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Specific Binding of PapI to Lrp-*pap* DNA Complexes

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Expression of pyelonephritis-associated pili (Pap) varies between transcriptionally active (ON) and inactive (OFF) phase states. Pap phase variation is controlled by the binding of leucine-responsive regulatory protein (Lrp) to two *pap* regulatory DNA regions, each containing a deoxyadenosine methylase site and designated GATC-I and GATC-II. Methylation of these GATC sites modulates binding of Lrp and plays an essential role in phase variation. PapI, an 8.8-kDa *pap*-encoded regulatory protein, plays a key role in the switch between OFF and ON transcription states. In the absence of PapI, Lrp binds to sites overlapping the *papBA* promoter and inhibits transcription. Addition of PapI results in a translocation of Lrp binding to sites over 100 bp upstream, resulting in the ON transcription state. Gel shift analysis using radiolabeled PapI shows that PapI binds with high specificity to Lrp-*pap* DNA complexes but binds only weakly to free Lrp. Protein cross-linking studies indicate that Lrp and PapI directly interact with each other. On the basis of these data, we present a hypothesis in which PapI facilitates the transition between OFF and ON transcription states by binding to Lrp and altering Lrp's affinity for the *pap* GATC-I and GATC-II regions.

Phase variation of pyelonephritis-associated pili (Pap) is controlled by the global regulatory leucine-responsive regulatory protein (Lrp), PapI, and deoxyadenosine methylase (Dam) (2, 4, 5, 16). These proteins act within the *papI-B* regulatory region, which includes two Dam target sites designated GATC-I and GATC-II (Fig. 1). Recent data indicate that in phase OFF cells, Lrp binds to sites 1, 2, and 3 near the *papBA* promoter and that this blocks methylation of the GATC-II site, which is located within Lrp binding site 2 (Fig. 1) (15). Binding of Lrp to sites 1, 2, and 3 appears to inhibit basal transcription, presumably via steric hindrance of RNA polymerase to the *papBA* promoter, since *pap* Lrp binding site 3 lies between the −35 and −10 consensus binding sites of the promoter (23).

The transition from phase OFF to ON requires PapI, a small (8.8-kDa) regulatory protein encoded by the *pap* operon (16). PapI is necessary, along with Lrp, for methylation protection of the GATC-I site (4). PapI reduces the affinity of Lrp for *pap* sites 1, 2, and 3 and increases the affinity of Lrp for sites 4 and 5, which are more than 100 bp upstream of GATC-II (15). This results in an alteration in the methylation pattern, since binding of Lrp at *pap* sites 4 and 5 protects the GATC-I site from methylation whereas the GATC-II site becomes methylated by Dam. Binding of Lrp, in the presence of PapI, at GATC-I appears to stimulate *pap* transcription about eightfold above the basal level (23). Analysis of Lrp activation mutants originally isolated by Platko and Calvo (17) indicates binding of Lrp to *pap* DNA is not sufficient for transcription activation. Instead, results indicate that Lrp, bound in the GATC-I region, might interact directly with the transcriptional apparatus to increase the affinity of RNA polymerase for the *papBAp* promoter and/or increase the rate of open complex formation (23).

In wild-type cells, PapI is required for *pap* transcription. However, certain *pap* mutants, such as those that carry mutations within Lrp binding site 3, display locked ON phenotypes which are independent of PapI yet still require Lrp (15). These results suggest that PapI may not directly contact the transcriptional apparatus but may act indirectly by modulation of Lrp binding specificity. Consistent with this hypothesis, PapI does not appear to bind specifically to *pap* DNA, in contrast to many transcriptional activators (16). Here we show that PapI binds specifically to the Lrp moiety of Lrp-*pap* DNA complexes, requiring both the GATC-I and GATC-II *pap* DNA regions for stable binding. We present a model for the phase OFF to ON transition in which PapI aids the release of Lrp bound to sites 1, 2, and 3 around GATC-II and enhances binding of Lrp to sites 4 and 5 near GATC-I, facilitating the translocation of Lrp to the GATC-I region.

