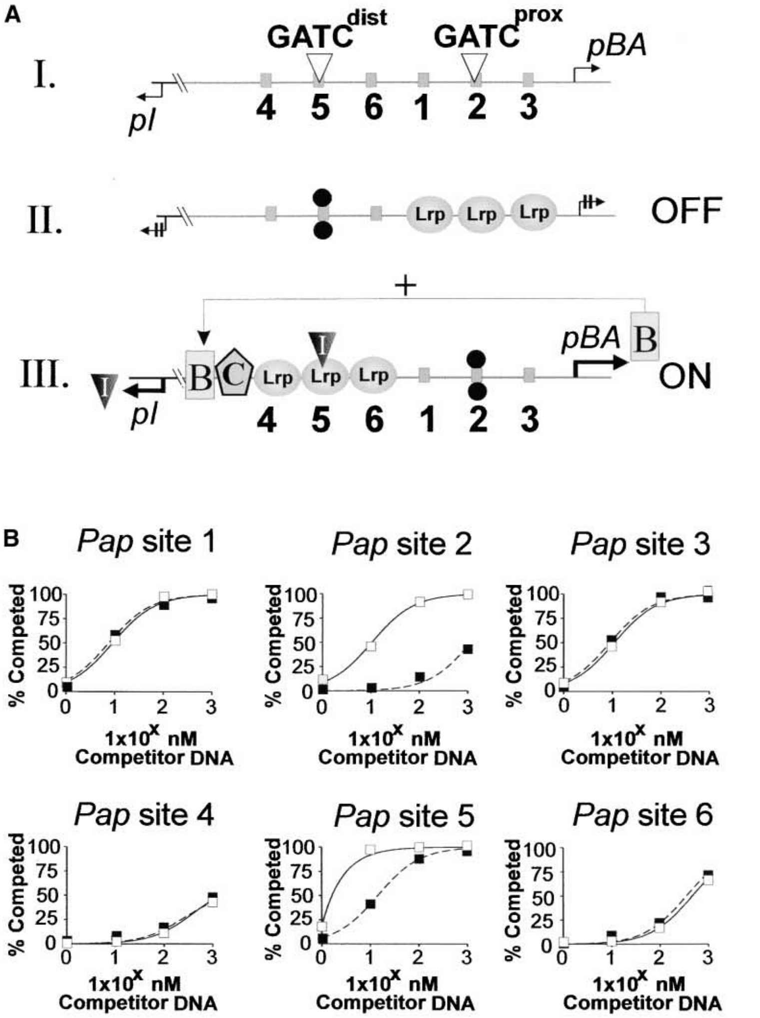
Introduction

Methylation of DNA in bacteria directly or indirectly regulates a number of important cellular events including timing of DNA replication (Lu et al., 1994), transposition (Roberts et al., 1985), DNA repair (Modrich, 1989), restriction of DNA by endonucleases (Bickle and Kruger, 1993), cell cycle progression (Reisenauer and Shapiro, 2002), virulence (Heithoff et al., 1999), and gene expression (Low et al., 2001). Many of these events are tied to chromosomal replication by the hemimethylated DNA state which is generated immediately following replication (parental DNA strand is methylated, daughter strand is nonmethylated). For example, timing of DNA replication is controlled by the SeqA protein, which binds to hemimethylated DNA sites in the origin of replication, inhibiting further rounds of initiation (Taghbalout et al., 2000). Methyl-directed mismatch repair is regulated by MutH, which binds specifically to hemimethylated DNA (Friedhoff et al., 2003) and nicks the nonmethylated (nascent) DNA strand, ensuring that the parental template strand is not altered.

In certain methylation-controlled events, binding of regulatory proteins to sequences that overlap DNA methylase target sites block methylation of these sites. This results in the formation of DNA methylation patterns, which consist of one or more stably nonmethylated DNA sequences. DNA methylation patterns, in turn, can modulate the binding of regulatory proteins, and thus control gene expression (Casadesus and D'Ari, 2002; Hernday et al., 2002). The first report of direct control of gene expression by DNA methylation patterns was a study on the pyelonephritis-associated pili (*pap*) operon of uropathogenic *Escherichia coli* (UPEC) (Braaten et al., 1994). Pap pili enable UPEC to bind to uroepithelial cells and play an important role in the pathogenesis of urinary tract infections (Kaack et al., 1993; Lund et al., 1988). The expression of Pap pili is under a phase variation control mechanism in which cells are either piliated (phase ON) or nonpiliated (phase OFF) (Blyn et al., 1989). The ON to OFF switch rate (about 10^{-2} per cell per generation in M9 minimal medium) is 100-fold higher than the OFF to ON rate (10^{-4} per cell per generation) (Blyn et al., 1989), resulting in a mostly phase OFF population. Pap phase variation provides a potential advantage of generating two different pili expression phenotypes within the cell population. In the host urinary tract, the Pap-expressing cells can bind to epithelial cell receptors, avoid clearance, and establish infection. The default is set toward the OFF state, which could serve to save cellular energy outside the host where pili expression may not be needed and could be deleterious.

Phase OFF and ON cells have distinctive, converse *pap* regulatory DNA methylation patterns (Blyn et al., 1990). Genetic studies showed that the global regulator leucine-responsive regulatory protein (Lrp), *pap*-encoded local regulator (PapI), and DNA adenine methylase (Dam) play important roles in formation of the phase ON DNA methylation pattern and activation of *papBA* transcription (Blyn et al., 1990; Braaten et al., 1991, 1994; Kaltenbach et al., 1995; Nou et al., 1993). Analysis of phase OFF cells indicated that Lrp is cooperatively bound to sites 1–3 proximal to and overlapping the *papBA* promoter, blocking both *pap* pilin transcription and Dam-mediated methylation of the promoter proximal GATC sequence within site 2, denoted GATC^{prox}. The promoter distal GATC site (GATC^{dist}) within site 5 is fully methylated since it is not occupied by Lrp (Figure 1A, panel II). Conversely, in phase ON cells, Lrp binds to promoter distal sites 4–6, forming a methylation pattern characteristic of transcriptionally active cells. In these phase ON cells, GATC^{dist} is protected from methylation by Lrp binding and the unbound GATC^{prox} site is fully methylated (Braaten et al., 1991, 1994) (Figure 1A, panel III). Formation of the phase ON state requires PapI, which has been shown to specifically bind to Lrp (Kaltenbach et al., 1995). Binding of Lrp at sites 4–6, together with cAMP-CAP binding upstream, activates the *papBA* pilin promoter resulting in Pap pilus expression (Goransson et al., 1989; Weyand et al., 2001). The *papB* regulatory gene expressed in phase ON cells initiates a positive feedback loop by the binding of PapB near the divergent *papI* promoter which upregulates PapI expression (Figure 1A, panel III) (Forsman et al., 1989; Hernday et al., 2002; Xia et al., 1998).

*Correspondence: low@lifesci.ucsb.edu



C

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	PapI Response
pap 2	A	A	C	G	A	T	C	T	T	T	T	A	A	C	C	C	A	C	Yes
pap 5	G	A	C	G	A	T	C	T	T	T	T	A	T	G	C	T	G	T	Yes
pap 1	T	T	A	G	T	G	T	T	T	T	G	T	T	C	T	A	G	T	No
pap 3	T	T	A	G	T	T	T	T	T	T	A	T	G	T	T	G	T	A	No
pap 4	T	A	C	G	G	A	C	T	T	T	C	T	T	T	T	G	G	C	No
pap 6	C	A	T	G	A	T	G	T	T	T	T	T	A	T	C	T	G	A	No
ilvIH 2	G	T	A	G	A	A	T	T	T	T	A	T	T	C	T	G	A	A	No
SELEX	-	Y	A	G	H	A	W	A	T	T	W	T	D	C	T	R	-	-	Not applicable

Figure 1. PapI Facilitates Binding of Lrp to *pap* DNA Sites 2 and 5 in the Regulatory Region

(A) Pap phase states. (AI) Organization of *pap* regulatory sequence. Six Lrp binding sites are located between the divergent *papBA* pilin and *papI* promoters. The *papBA* proximal GATC site (GATC^{prox}) is located within Lrp binding site 2 and the *papBA* distal GATC site (GATC^{dist}) is located within Lrp binding site 5. (AII) In phase OFF cells, Lrp binds cooperatively and with highest affinity to *pap* sites 1–3 overlapping the *papBA* promoter, blocking *pap* transcription and methylation (denoted by a black circle) of GATC^{prox}. (AIII) In phase ON cells, PapI-Lrp is bound to sites 4–6, blocking methylation of GATC^{dist} and along with CAP (denoted by a pentagon) facilitating activation of *papBA* pilin transcription which initiates the PapB-positive feedback loop (PapB is denoted by a rectangle).

(B) Competition gel shift analysis. Lrp (5 nM) was incubated with ^{32}P-labeled *ilvIH* Lrp binding site 2 (100 pM) (Wang and Calvo, 1993a) in the presence (open squares) and absence (closed squares) of PapI (100 nM). The indicated levels of duplex 31- or 32-mer *pap* oligonucleotides each containing one of the six Lrp binding sites was added in oligonucleotide binding buffer (OBB) (see Experimental Procedures). Protein-DNA complexes were separated by electrophoretic mobility shift (EMSA). The fraction of ^{32}P-*ilvIH* site 2-Lrp complexes competed ([fraction DNA bound with no competitor − fraction DNA bound with X nM competitor] / fraction bound with no competitor) at each *pap* competitor DNA concentration is shown on the *y* axis.

(C) Lrp binding site comparison. The DNA sequences of the six *pap* Lrp binding sites, *ilvIH* site 2, and the SELEX consensus Lrp binding site (Cui et al., 1995) are shown (Y = C or T, H = not G, W = A or T, D = not C, R = A or G). The GxxxxTT sequence in common between all sequences is highlighted, as well as the ACGATC sequence common to *pap* sites 2 and 5.

DNA methylation plays both positive and negative roles in controlling the reversible switch between Pap pili^{+} and pili^{-} expression states (Braaten et al., 1994; Hernday et al., 2002). Methylation of GATC^{dist}, which occurs in phase OFF cells, inhibits the switch to ON since a GCTC^{dist} mutation which blocks methylation by Dam results in a phase-locked ON phenotype (Braaten et al., 1994). In contrast, methylation of GATC^{prox} is essential for transcription since a GCTC^{prox} mutation that blocks methylation results in a phase-locked OFF phenotype. Moreover, a GCTC^{dist}/GCTC^{prox} double mutant is also locked OFF, showing that methylation of GATC^{prox} is required for phase-locked ON cells (Braaten et al., 1994). Although it is clear that Dam and PapI are essential for transition to the phase ON state, the mechanism(s) by which this occurs was unknown. Data presented here show that PapI increases the affinity of Lrp for the sequence ACGATC found in both promoter proximal and distal sites. However, binding of Lrp at promoter proximal sites blocks *pap* pilin transcription, explaining why PapI alone is not sufficient for transition to the phase ON state but also requires Dam. Dam methylation at GATC^{prox} specifically blocks the PapI-dependent increase in affinity of Lrp for promoter proximal sites 1–3, favoring binding of PapI-Lrp to distal sites 4–6, which we show is required for transcription activation.

Results

PapI Increases the Affinity of Lrp for Pap Sites 2 and 5 via Conserved ACGATC Sequence

We initially examined the effects of PapI on binding of Lrp to nonmethylated pap regulatory DNA, since DNA methylation could inhibit these binding interactions and complicate interpretation of the data. Competitive gel shift analysis was carried out using each of the six *pap* Lrp binding sites (see Figure 1A) in the presence and absence of PapI (Figure 1B). The results showed that PapI increased the affinity of Lrp for *pap* sites 2 and 5, but had no effect on any of the other four Lrp binding sites. *Pap* Lrp binding sites 2 and 5 share the sequence ACGATC, which differs from the other four *pap* sites (Figure 1C) and the *ilvIH* Lrp binding site 2 (Kaltenbach et al., 1995), which do not display PapI-dependent Lrp binding. All Lrp binding sites share the sequence GxxxxTT with the Lrp binding consensus determined by SELEX (Cui et al., 1995) (Figure 1C).

PapI does not bind specifically to *pap* DNA by itself based on gel shift analysis (Kaltenbach et al., 1995) and DNA crosslinking (our unpublished data). Therefore, to identify the base pairs important for the observed PapI-mediated increase in Lrp affinity for *pap* Lrp binding sites 2 and 5 (Figure 1B), missing contact footprinting was performed in the presence and absence of PapI (see Figure 2A). The results indicated that the absence of certain bases in the top and bottom strands of *pap* site 5 (indicated by underline), including those overlapping the $GATC^{dist}$ site, disrupted PapI-dependent Lrp binding as evidenced by a lower bound/free ratio for PapI/Lrp (open box) compared with Lrp alone (filled box) (Figure 2A).

Since deletion of bases can indirectly affect protein-DNA interaction via structural effects (Papp and Chattoraj, 1994), we analyzed Lrp and PapI-Lrp binding to a series of *pap* site 5 DNAs (see Figure 2B), each containing a different methylated base, using an electrophoretic mobility shift assay (EMSA). Methylation of bases in the sequence 5′-GxCGAT-3′ overlapping $GATC^{dist}$ in the top strand and 3′-TGCTAG-5′ in the bottom strand significantly reduced PapI-dependent Lrp binding compared with binding of Lrp alone (Figure 2B). In contrast, methylation of the TTTA sequence identified by missing contact footprinting (Figure 2A) did not affect Lrp binding in the presence or absence of PapI (Figure 2B) nor did mutation of the TTTA sequence to CCCA (our unpublished data). These results indicate that the ACGATC sequence identified by both missing contact footprinting and methylation interference (Figure 2) is required for PapI-dependent binding of Lrp whereas the upstream TTTA sequence is not.

Methylation of the bottom strand cytosine complementary to the guanine of GATC (denoted $^{me}C9$ in Figure 2) blocked formation of the ternary PapI-Lrp-*pap* site 5 complex without affecting Lrp binding (Figure 3A, compare lanes 2 and 4 with lanes 6 and 8). These results support the hypothesis that enhancement of Lrp binding to site 5 occurs via formation of a PapI-dependent ternary complex with Lrp and *pap* DNA. Although PapI has no measurable specific binding to *pap* DNA in the absence of Lrp, it does bind specifically to Lrp in the ternary complex (Kaltenbach et al., 1995). Therefore, it is possible that binding of PapI to *pap* ACGATC sequences contributes binding energy which stabilizes the PapI-Lrp-*pap* sites 2 and 5 ternary complexes. Alternatively, binding of PapI to Lrp might alter Lrp conformation, enabling a cryptic Lrp domain to interact with *pap* DNA. We used photoaffinity crosslinking to determine if PapI is located near *pap* DNA in the ternary complex, placing a photoactivatible 9 Å azidophenacyl crosslinker 3 bases from the presumptive PapI binding sequence ACGATC (see Figure 2A, top DNA strand). The results using nonmethylated *pap* site 5 showed that both PapI and Lrp were crosslinked to *pap* DNA in the ternary complex (Figure 3B). Moreover, analysis using *pap* site 5 DNA methylated at C9 ($^{me}C9$, Figure 2) showed that the amount of azidophenacyl crosslinked PapI was significantly reduced with no effect on the level of crosslinked Lrp (Figure 3B). These results indicate that PapI is located near the *pap* ACGATC sequence in the PapI-Lrp-*pap* site 5 ternary complex, and may bind specifically to this sequence.

Methylation of $GATC^{prox}$ Is Required for Phase OFF to ON Switching via Inhibition of PapI-Dependent Binding of Lrp to Sites 1, 2, and 3 Proximal to the Pilin Promoter

PapI is required for activation of transcription and formation of the phase ON DNA methylation pattern (Braaten et al., 1994). The observation that PapI (100 nM) increases Lrp's affinity for *pap* site 2 (Figure 1B) presents an apparent paradox since this should block *pap* transcription due to its close proximity to the *papBA* pilin promoter (Weyand and Low, 2000). Further analysis showed that at low PapI levels significant enhancement of Lrp binding occurred at sites 4–6 ($CGATC^{dist}$) but not at sites 1–3 ($CGATC^{prox}$) (Figure 4A). At 5 nM PapI, the affinity of Lrp was 4-fold higher for *pap* sites 4–6 (K_d = 0.25 nM) compared to sites 1–3 (K_d = 1.0 nM). Conversely, in the absence of PapI, the affinity of Lrp for sites 1–3 (K_d = 1.2 nM) was about 2-fold higher than for sites 4–6 (K_d = 2.5 nM) (Figure 4B). Thus, binding of Lrp at sites 4, 5, and 6 should be favored at low PapI levels, resulting in activation of *papBA* transcription. This, in turn, would increase the PapI level via the PapB-mediated positive feedback loop (Figure 1A, panel III) (Forsman et al., 1989). High PapI levels could potentially shut off *pap* transcription by increasing the binding of PapI-Lrp complexes at promoter proximal sites 1–3 (Figure 4A, $CGATC^{prox}$). These results suggested the possibility that an additional factor(s) may be required to prevent PapI-mediated binding of Lrp to sites 1–3.