MATERIALS AND METHODS

Cloning and expression of recombinant GST-PapI. The *papI* gene was amplified by PCR from plasmid pDAL262 (Table 1), using oligonucleotide primer 130 (5′-CGATGAGTGAATATATGAA-3′), which adds a *Bam*HI site in frame at the 5′ end of *papI*, and oligonucleotide primer 139 (5′-CACGAATTCTTATTA AGTTGTGGAAGA-3′), which adds an *Eco*RI site to the 3′ end. The resulting PCR-amplified fragment was ligated into *Bam*HI-*Eco*RI-digested vector pGEX-2TK, which contains coding sequences for glutathione *S*-transferase (GST), a heart muscle kinase recognition site, and a thrombin cleavage site (Pharmacia Biotech). To express recombinant GST-PapI protein, cultures (5 ml) were incubated at 28°C in Luria-Bertani broth to an optical density at 600 nm of 0.8. Isopropylthiogalactoside was added to a final concentration of 0.5 mM, and cultures were incubated 1 to 3 h at 28°C. Bacteria were harvested by centrifugation, washed once in phosphate-buffered saline, pH 7.4 (PBS), and resuspended in 3 ml of PBS containing proteinase inhibitors (pepstatin [1 μg/ml], chymostatin [1 μg/ml], antipain [1 μg/ml], leupeptin [1 μg/ml], and phenylmethylsulfonyl fluoride [10 μg/ml]) (PBS-PI). Bacterial cells were broken by sonication for 20 min in a cup horn sonicator (Heat System-Ultrasonics Inc.) at a setting of 6, using polycarbonate tubes. Sonic extracts were clarified by centrifugation for 20 min at 8,000 × *g* at 4°C. Supernatants were transferred to clean microcentrifuge (1.7 ml) tubes and frozen at −70°C in 10% glycerol–1% Triton X-100 until needed.

Purification of PapI. A 50% slurry of PBS-washed glutathione agarose beads (60 μl) was added to 1.7 ml of cleared sonic extract, prepared as described above, in a microcentrifuge tube and incubated at room temperature for 2 min. The beads, containing GST-PapI fusion protein, were washed four times in PBS-PI, and 5 μl of 10× heart muscle kinase buffer (200 mM Tris-HCl [pH 7.5], 10 mM dithiothreitol [DTT], 1 M NaCl, 120 mM $MgCl_2$), 5 μl of [γ-^{32}P]ATP (New England Nuclear), and 10 μl of heart muscle kinase (Sigma P2645; 6 U/μl in 40 mM DTT) were added to 30 μl of beads. After a 20-min incubation at room temperature, beads were washed four times with thrombin cleavage buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 2.5 mM $CaCl_2$), 14 U of thrombin was added, and incubation was carried out at room temperature for 1 to 2 h. Beads were centrifuged at 5,000 × *g* for 5 s, and the supernatant solution containing ^{32}P-PapI was collected and stored in PBS containing 10 mM DTT. This radiolabeled PapI contained a Gly-Ser-Arg-Arg-Ala-Ser-Val sequence on its amino terminus, which includes the phosphorylation site. PapI retained activity, as

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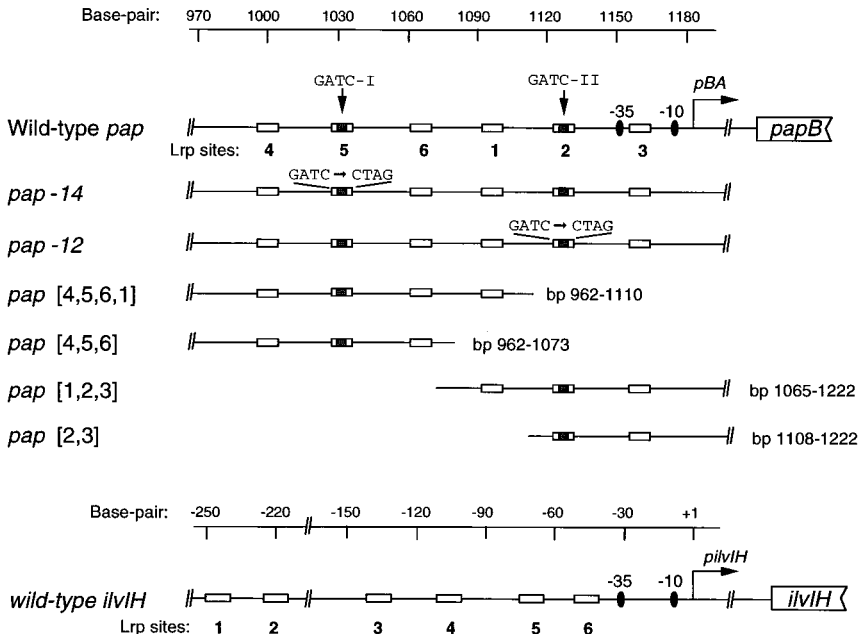