Since methylation of $GATC^{prox}$ is essential for transition to the phase ON transcription state (Braaten et al., 1994), we hypothesized that methylation of $GATC^{prox}$ might block PapI-dependent binding of Lrp at sites 1–3. Our results showed that methylation of $GATC^{prox}$ does block PapI-dependent Lrp binding to sites 1–3, but has no effect on binding of Lrp alone (Figure 5A). To determine if this disruption of PapI-dependent binding is essential for transition to the phase ON state, we mutated the wild-type *C*$GATC^{prox}$ sequence to *T*$GATC^{prox}$ to specifically inhibit PapI-dependent Lrp binding. We reasoned that

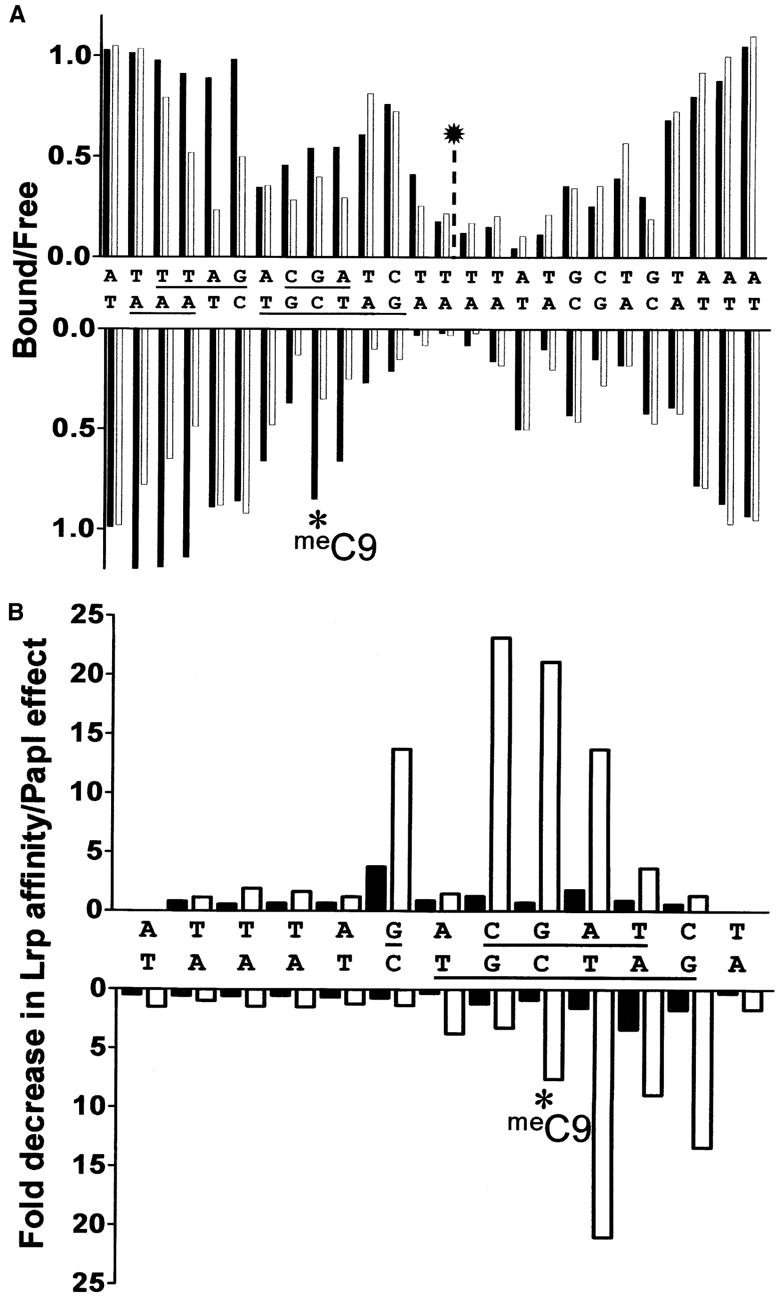


Figure 2. Identification of the PapI Response Element in *pap* Site 5

(A) Missing contact footprinting. Missing contact footprinting (Brunelle and Schleif, 1987) was carried out on *pap* site 5 as described in Experimental Procedures. The effects of deletion of bases on binding of Lrp (filled bar) and PapI-Lrp (open bar) is shown as "bound/free" on the *y* axis (a bound/free value of 1.0 indicates no effect of base deletion on binding). The symbol in the middle of the top DNA strand represents an azidophenacyl moiety inserted between thymidines and the methylated cytosine on the bottom strand (meC9) shows the position of the methylated cytosine used in Figure 3.

(B) Methylation scanning. 24 50-mer oligonucleotides (same sequence as in Figure 2A above) were constructed, each containing a single methylated base: 6-methyl-G, 5-methyl-C, 6-methyl-A, or 4-methyl-T. Oligonucleotides (50 pM) were incubated with Lrp in the presence and absence of PapI and analyzed by EMSA. Binding was compared to results obtained with unmethylated oligonucleotides. The *y* axis shows the fold decrease in both Lrp affinity (closed bar) and the "PapI effect" (open bars) upon methylation of each base. The "PapI effect" is the fold increase in Lrp affinity for *pap* site 5 DNA upon addition of PapI (100 nM).

under conditions in which PapI-dependent binding of Lrp to sites 1–3 was blocked, switching from OFF to ON should occur in the absence of Dam. Analysis of the TGATC^{prox} mutant showed that PapI-dependent Lrp binding to sites 1–3 was completely inhibited (Figure 4A) but binding of Lrp was unaffected in vitro based on EMSA (our unpublished data). The effects of the TGATC^{prox} mutation on binding of Lrp to sites 1–3 in vivo was determined by Southern blotting with a radiolabeled *pap* probe following digestion with MboI, which digests only nonmethylated GATC sites (Braaten et al., 1994). For this analysis, a *papI* null mutant *E. coli* isolate was used to measure Lrp binding in the absence of the PapI coregulator. It was found that 77% of *pap* DNAs from wild-type (CGATC^{prox}) *E. coli* and 60% of DNAs from the TGATC^{prox} mutant contained a nonmethylated GATC^{prox} site. Neither wild-type nor TGATC^{prox} mutant *pap* DNAs contained nonmethylated GATC^{dist} sites which would form as a result of Lrp binding to sites 4–6 under conditions in which binding of Lrp to sites 1–3 is inhibited (Nou et al., 1995) (data not shown). Together, these results strongly indicate that the TGATC^{prox} mutation specifically inhibits PapI-dependent binding of Lrp to sites 1–3.

Switch frequency analysis of *E. coli* containing the TGATC^{prox} mutation showed that the OFF to ON rate (5.6×10^{-4}/cell/generation) was about 7-fold higher than that of wild-type cells (8.2×10^{-5}/cell/generation) (Figure 4C). Notably, in a *dam* null mutant background, cells

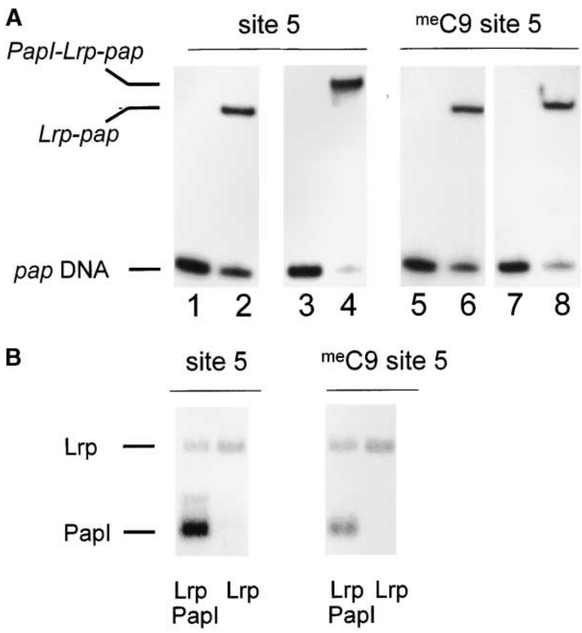


Figure 3. Formation of PapI-Lrp-*pap* Site 5 Ternary Complex

(A) Analysis of PapI-Lrp-*pap* site 5 ternary complexes by EMSA. ^{32}P-labeled *pap* site 5 DNA was incubated with Lrp in the presence and absence of PapI, then analyzed by EMSA. The left panel shows results obtained with nonmethylated DNA; the right panel shows results obtained with DNA methylated at C9 (see ^{me}C9 in Figure 2). All lanes contained *pap* site 5 DNA with the following additions: lanes 1 and 5, no addition; lanes 2 and 6, 2 nM Lrp; lanes 3 and 7, PapI (100 nM); lanes 4 and 8, PapI (100 nM) and Lrp (2 nM). The locations of Lrp-*pap* and PapI-Lrp-*pap* complexes are indicated.