FIG. 1. The *pap* regulatory region located between the *papI* and *papB* genes. At the top, base pair locations are shown relative to the *Hin*dIII site of the *pap-17* sequence of *E. coli* C1212 (3). The six Lrp binding sites in *pap* are shown as open boxes. Lrp binding sites 5 and 2 contain the GATC-I and GATC-II sites, respectively, which are shown as shaded regions within the Lrp binding sites. Mutant *pap* derivatives *pap-14* and *pap-12*, shown below wild-type *pap*, contain six base pair substitution mutations (GCTAGC) spanning the GATC-I and GATC-II sequences, respectively (15). *pap* DNA sequences followed by brackets (e.g., *pap* [4, 5, 6, 1]) represent *pap* deletion derivatives containing the *pap* base pairs shown at the right of each construct. The *ilvIH* regulatory region, including Lrp binding sites, is from Wang and Calvo (24). Base pair locations are relative to the *ilvIH* transcript start site. The consensus RNA polymerase binding sites for *pap* (2) and *ilvIH* (24) are shown as filled ovals.

evidenced by the appearance of a DNase I footprint at the *pap* GATC-I region in the presence of Lrp (16). With increasing storage time, we observed the formation of PapI aggregates with concomitant loss of PapI activity. Unlabeled, phosphorylated PapI was prepared as described for labeled PapI except that 1 μM ATP was used in the labeling reaction. Although absolute PapI concentrations were not measured, relative protein concentrations were determined after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by silver staining (Sigma).

Gel retardation and quantitative DNase I footprint analysis. Gel retardation analyses were carried out as described previously, using high-ionic-strength gels (16).

The DNase I footprint analyses shown in Fig. 5B and C were carried out as follows. End-labeled ^{32}P-*pap* DNA (125,000 cpm, 0.3 nM) was incubated in a total volume of 20 μl with 3 μg of herring sperm DNA and 2 μg of acetylated bovine serum albumin (New England Biolabs) in binding buffer (60 mM Tris-HCl [pH 7.5], 40 mM KCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT). After incubation for 15 min at room temperature, 2 μl of DNase I (2 μg/ml) containing 22 mM $CaCl_2$ and 22 mM $MgCl_2$ was added for 2.5 min. The reaction was stopped by addition of 120 μl of stop/precipitation buffer (4% glycerol, 5 mM EDTA, 0.6 M ammonium acetate, 80% ethanol, 2 mg of yeast tRNA per ml) to each sample. Samples were incubated for 20 min at −65°C and centrifuged at 17,000 × *g* to obtain a pellet. Pellets were washed once on 70% ethanol, vacuum dried, and resuspended in 4 μl of formamide loading buffer (95% formamide, 10 mM EDTA [pH 8.0], 0.1% [wt/vol] bromophenol blue, 0.1% [wt/vol] xylene cyanol). After heating at 90°C for 3 min, samples were analyzed by PAGE as described previously (16). Quantitation of radioactivity was carried out with a Bio-Rad model GS-250 imager system.

Preparation of DNA probes. DNA probes were constructed by PCR amplification and were purified by using a Qiaquick spin column (Qiagen). Full-length wild-type *pap* (259 bp), *pap-14*, and *pap-12* DNAs were amplified by using oligonucleotides 14 (5′-TTGATGTGTATCACATTTTGCG-3′) and 74 (5′-GCCCCTGGATATATGCTTCC-3′). Plasmids pDAL337, pDAL337-14, and pDAL337-12 were used as templates for wild-type *pap*, *pap-14*, and *pap-12*, respectively (Table 1). *pap*-Lrp binding site deletion probes were constructed by using pDAL337 as the template. Oligonucleotide primers 14 and 118 (5′-TTAAACTAGAACAAAACAC-3′) were used to construct *pap* [4, 5, 6, 1]. Oligonucleotide primers 14 and 102 (5′-AGATAAAAACATCATGGCAAA-3′) were used to construct *pap* [4, 5, 6]. *pap* [1, 2, 3] was constructed by using primers 74