(B) Photoaffinity crosslinking analysis of PapI-Lrp-*pap* ternary complexes. An azidophenacyl photoaffinity crosslinker was inserted between thymines (T14 and T15) as shown in Figure 2A using a phosphorothioate derivative. An adjacent ^{32}P was inserted by primer extension using radiolabeled dTTP as described (Bartlett et al., 2000). Lrp (100 nM) was incubated with 20,000 cpm azidophenacyl derivatized *pap* site 5 DNA in the presence and absence of PapI (200 nM). Samples were irradiated, digested with nucleases, and analyzed by SDS-PAGE as described (Kim et al., 1999). The migration positions of Lrp and PapI are shown at left. Nonmethylated *pap* site 5 was analyzed in the left panel and *pap* site 5 methylated at C9 (see ^{me}C9 in Figure 2) was analyzed in the right panel.

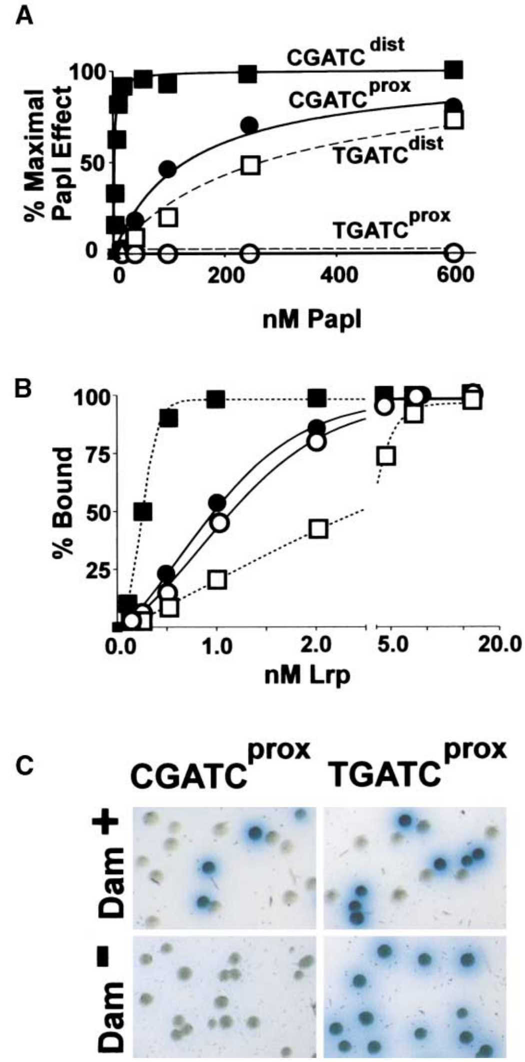


Figure 4. Pap Phase Variation Is Controlled by Differential Effects of DNA Methylation at GATC^{prox} and GATC^{dist} on Binding of Lrp and PapI-Lrp

(A) PapI response. The effects of different PapI levels on binding of Lrp to wild-type and TGATC mutant *pap* sites 1–3 and 4–6 was determined by EMSA analysis. The percent maximal PapI effect shown on the *y* axis was determined by the formula: (% bound at *x* nM PapI − % bound at 0 nM PapI)/(100 − % bound at 0 nM PapI), under conditions in which the Lrp concentration was sufficient to shift one-half of the *pap* DNA probe (Lrp = 2 nM for *pap* sites 4–6 and 1 nM for *pap* sites 1–3).

(B) Determination of Lrp affinity for *pap* sites 4–6 and 1–3 in the presence of limiting PapI. Lrp was incubated with ^{32}P-labeled *pap* sites 1–3 (circles) and 4–6 (squares) DNA probes (see Experimental Procedures) in the presence (filled symbols) and absence (open symbols) of 5 nM PapI. Lrp binding was measured by EMSA.

(C) Phase variation analysis of the TGATC^{prox} mutant. Dam^{+} *E. coli* (top panels) and Dam^{-} (*dam-16::Tn9*) (Parker and Marinus, 1988) *E. coli* (bottom panels) containing a chromosomal wild-type *papBA-lac* fusion (left panels) or TGATC^{prox} mutant *papBA-lac* fusion (right panels) were analyzed by plating on M9 minimal medium/glycerol with the Lac indicator X-Gal as described (Blyn et al., 1989).

were locked in the phase ON state, showing that methylation is not required for *pap* transcription under conditions in which PapI-dependent binding of Lrp to *pap* site 2 containing GATC^{prox} is blocked. These results strongly support the conclusion that methylation at GATC^{prox} is required for the phase OFF to ON transition by specifically inhibiting PapI-dependent Lrp binding to sites 1–3.

Binding of PapI-Lrp to Sites 4, 5, and 6 Is Required for Transition to the Phase ON State: Evidence for Intrinsic Switch Bias Based on Analysis of Hemimethylated DNA Intermediates

In contrast to the positive role of methylation of GATC^{prox} in stimulating OFF to ON switching (Figure 4), methylation of GATC^{dist} is required to maintain cells in the phase OFF state (Braaten et al., 1994). Quantitative analysis showed that methylation of GATC^{dist} reduces the affinity of Lrp for sites 4–6 by about 8-fold (Figure 5B). This is in contrast to methylation of GATC^{prox} which had no effect on Lrp binding to sites 1–3 (compare Figures 5A and 5B). These results support the hypothesis that methylation of GATC^{dist} helps stabilize the phase OFF state

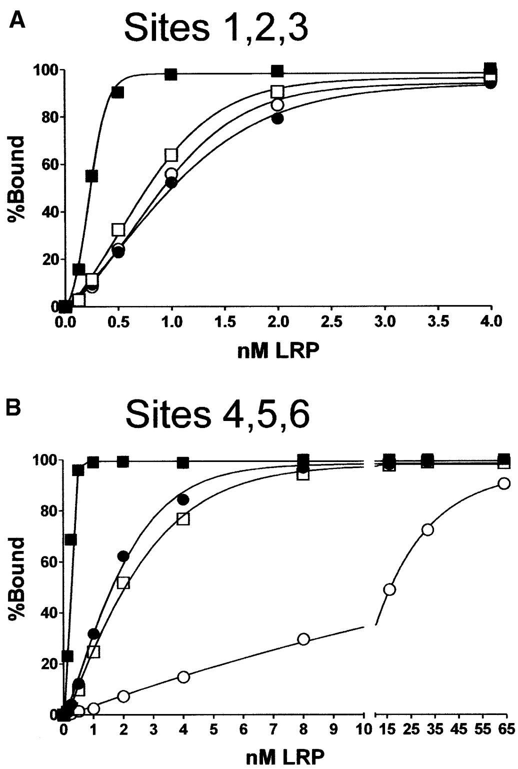


Figure 5. Effects of Fully Methylated *pap* DNA on PapI and Lrp Binding

(A) Effect of GATC^{prox} methylation on Lrp and PapI-Lrp binding to *pap* sites 1–3. Binding of Lrp to nonmethylated (squares) and fully methylated (circles) *pap* sites 1–3 was determined in the presence (solid symbol) and absence (open symbol) of PapI.

(B) Effect of GATC^{dist} methylation on Lrp and PapI-Lrp binding to *pap* sites 4–6. Same as (A) above but *pap* sites 4–6 were analyzed.

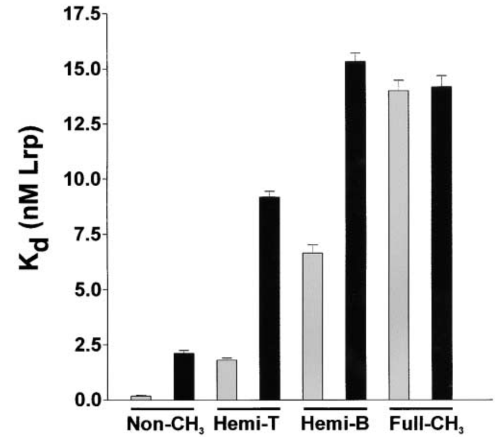


Figure 6. Differential Effects of Hemimethylation on PapI-Dependent Binding of Lrp to Sites 4, 5, and 6

Radiolabeled *pap* site 4–6 DNA containing a nonmethylated GATC^{dist} (Non-CH_3), fully methylated GATC^{dist} (Full-CH_3), and the two hemimethylated forms Hemi-T (methylated on top strand) and Hemi-B (methylated on bottom strand) were constructed and analyzed by EMSA as described in Experimental Procedures. Black bars show the affinity of Lrp alone and lightly shaded bars show the affinity of Lrp in the presence of 5 nM PapI.

by inhibiting binding of Lrp to sites 4–6 (Nou et al., 1993). However, methylation of GATC^{dist} also reduced PapI-dependent Lrp binding to sites 4–6 (Figure 5B), raising the question of how transition to the phase ON state can occur. We explored this question by first determining if PapI-dependent binding of Lrp to sites 4–6 is necessary to obtain phase ON cells using a TGATC^{dist} mutant. Similar to TGATC^{prox}, the TGATC^{dist} mutant showed greatly reduced (>150-fold) PapI-dependent enhancement of Lrp binding to sites 4–6, with less than a 2-fold reduction on Lrp binding (Figure 4A). *E. coli* containing the TGATC^{dist} mutation were phase-locked OFF (not shown), indicating that transition to the phase ON state requires PapI-dependent binding of Lrp at sites 4–6.

We hypothesized previously that transition to the phase ON state is blocked by the fully methylated GATC^{dist} site present in phase OFF cells (Braaten et al., 1994). This hypothesis was based in part on the observation that overexpression of Dam by just 4-fold prevents the OFF to ON switch and *E. coli* containing a GCTC^{dist} mutation that prevents methylation by Dam is locked ON (Braaten et al., 1994). Thus, it seems likely that the OFF to ON switch requires DNA replication to generate hemimethylated GATC^{dist} intermediates, which should bind to PapI-Lrp with a higher affinity than DNA with a fully methylated GATC^{dist}. This hypothesis was tested by constructing *pap* site 4–6 DNAs methylated at GATC^{dist} on the top (Hemi-T) or bottom (Hemi-B) strands (see Experimental Procedures) and measuring the affinity of Lrp/PapI by EMSA. The results showed that the affinity of PapI-Lrp for the hemimethylated *pap* DNAs was significantly higher (up to 8-fold) than for fully methylated DNA (Figure 6). Notably, PapI/Lrp could discriminate between the two hemimethylated *pap* DNA substrates. The affinity of PapI-Lrp for Hemi-T (K_d = 1.8 nM) was about 4-fold higher than for Hemi-B (K_d = 6.7 nM) (Figure 6). Similar differences in PapI-dependent Lrp binding were observed at a near saturating PapI level (100 nM) (data not shown). These results support the hypothesis that the switch to ON involves binding of PapI-Lrp to a hemimethylated intermediate present for a short time following DNA replication. Moreover, the data predict that the two daughter cells generated by DNA replication may have different switch potentials, which would constitute a simple differentiation mechanism (see Discussion).

Discussion

The results presented here show how PapI and Dam work together to stimulate switching from the phase OFF to phase ON *pap* transcription states. We find that PapI increases the affinity of Lrp for both *pap* pilin promoter proximal and distal sites (Figure 1B) via the ACGATC sequence present in *pap* Lrp binding sites 2 and 5 (Figure 1C). Switch directionality is effected by Dam methylation at GATC^{prox}, which inhibits PapI-dependent binding of Lrp to site 2, thus favoring binding of PapI-Lrp to sites 4–6 and formation of the phase ON state. Dam is not required for *pap* pilin transcription under conditions in which PapI-dependent binding of Lrp to site 2 is blocked by a TGATC^{prox} mutation. In a *dam*$^-$ host, wild-type CGATC^{prox} cells are locked OFF whereas mutant TGATC^{prox} cells are locked ON (Figure 4). This result strongly indicates that the reason Dam is required for

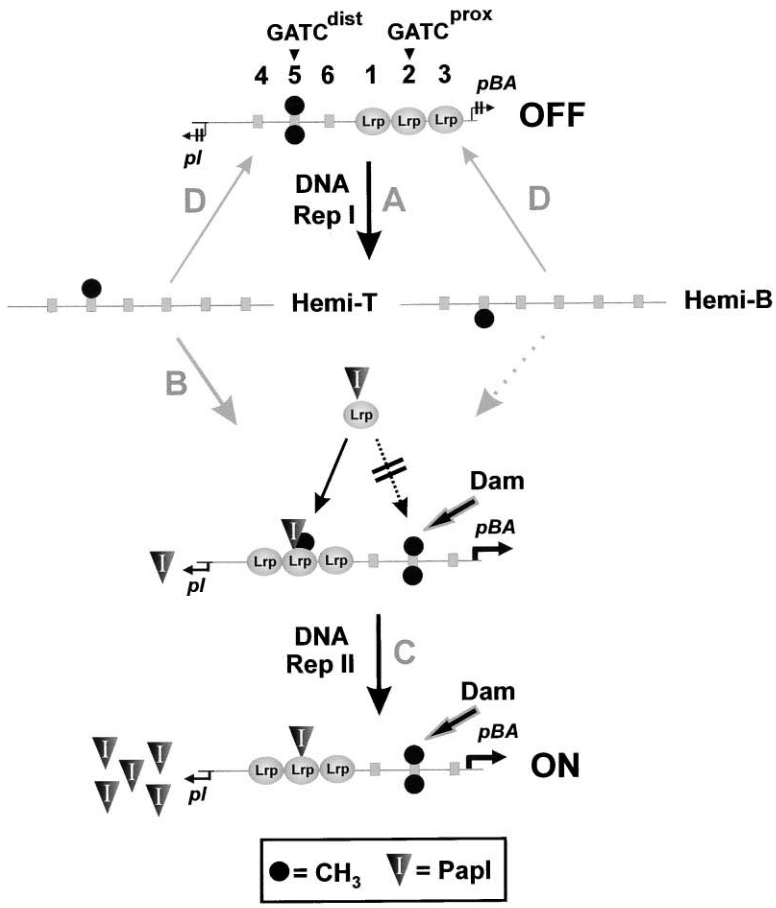


Figure 7. Proposed Mechanism for the Pap Phase OFF to ON Switch

(A) DNA replication dissociates Lrp from sites 1–3 and generates two hemimethylated GATC^dist DNAs: one methylated on the top strand (Hemi-T) and the other methylated on the bottom strand (Hemi-B).

(B) The switch from OFF to ON requires PapI and Dam. PapI facilitates binding of Lrp at hemimethylated site 5 and methylation at GATC^prox by Dam blocks PapI-dependent Lrp binding at site 2. The dotted arrow indicates that there is likely a lower probability for the Hemi-B intermediate to transition to the phase ON state compared to Hemi-T (see Discussion).

(C) Transition to the phase ON methylation pattern requires an additional round of DNA replication followed by complete methylation of GATC^prox. Rebinding of PapI-Lrp at sites 4–6 is facilitated by the PapB positive feedback loop (see Figure 1A, panel III), which increases the PapI level.

(D) Binding of Lrp to sites 1–3 and full methylation of hemimethylated GATC^dist regenerates the OFF state.

transition to the phase ON transcription state is to block PapI-dependent Lrp binding to site 2. The observation that the OFF to ON switch rate increased by 7-fold in Dam^+ cells containing the TGATC^prox mutation suggests that the Dam level normally present in cells is not as efficient at inhibiting PapI-dependent binding of Lrp at site 2 as the TGATC^prox mutation, which blocked PapI-dependent Lrp binding in vitro (Figure 4A). These results strongly support a model in which competition between PapI-Lrp and Dam at GATC^prox is an important factor in determining the OFF to ON switch rate (Figure 7B).

Based on our data, the decision to switch either ON or OFF is a two-step process involving an initial stochastic event in which binding of Lrp occurs to repressive sites 1–3 or activation sites 4–6. The probability of Lrp binding to these sites is dictated by the PapI level (Figure 4A). The second step is Dam methylation, which occurs at whichever GATC site is unoccupied following the initial stochastic binding step (Figure 5). Methylation locks a given cell into whatever initial binding state was present which prevents the alternate Lrp binding state. In phase OFF cells, Lrp should have a higher affinity for sites 1–3 than 4–6 based on in vitro analysis (Figure 4B). This difference in Lrp affinity is amplified by a phenomenon denoted as "mutual exclusion" in which binding of Lrp at sites 1–3 exerts a negative effect on Lrp binding at sites 4–6 by a mechanism that requires DNA supercoiling (Hernday et al., 2002). Stabilization of the OFF state is achieved by methylation of GATC^dist, which further decreases the affinity of Lrp for sites 4–6 (Figure 5B). In phase ON cells, PapI increases Lrp's affinity for sites 4–6 to a level higher than for sites 1–3 (Figure 4B). Mutual exclusion now works in the other direction to decrease Lrp binding at sites 1–3 (Hernday et al., 2002). Methylation at GATC^prox further stabilizes the phase ON state by preventing PapI-dependent Lrp binding to sites 1–3 (Figure 4A). These in vitro observations are supported by in vivo studies in which the levels of PapI and Dam were varied under specific conditions. For example, elimination of DNA methylation at GATC^dist by a GCTC^dist mutation destabilizes the phase OFF state by enabling Lrp binding to sites 4–6, causing a phase-locked ON phenotype (Braaten et al., 1994). Introduction of a plasmid overexpressing PapI into the *E. coli* GCTC^dist mutant caused a switching phenotype with both phase OFF and phase ON colonies present. Introduction of a second plasmid overexpressing Dam turned these cells back to the phase-locked ON phenotype. These results indicate that high levels of PapI enable PapI-Lrp to compete with Dam for binding at GATC^prox, switching some cells off. The balance can be shifted back to the phase-locked ON state by increasing the Dam level which outcompetes PapI-Lrp at GATC^prox. This conclusion is supported by the observation that in a GCTC^dist-TGATC^prox double mutant in which PapI-dependent Lrp binding to GATC^prox is blocked, cells remain phase-locked ON even when PapI is overexpressed (our unpublished data).