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
MC4100	F$^-$ *araD139* Δ(*lacIPOZYA-argF*)*U169 rpsL thi-1*	8
DL812	MC4100 *fanABC′lacZYA*	6
DL1530	MC4100 λ366 lysogen (*daa*$^+$)	21
UT5600	Δ*lacZ lacI*^{q} *ompT* Δ*leu trpE thi-1* Δ*fepA-ompT*	20
DL2138	UT5600(pDAL414)	This study
DL2285	UT5600(pDAL435)	This study
Plasmids		
pTZ18R	*amp* pMB1 replicon	U.S. Biochemical
pTZ19U	*amp* pMB1 replicon	U.S. Biochemical
pGEX2TK	GST fusion *amp* ColE1 replicon	Pharmacia Biotech
pDAL262	pTZ18R containing *papI*	16
pDAL337	pTZ19U containing a 1.76-kb *papIB* regulatory sequence	5
pDAL435	pGEX2TK *papI-gst* (contains protein kinase site)	This study
pCV112	pUC13 containing *ilvIH* regulatory region	19

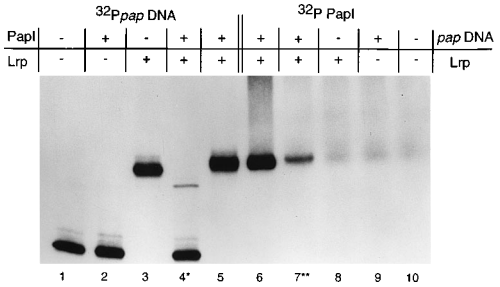

FIG. 2. PapI binds specifically to Lrp-*pap* DNA complexes. ^{32}P-*pap* DNA (0.3 nM; lanes 1 to 5) and ^{32}P-PapI (300,000 cpm, absolute concentration not determined; lanes 6 to 10) were incubated with combinations of unlabeled phosphorylated PapI (same amount as ^{32}P-PapI), Lrp (270 nM), and *pap* DNA (0.3 nM) as indicated at the left and right. Protein-DNA complexes were separated by native gel electrophoresis and visualized by autoradiography (see Materials and Methods). A 50-fold molar excess of unlabeled *pap* DNA competitor was added to the sample shown in lane 4 and a 10-fold molar excess of unlabeled phosphorylated PapI protein was added to the sample shown in lane 7 to show binding specificity to *pap* DNA and PapI, respectively (indicated by asterisks). ^{32}P-PapI does not migrate into the gel under the conditions used here (lane 10) unless it is in complex with Lrp-*pap* DNA complexes (lane 6). The 259-bp *pap* DNA probe used here contained bp 962 to 1221.

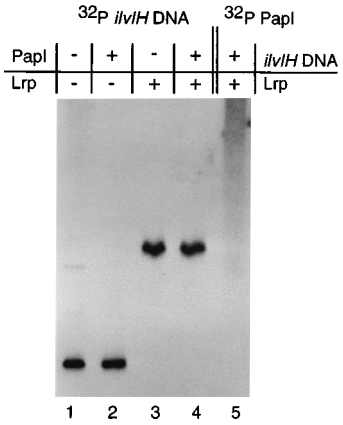

FIG. 3. PapI does not bind to Lrp-*ilvIH* DNA complexes. ^{32}P-*ilvIH* DNA (0.27 nM; lanes 1 to 4) and ^{32}P-PapI (300,000 cpm; lane 5) were added with the combinations of unlabeled phosphorylated PapI (same level as ^{32}P-PapI), Lrp (270 nM), and *ilvIH* DNA (0.27 nM) shown at the top. The 284-bp *ilvIH* DNA probe used here contained bp −311 to −27 (24). Analysis was carried out as described for Fig. 2.