Transition from the OFF to ON state presents two major problems. First, methylation of GATC^prox is essential for transition to the ON state (Figure 4C), and yet in phase

OFF cells Lrp blocks methylation of GATC^{prox} (Weyand and Low, 2000). Second, methylation of GATC^{dist}, which occurs in phase OFF cells (Blyn et al., 1990), inhibits PapI-dependent binding of Lrp to sites 4–6 (Figure 5B), which is required for transition to the phase ON state (Figure 4A). A potential solution to both of these problems is provided by DNA replication which generates two different hemimethylated GATC^{dist} DNAs and displaces Lrp from sites 1–3 (Figure 7A). This would provide an opportunity for PapI-dependent binding of Lrp at hemimethylated site 5 as well as methylation of GATC^{prox} by Dam, both of which must occur to switch to the ON state (Figure 7B). To complete the transition to the phase ON DNA methylation pattern an additional round of DNA replication, rebinding of PapI-Lrp to a nonmethylated GATC^{dist} site and full methylation of GATC^{prox} is required (Figure 7C). If methylation of the hemimethylated GATC^{dist} strand and binding of Lrp or PapI/Lrp to sites 1–3 occurred first, however, this would block switching (Figure 7D).

Our analysis of binding of PapI/Lrp to hemimethylated *pap* site 4–6 DNAs showed that the affinity of PapI-Lrp for DNA methylated on the top strand (Hemi-T) was 4-fold higher than for DNA methylated on the bottom strand (Hemi-B) (Figure 6). This result suggests that the probability of switching ON is higher for daughter cells containing Hemi-T than Hemi-B, although it is not necessarily 4-fold higher. Since binding of PapI/Lrp to sites 4–6 is highly cooperative, there may not be a linear relationship between affinity of PapI/Lrp for sites 4–6 and switch rate. This speculation is supported by our previous observation that increasing the Dam level by just 4-fold locks cells in the phase OFF state (Braaten et al., 1994). To obtain a rough idea of the affinities of PapI/Lrp for sites 4–6 necessary for phase ON switching, we included a nonmethylated control DNA. It was shown previously that a GCTC^{dist} mutant (which cannot be methylated by Dam and does not significantly alter Lrp affinity) is phase-locked ON even in the absence of PapI (Braaten et al., 1994). Therefore, we approximate the K_d for Lrp binding which should be sufficient for switching at about 2 nM, the value obtained with nonmethylated DNA in the absence of PapI (Figure 6). Using this criterion it appears that Hemi-T daughter cells should be capable of switching ON while we predict that Hemi-B cells will switch ON at a lower rate if at all (see Figure 7B). We will test this hypothesis which, if true, might function to maximize phenotypic diversity and increase the fitness of bacterial populations in diverse environments. There is a precedent for differentiation of daughter cells with regards to probability of Tn*10* transposition, where it was shown that one hemimethylated intermediate was much more active than the other (Roberts et al., 1985).

Methylation of the GATC^{dist} and GATC^{prox} sites had converse effects on binding of Lrp and PapI-Lrp. Methylation of GATC^{dist} preferentially inhibits Lrp binding to sites 4–6, whereas methylation of GATC^{prox} only inhibits PapI-dependent Lrp binding at sites 1–3 (Figure 5A). We propose that these intrinsic differences in methylation responsiveness are important for the observed properties of the Pap phase switch. The PapI dependence of the switch is likely due to preferential binding of PapI-Lrp at hemimethylated GATC^{dist} compared with Lrp (Figure 6). The methylation dependence of the switch is due to the block in binding of PapI/Lrp by methylation of GATC^{prox} within the PapI response element (Figures 1C and 5A). Notably, methylation of GATC^{prox} did not affect binding of Lrp to sites 1–3. The predicted consequence of this is that in cells in which methylation of GATC^{prox} has occurred but PapI/Lrp binding at GATC^{dist} has not, Lrp should still bind to sites 1–3 to repress *pap* transcription and maintain a phase OFF transcription state. If, however, Lrp binding at sites 1–3 were blocked by methylation of GATC^{prox}, cells could be in a state in which both GATC sites are fully methylated and all Lrp binding sites are unoccupied. These cells would have a leaky switch phenotype due to endogenous transcription from the *papBA* promoter (van der Woude et al., 1995).

The "core" Lrp binding sites 2 and 5 confer PapI responsiveness to *pap* sites 1–3 and 4–6, respectively (Figure 1B), as discussed above. In addition, the Dam target site GATC present in sites 2 and 5 confers potential methylation sensitivity of PapI/Lrp binding. Analysis of binding of PapI-Lrp to hemimethylated *pap* site 5 DNAs showed that the affinity of PapI-Lrp for *pap* site 5 methylated on the top strand was 2.4-fold higher than for *pap* site 5 methylated on the bottom strand at saturating PapI (100 nM). Similarly, the affinity of PapI-Lrp for *pap* sites 4–6 methylated on the top strand was 2.7-fold higher than for DNA methylated on the bottom strand at 100 nM PapI (our unpublished data). Thus, the difference in affinities of PapI-Lrp for *pap* sites 4–6 Hemi-T and Hemi-B DNAs (see Figure 6) appears to be dictated by *pap* site 5 without significant influence from the flanking sites 4 and 6. Together, these results indicate that the main role of the flanking sites 4 and 6 is to increase the affinity of Lrp/PapI by enabling further cooperative binding of Lrp around the core site 5. The sequence of *pap* site 2 shares 12/18 identical base pairs with *pap* site 5 (Figure 1C), yet sites 1–3 display an altered methylation responsiveness compared to sites 4–6 (Figure 5). The mechanism by which this occurs is unknown. Although the affinity of Lrp for *pap* site 2 is too low to measure directly by EMSA, competition binding analysis indicated that methylation of site 2 blocked PapI-dependent Lrp binding without affecting binding of Lrp. Thus, the binding properties of sites 1–3 are dictated by the core site 2. Together, these results indicate that the PapI and methylation responses observed for sites 1–3 and 4–6 are primarily controlled by core sites 2 and 5, respectively.

The results presented here explain the conservation of DNA sequences around the *pap* GATC^{prox} and GATC^{dist} sites (previously denoted as GATC box sequence) with many different non-*pap* pili operons which each contain PapI homologs (van der Woude et al., 1996). All of these GATC box sequences CGATCTTTT contain the core PapI-response element identified here (CGATC), the conserved Lrp binding sequences TTTT which we have identified by DNA footprint analysis (Nou et al., 1995), and GATC sequence to allow DNA methylation by Dam. It is interesting to note that certain operons including *fae* encoding K88 fimbriae appear to be regulated in a reverse manner to *pap*: transcription is normally ON but is turned OFF when the PapI homolog FaeA is expressed (Huisman and de Graaf, 1995). Examination of *fae* regulatory DNA indicates that multiple consensus PapI response elements are present in the promoter proximal region, consistent with the observation that FaeA facilitates movement of Lrp to promoter proximal sites in the *fae* regulatory region, shutting off transcription (Huisman and de Graaf, 1995).

The Pap regulatory system is unique in its design for programmed switching between different DNA methylation patterns at a specific genomic locus. In eukaryotes, DNA methylation at CpG has been shown to globally silence gene expression, but switching between methylation patterns at specific genes has not been described (Ng et al., 2000). Moreover, it is unclear how DNA methylation patterns are generated in eukaryotes (Bird, 2002). Our work here shows that the local regulator PapI, along with Dam methylase, act to direct binding of the global regulator Lrp between two *pap* regulatory DNA sites. Binding of the PapI and Lrp proteins to DNA, in turn, dictate the *pap* DNA methylation pattern by specifically blocking methylation by Dam. This simple yet highly sophisticated epigenetic system provides a mechanism for transition between and maintenance of heritable phase ON and phase OFF transcription states.

Experimental Procedures

Competition Binding Analysis

The competition binding analysis shown in Figure 1B was carried out as follows. ^{32}P-end-labeled *ilvIH* Lrp binding site 2 (100 pM of the double-stranded 31-mer, top strand = CTAGATTGAATGTAG AATTTTATTCTGAATG) (Wang and Calvo, 1993b) was incubated in oligonucleotide binding buffer (OBB) (20 mM Tris-HCl, pH 8.0, 75 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 12.5% glycerol plus 0.1 mg/ml BSA) with Lrp (1 nM) in the presence and absence of PapI (100 nM). The indicated concentrations of each *pap* Lrp binding site (1–6) were added together with the ^{32}P-*ilvIH* site 2 DNA probe, prior to addition of Lrp/PapI. Following a 20 min incubation, protein-DNA complexes were separated using electrophoretic mobility shift analysis (EMSA) on 9% acrylamide gels in 0.5× TGE buffer (12.5 mM Tris, 95 mM Glycine, 5 mM EDTA, pH 7.3) containing 2.5% glycerol. Samples were loaded onto gels while running at 8V/cm at 23°C. The *pap* oligonucleotide sequences used were (top strand of each duplex indicated) Lrp Site 1 (5′ to 3′): CTTGCTATTAGTGTTTTGTTC TAGTTTAATT; Lrp site 2: TGATTTAAACGATCTTTTAACCCACAAAA CAA; Lrp site 3:AGTTAAATTTAG TTTTTTATGTTGTAAATAT; Lrp site 4: ATTTTTACGGACTTTCTTTTCGCAGAA AAAT; Lrp site 5: TCATT TAGACGATCTTTTATGCTGTAAATTCA; Lrp site 6: ATTCAATTTGC CATGATGTTTTTATCTGAGTA. Complementary bottom strand sequences were annealed by denaturation at 95°C and cooling to 23°C at 1°C/min.

Electrophoretic Mobility Shift Analyses

Analysis of Lrp/PapI binding to methylated and nonmethylated *pap* site 5 DNAs (Figure 2B) was carried out in 1× OBB containing 0.1 mg/ml BSA and 50 pM oligonucleotide. Analysis of Lrp/PapI binding to *pap* regulatory DNA "half-sites" 1–3 and 4–6 (Figures 3–6) was carried out in EMSA buffer (10 mM Tris, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 20 μg/ml BSA, 5 μg/ml poly dI/dC) containing 10 pM ^{32}P-labeled DNA. For *pap* sites 1–3 the primer pair 5′-TACTCTTCACGCAATAAGTTAAAT-3′ and 5′-TATCTGAGTACCCT CTTGCTATTA-3′ were used with a wild-type *pap* DNA template to generate a 123 base pair DNA fragment by PCR. For *pap* sites 4–6 the primer pair 5′-ACATTTTGCGTTTTATTTTTCTGC-3′ and 5′-TAA TAGCAAGAGGGTACTCAGATA-3′ were used to generate a 116 base pair DNA fragment by PCR (see above). Protein-DNA complexes were resolved on 6% acrylamide gels (29:1 acrylamide/Bisacrylamide ratio) containing 2.5% glycerol in 0.5× TGE buffer.

Missing Contact Footprint Analysis

Single-stranded pap site 5 oligonucleotide (40 pM) (5′-GCAGCAATC TCATTTAGACGATCTTTTATGCTGTAAATTCATAGACGCAT-3′) and its complementary sequence were end labeled with ^{32}P and used to construct duplex 50-mer labeled on the top or bottom strands by annealing with the unlabeled complementary sequence. Following limited depurination or depyrimidination as described (Brunelle and Schleif, 1987), DNAs (5 × 10^5 cpm/100 μl binding reaction) were incubated with PapI and Lrp under conditions in which 50% of DNA was in complex following separation on a 6% acrylamide gel in 0.5× TGE buffer. (Lrp alone, 11 nM; PapI + Lrp, 2 nM Lrp and 200 nM PapI). Bound and free *pap* DNA were extracted from the gel with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for 15 min at 65°C and recovered using Quiaquick (Quiagen, CA). DNAs were cleaved with piperidine and analyzed on 20% acrylamide gels containing 7 M urea in 1× TBE buffer (90 mM Tris-Borate, pH 8.85, 2 mM EDTA).

UV Crosslinking of DNA-Protein Complexes

A truncated *pap* site 5 oligonucleotide (10 pmol, 5′-GCAGCAATCT CATTTAGACGATCTTT-3′) containing phosphorothioate between the 3′ terminal thymidines was annealed to the complementary full-length *pap* site 5 oligonucleotide 5′-ATGCGTCTATGAATTTACAG CATAAAAGATCGTCTAAATGAGATTGCTGC-3′. Radiolabeling of the phosphate adjacent to the phosphorothioate of *pap* site 5 sequence was carried out using [α^{32}-P]dTTP using the 3′-5′exonuclease-negative Klenow fragment of DNA polymerase (New England BioLabs, MA) for 10 min at 37°C. Unlabeled dNTPs were then added for an additional 10 min incubation to complete the extension and generate a duplex oligonucleotide, which was precipitated and suspend in 55 μl 100 mM potassium phosphate buffer, pH 7.0. All subsequent steps were performed in the dark with a single 25 W red light bulb. Azidophenacyl bromide (55 μl of a 20 mM solution in methanol) was added and incubated 3 hr at 37°C for derivitization of phosphorothioate. Binding reactions contained 20,000 cpm derivitized DNA in 40 μl 1× OBB without DTT, Lrp (100 nM), and when indicated PapI (200 nM). Following a 20 min incubation at 23°C, 10 μl was analyzed by EMSA and the remainder was irradiated for 3 min using a 366 nm hand-held UV lamp (4 W) at a 1 cm distance. Nuclease digestion was performed as described (Kim et al., 1999) and samples were analyzed by SDS-PAGE. Bands corresponding to PapI and Lrp were identified by comparison with purified Lrp and PapI standards run on the same gel and stained with Coomassie blue R-250.

Pap Phase Variation Analysis

The analysis of *pap* gene expression shown in Figure 4C was carried out as follows. The $TGATC^{prox}$ and $TGATC^{dist}$ mutations were introduced into the *pap* operon in plasmid pDAL337 by in vitro mutagenesis using mutant oligonucleotide primers as described (Braaten et al., 1994). Mutant *pap* sequences were recombined into the chromosome of *E. coli* K-12 (isolate MC4100) by in vivo recombination into phage λRS45 and integration at *attB*, and single copy lysogens containing *pap-lac* were isolated (Simons et al., 1987). Mutant *pap* sequences were checked by DNA sequence analysis. The *dam-16*::Cam^R allele was introduced by phage P1 transduction as described to knock out Dam activity (Braaten et al., 1994). Pap phase variation was analyzed on M9 minimal medium containing glycerol as sole carbon source and the indicator X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and Pap switch rates were calculated as described (Blyn et al., 1989).

Construction of Hemimethylated and Fully Methylated DNA Probes

Two *pap* DNA 4–6 half-sites were constructed which were biotinylated on the top or bottom strands using oligonucleotides 5′-Biotin (C6 spacer)-ACATTTTGCGTTTTATTTTTCTGC-3′ and 5′-Biotin (C6 spacer)-TAATAGCAAGAGGGTACTCAGATA-3′, respectively, in a PCR reaction with the corresponding nonbiotinylated primer oligonucleotide and *pap* DNA template. One-half of each DNA preparation was fully methylated at $GATC^{dist}$ by incubation with purified Dam (van der Woude et al., 1998), 80 μM S-adenosyl methionine in 50 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 10 mM EDTA. Methylated and nonmethylated biotinylated DNAs were immobilized and washed on M-280 Dynabeads (Dynal Biotech, NY) according to the manufacturer's instructions. Bound DNA was denatured by addition of freshly made 0.1 N NaOH for 5 min at 23°C and the complementary nonbiotinylated DNA strand was collected. Eluted DNA solutions were neutralized as described (Slominska et al., 2003). The appropriate methylated and complementary nonmethylated DNA strands were annealed to construct hemimethylated DNAs, which were tested by digestion with MboI, which cuts fully nonmethylated but not hemimethylated GATC sites. Lrp and PapI-Lrp binding was analyzed using "half-site" EMSA conditions (see above).

Acknowledgments

We thank Stephanie Snyder and John Perona for a gift of highly purified PapI, M. Bartlett and E.P. Geiduschek for advice on photocrosslinking, and Brian Matsumoto and Olympus America, Inc. for colony imaging. We also thank Josep Casadesus and Peggy Cotter for helpful comments. We are very grateful to the National Institutes of Health (AI 23348 to D.L.) for continuing support of this project from its inception.

Received: June 16, 2003
Revised: August 15, 2003
Accepted: August 21, 2003
Published: October 23, 2003

References

Bartlett, M.S., Thomm, M., and Geiduschek, E.P. (2000). The orientation of DNA in an archaeal transcription initiation complex. Nat. Struct. Biol. *7*, 782–785.