and 97 (5′-TTTATCTGAGTACCCTCTTG-3′), whereas *pap* [2, 3] was constructed by using primers 74 and 94 (5′-AATGTTCGAATTTTGTTTTGT GGG-3′). *daa* DNA was amplified from *Escherichia coli* DL1530 chromosomal DNA by using primers 159 (5′-CAATAACAGTCACTCATTCC-3′) and 160 (5′-CGTCAACGGCACAAATAC-3′), which amplifies a 199-bp DNA fragment containing bp 787 to 985 of the *daa* regulatory region (1). *ilvIH* regulatory DNA containing all six Lrp binding sites was PCR amplified from plasmid pCV112 by using primers 99 (5′-ATTAGTCTAGATTTGCAAACGC-3′) and 100 (5′-GCT TAAGGAATTCGCAGCCAGA-3′) (24). *fan* regulatory DNA was amplified from *E. coli* DL812 by using primers 121 (5′-TGTGGGATTAACTGCGCGT -3′) and 122 (5′-CATAAACTGCCAGGAATTG-3′) followed by cleavage with *Bam*HI and *Acc*I to remove vector ends. Radiolabeling of PCR products was carried out by ^{32}P labeling one primer with T4 polynucleotide kinase (New England Biolabs) prior to PCR amplification. Radiolabeled DNAs were quantitated by phosphoimager analysis (Bio-Rad).

Chemical cross-linking. Chemical cross-linking was carried out as described previously (13), with the following modifications. ^{32}P-PapI (100,000 cpm) was incubated with Lrp (2.7 μM) in the presence and absence of *pap* or *ilvIH* DNAs (30 nM) for 10 min in cross-linking buffer (80 mM triethanolamine-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.1 mM DTT) in a total volume of 20 μl. Dimethylsuberimidate (DMSI; 10 mg/ml in cross-linking buffer, freshly made) was added, and samples were incubated for 50 min at room temperature. SDS-PAGE loading buffer (0.0625 M Tris-HCl [pH 6.8], 1% SDS, 15% glycerol, 15 mM DTT) was added, and samples were heated to 90°C for 5 min. After separation on a 20% acrylamide gel (Hoefer), radioactive protein complexes were detected by autoradiography.

RESULTS

Binding of radiolabeled PapI to Lrp-*pap* DNA complexes. To radiolabel PapI, we introduced a five-amino-acid protein kinase recognition site at its N terminus, using a GST fusion vector (11). Affinity-purified recombinant GST-PapI protein was radiolabeled with [γ-^{32}P]ATP and heart muscle protein kinase and cleaved with thrombin to release ^{32}P-PapI (see Materials and Methods). Addition of the ^{32}P-PapI protein to Lrp and *pap* DNA resulted in the formation of a complex on a native polyacrylamide gel (Fig. 2, lane 6). This complex was retarded slightly more than Lrp-DNA complexes (lane 3) but had the same mobility as the complex between unlabeled phosphorylated PapI, Lrp, and radiolabeled *pap* DNA (lane 5). In the absence of either Lrp or *pap* DNA, complex formation with ^{32}P-PapI was not observed (lanes 8 to 10). Binding of radiolabeled PapI to Lrp-*pap* DNA complexes is specific, as evidenced by competition with unlabeled PapI (lane 7). As shown previously, binding of Lrp-PapI to ^{32}P-labeled *pap* DNA is specific, as evidenced by competition with unlabeled *pap* DNA (lane 4). In addition, PapI alone does not bind specifically to *pap* DNA, as evidenced by the absence of a shift in migration of ^{32}P-labeled *pap* DNA after PapI addition (lane 2). Results similar to those shown in Fig. 2 were obtained by using gel filtration chromatography to separate free and bound ^{32}P-PapI. Under these conditions, PapI formed a complex with Lrp-*pap* DNA but did not bind to free Lrp (12). Together, these data show that PapI binds specifically to Lrp-*pap* DNA complexes.

To further explore PapI binding specificity, we determined if PapI could bind to complexes of Lrp with the *ilvIH* regulatory region, which lacks GATC box motifs (18). Lrp binds to six *ilvIH* DNA binding sites with affinities higher than or equal to that of *pap* (24) (Fig. 1). Lrp formed a complex with *ilvIH* DNA, as evidenced by retarded migration of the radiolabeled DNA probe (Fig. 3, lane 3). Addition of PapI did not alter the mobility of the Lrp-*ilvIH* DNA complex (lane 4). When this experiment was repeated with a wide Lrp concentration range (1.35 to 675 nM), the same results were obtained (data not shown). Moreover, complex formation with ^{32}P-PapI was not detected (lane 5), indicating that PapI does not bind to Lrp-*ilvIH* DNA complexes.