Bickle, T.A., and Kruger, D.H. (1993). Biology of DNA restriction. Microbiol. Rev. *57*, 434–450.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev. *16*, 6–21.

Blyn, L.B., Braaten, B.A., White-Ziegler, C.A., Rolfson, D.H., and Low, D.A. (1989). Phase-variation of pyelonephritis-associated pili in *Escherichia coli*: evidence for transcriptional regulation. EMBO J. *8*, 613–620.

Blyn, L.B., Braaten, B.A., and Low, D.A. (1990). Regulation of *pap* pilin phase variation by a mechanism involving differential *dam* methylation states. EMBO J. *9*, 4045–4054.

Braaten, B.A., Blyn, L.B., Skinner, B.S., and Low, D.A. (1991). Evidence for a methylation-blocking factor (*mbf*) locus involved in *pap* pilus expression and phase variation in *Escherichia coli*. J. Bacteriol. *173*, 1789–1800.

Braaten, B.A., Nou, X., Kaltenbach, L.S., and Low, D.A. (1994). Methylation patterns in *pap* regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. Cell *76*, 577–588.

Brunelle, A., and Schleif, R.F. (1987). Missing contact probing of DNA-protein interactions. Proc. Natl. Acad. Sci. USA *84*, 6673–6676.

Casadesus, J., and D'Ari, R. (2002). Memory in bacteria and phage. Bioessays *24*, 512–518.

Cui, Y., Wang, Q., Stormo, G.D., and Calvo, J.M. (1995). A consensus sequence for binding of Lrp to DNA. J. Bacteriol. *177*, 4872–4880.

Forsman, K., Goransson, M., and Uhlin, B.E. (1989). Autoregulation and multiple DNA interactions by a transcriptional regulatory protein in *E. coli* pili biogenesis. EMBO J. *8*, 1271–1277.

Friedhoff, P., Thomas, E., and Pingoud, A. (2003). Tyr212: a key residue involved in strand discrimination by the DNA mismatch repair endonuclease MutH. J. Mol. Biol. *325*, 285–297.

Goransson, M., Forsman, P., Nilsson, P., and Uhlin, B.E. (1989). Upstream activating sequences that are shared by two divergently transcribed operons mediate cAMP-CRP regulation of pilus-adhesin in *Escherichia coli*. Mol. Microbiol. *3*, 1557–1565.

Heithoff, D.M., Sinsheimer, R.L., Low, D.A., and Mahan, M.J. (1999). An essential role for DNA adenine methylation in bacterial virulence. Science *284*, 967–970.

Hernday, A., Krabbe, M., Braaten, B., and Low, D. (2002). Self-perpetuating epigenetic pili switches in bacteria. Proc. Natl. Acad. Sci. USA *99*, 16470–16476.

Huisman, T.T., and de Graaf, F.K. (1995). Negative control of fae (K88) expression by the 'global' regulator Lrp is modulated by the "local" regulator FaeA and affected by DNA methylation. Mol. Microbiol. *16*, 943–953.

Kaack, M.B., Martin, L.N., Svenson, S.B., Baskin, G., Steele, R.H., and Roberts, J.A. (1993). Protective anti-idiotype antibodies in the primate model of pyelonephritis. Infect. Immun. *61*, 2289–2295.

Kaltenbach, L.S., Braaten, B.A., and Low, D.A. (1995). Specific binding of PapI to Lrp-pap DNA complexes. J. Bacteriol. *177*, 6449–6455.

Kim, T., Lagrange, T., Naryshkin, N., Reinberg, D., and Ebright, R. (1999). Site specific protein-DNA photocrosslinking. In Protein-Dna Interactions: A Practical Approach, A. Travers and M. Buckle, eds. (Oxford: IRL Press).

Low, D.A., Weyand, N.J., and Mahan, M.J. (2001). Roles of DNA adenine methylation in regulating bacterial gene expression and virulence. Infect. Immun. *69*, 7197–7204.

Lu, M., Campbell, J.L., Boye, E., and Kleckner, N. (1994). SeqA: a negative modulator of replication initiation in *E. coli*. Cell *77*, 413–426.

Lund, B., Lindberg, F., Marklund, B.I., and Normark, S. (1988). Tip proteins of pili associated with pyelonephritis: new candidates for vaccine development. Vaccine *6*, 110–112.

Modrich, P. (1989). Methyl-directed DNA mismatch correction. J. Biol. Chem. *264*, 6597–6600.

Ng, H.H., Jeppesen, P., and Bird, A. (2000). Active repression of methylated genes by the chromosomal protein MBD1. Mol. Cell. Biol. *20*, 1394–1406.

Nou, X., Skinner, B., Braaten, B., Blyn, L., Hirsch, D., and Low, D. (1993). Regulation of pyelonephritis-associated pili phase-variation in *Escherichia coli*: binding of the PapI and the Lrp regulatory proteins is controlled by DNA methylation. Mol. Microbiol. *7*, 545–553.

Nou, X., Braaten, B., Kaltenbach, L., and Low, D.A. (1995). Differential binding of Lrp to two sets of *pap* DNA binding sites mediated by Pap I regulates Pap phase variation in *Escherichia coli*. EMBO J. *14*, 5785–5797.

Papp, P.P., and Chattoraj, D.K. (1994). Missing-base and ethylation interference footprinting of P1 plasmid replication initiator. Nucleic Acids Res. *22*, 152–157.

Parker, B., and Marinus, M.G. (1988). A simple and rapid method to obtain substitution mutations in *Escherichia coli*: isolation of a *dam* deletion/insertion mutation. Gene *73*, 531–535.

Reisenauer, A., and Shapiro, L. (2002). DNA methylation affects the cell cycle transcription of the CtrA global regulator in *Caulobacter*. EMBO J. *21*, 4969–4977.

Roberts, D., Hoopes, B.C., McClure, W.R., and Kleckner, N. (1985). IS*10* transposition is regulated by DNA adenine methylation. Cell *43*, 117–130.

Simons, R.W., Houman, F., and Kleckner, N. (1987). Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene *53*, 85–96.

Slominska, M., Wahl, A., Wegrzyn, G., and Skarstad, K. (2003). Degradation of mutant initiator protein DnaA204 by proteases ClpP, ClpQ and Lon is prevented when DNA is SeqA-free. Biochem. J. *370*, 867–871.

Taghbalout, A., Landoulsi, A., Kern, R., Yamazoe, M., Hiraga, S., Holland, B., Kohiyama, M., and Malki, A. (2000). Competition between the replication initiator DnaA and the sequestration factor SeqA for binding to the hemimethylated chromosomal origin of *E. coli* in vitro. Genes Cells *5*, 873–884.

van der Woude, M.W., Kaltenbach, L.S., and Low, D.A. (1995). Leucine-responsive regulatory protein plays dual roles as both an activator and a repressor of the *Escherichia coli pap* fimbrial operon. Mol. Microbiol. *17*, 303–312.

van der Woude, M., Braaten, B., and Low, D. (1996). Epigenetic phase variation of the *pap* operon in *Escherichia coli*. Trends Microbiol. *4*, 5–9.

van der Woude, M., Hale, W.B., and Low, D.A. (1998). Formation of DNA methylation patterns: nonmethylated GATC sequences in *gut* and *pap* operons. J. Bacteriol. *180*, 5913–5920.

Wang, Q., and Calvo, J.M. (1993a). Lrp, a global regulatory protein of *Escherichia coli*, binds co-operatively to multiple sites and activates transcription of *ilvIH*. J. Mol. Biol. *229*, 306–318.

Wang, Q., and Calvo, J.M. (1993b). Lrp, a major regulatory protein in *Escherichia coli,* bends DNA and can organize the assembly of a higher-order nucleoprotein structure. EMBO J. *12*, 2495–2501.

Weyand, N.J., and Low, D.A. (2000). Regulation of Pap phase variation. Lrp is sufficient for the establishment of the phase off *pap* DNA methylation pattern and repression of *pap* transcription in vitro. J. Biol. Chem. *275*, 3192–3200.

Weyand, N.J., Braaten, B.A., van der Woude, M., Tucker, J., and Low, D.A. (2001). The essential role of the promoter proximal subunit of CAP in Pap phase variation: Lrp- and helical phase-dependent activation of *papBA* transcription by CAP from -215. Mol. Microbiol. *39*, 1504–1522.

Xia, Y., Forsman, K., Jass, J., and Uhlin, B.E. (1998). Oligomeric interaction of the PapB transcriptional regulator with the upstream activating region of pili adhesin gene promoters in *Escherichia coli*. Mol. Microbiol. *30*, 513–523.