To determine if PapI binds directly to Lrp, we cross-linked complexes with DMSI, which links ε-NH_2-lysine residues in proteins (13). Incubation of ^{32}P-PapI with Lrp and *pap* DNA resulted in complexes of 28 and 50 kDa after DMSI addition (Fig. 4, lane 6). Larger complexes were also observed. PapI-Lrp complex formation was much weaker in the absence of *pap* DNA (lane 4) or in the presence of *ilvIH* DNA (data not shown), indicating that PapI binds strongly to Lrp in complex with *pap* DNA but interacts weakly with Lrp in solution. Radioactive material migrating more slowly than PapI (lanes 1, 3, and 5) is probably denatured, aggregated PapI which is shifted to very high molecular weight complexes that do not enter the gel after DMSI addition (Materials and Methods).

Both the *pap* GATC-I and GATC-II DNA regions are required for Lrp-PapI complex formation. To further define the regions of *pap* regulatory DNA required for enhancement of PapI-Lrp binding, the abilities of various mutant *pap* derivatives to facilitate the formation of stable Lrp-PapI complexes were determined. None of the four *pap* DNA fragments containing only a single GATC site enhanced Lrp-PapI complex formation (Fig. 1; Table 2). Although the affinity of Lrp for

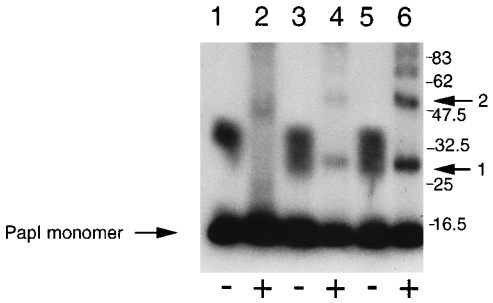

FIG. 4. Chemical cross-linking of PapI and Lrp. Lanes 1 and 2 contain ^{32}P-PapI (100,000 cpm), lanes 3 and 4 contain ^{32}P-PapI and Lrp (2.7 μM), and lanes 5 and 6 contain ^{32}P-PapI, Lrp, and *pap* regulatory DNA (30 nM). Lanes 2, 4, and 6 have the chemical cross-linker DMSI added, as indicated by +; lanes 1, 3, and 5 do not have DMSI added, indicated by −. Samples were analyzed by SDS-PAGE as described in Materials and Methods. Sizes are indicated in kilodaltons.

each of these DNA fragments was less than that for wild-type *pap*, the amount of Lrp used in this experiment (270 nM) was sufficient for occupancy of all Lrp binding sites (15). These results indicate that stable complex formation between Lrp and PapI requires both of the Lrp binding regions surrounding the GATC-I and GATC-II sites. The GATC sequences themselves appear to be important for stable PapI-Lrp-*pap* DNA complex formation, since substitution mutations of the *pap* GATC sequences (mutants *pap-12* and *pap-14*; Fig. 1) reduced Lrp-PapI complex formation by two- to fourfold (Table 2). Together, these results show that stable complex formation between PapI and Lrp requires both of the GATC box regions of *pap*. Neither *ilvIH* nor *fan* regulatory DNA, which both bind Lrp but lack GATC box sites (6), was able to enhance formation of PapI-Lrp complexes. In contrast, *daa* DNA, which contains GATC box regions similar to *pap* and codes for DaaF, a PapI homolog (1, 22), enhanced binding of PapI to Lrp (Table 2).

To determine the relationship between the binding of PapI to Lrp-*pap* DNA complexes (Fig. 2) and the PapI-mediated translocation of Lrp binding from GATC-II to GATC-I, we measured the Lrp dose response for each event. ^{32}P-PapI was added to *pap* DNA along with increasing levels of Lrp, and the amounts of ^{32}P-PapI in complex were determined (Fig. 5A). At the same time, DNase I footprint analysis was carried out to measure the binding of Lrp to the GATC-I and GATC-II regions, using the same level of unlabeled phosphorylated PapI (Fig. 5B and C). PapI reduced the affinity of Lrp for the GATC-II region and increased the affinity of Lrp for the GATC-I region, as described previously (15). In the presence of PapI, Lrp bound with highest affinity to the GATC-I region, reaching saturation at about 150 nM Lrp (Fig. 5B). At this Lrp concentration, stable Lrp-PapI complexes were not detected (Fig. 5A). Instead, the formation of Lrp-PapI complexes more closely paralleled the binding of Lrp, in the presence of PapI, to GATC-II (Fig. 5C). Under these conditions, both the GATC-I and GATC-II sites were bound by Lrp (compare Fig. 5A, B, and C), supporting the hypothesis that stable complex formation between PapI and Lrp requires binding of Lrp to both *pap* GATC-I and GATC-II regions.

DISCUSSION

The results presented here show that PapI binds specifically to Lrp-*pap* DNA complexes (Fig. 2). PapI did not bind to Lrp-*ilvIH* or Lrp-*fan* DNA, which both lack DNA sequences similar to the *pap* GATC boxes. In support of these in vitro data, transcription of the *ilvIH* operon was not affected by PapI in vivo (12). Moreover, *pap* DNA fragments containing only the GATC-I or GATC-II site did not facilitate the formation of Lrp-PapI complexes (Fig. 1; Table 2). In contrast, the *daa* regulatory region, which contains *pap*-like GATC boxes with spacing identical to that of *pap* (102 bp) (22), did support formation of Lrp-PapI complexes (Table 2). Thus, binding of Lrp to *pap*-like operons which contain GATC box motifs is qualitatively different from binding of Lrp to other members of the Lrp regulon. One possible reason for this difference in Lrp-PapI binding is that the spacing between Lrp binding sites of *ilvIH* differs from that of *pap* (Fig. 1) (24), which could result in differential phasing of the PapI binding site of Lrp. Alternatively, the PapI binding site of Lrp may be exposed upon binding of Lrp to *pap* DNA but not to *ilvIH* DNA, since the Lrp binding consensus for *ilvIH*, AGAATTTTATTCT (24), differs from that of *pap*, Gnn(n)TTT (15). In any case, the result of this specificity in Lrp-PapI-*pap* DNA complex formation is that PapI acts on only a subset of the Lrp regulon, even though its activity requires interaction with the global regulator Lrp.

Although PapI binds specifically to Lrp-*pap* DNA, our data show that the translocation of Lrp from the GATC-II region to the GATC-I region, mediated by PapI, occurred at Lrp concentrations significantly below those required for the formation of stable Lrp-PapI-*pap* DNA complexes (Fig. 5). Instead, Lrp-PapI-*pap* DNA complex formation occurred at higher Lrp levels sufficient for binding of both *pap* GATC regions by Lrp

TABLE 2. Lrp-PapI complex formation with different Lrp-DNA complexes

DNA type	Description	% ^{32}P-PapI bound to Lrp[a]
Wild-type *pap* regulatory region	GATC-I and GATC-II present	100
pap, Lrp sites 4, 5, 6, 1	GATC-I present[b]	3
pap, Lrp sites 4, 5, 6	GATC-I present	0
pap, Lrp sites 1, 2, 3	GATC-II present	1
pap, Lrp sites 2, 3	GATC-II present	0
pap-14	GATC-I substitution mutation	24
pap-12	GATC-II substitution mutation	41
daa regulatory region	Contains *pap* GATC box motifs[c]	110
ilvIH regulatory region	Binds Lrp, no homology to *pap*	2
fan regulatory region	Binds Lrp, no homology to *pap*	3

[a] Gel shift analysis was performed as described in Materials and Methods with ^{32}P-PapI protein and quantitated by phosphoimager scanning. Values are relative to that for wild-type *pap*, which was set to 100%. Concentrations of reactants were as follows: *pap* DNAs (both wild-type and mutant derivatives), 0.3 nM; *ilvIH* DNA, 0.27 nM; *fan* and *daa* DNAs, 3 nM. ^{32}P-PapI was used at the same level as in Fig. 2 and 3.

[b] The four *pap* DNA fragments analyzed here containing various combinations of Lrp binding sites are shown in Fig. 1.

[c] The *daa*, *ilvIH*, and *fan* regulatory DNAs are described in Materials and Methods.

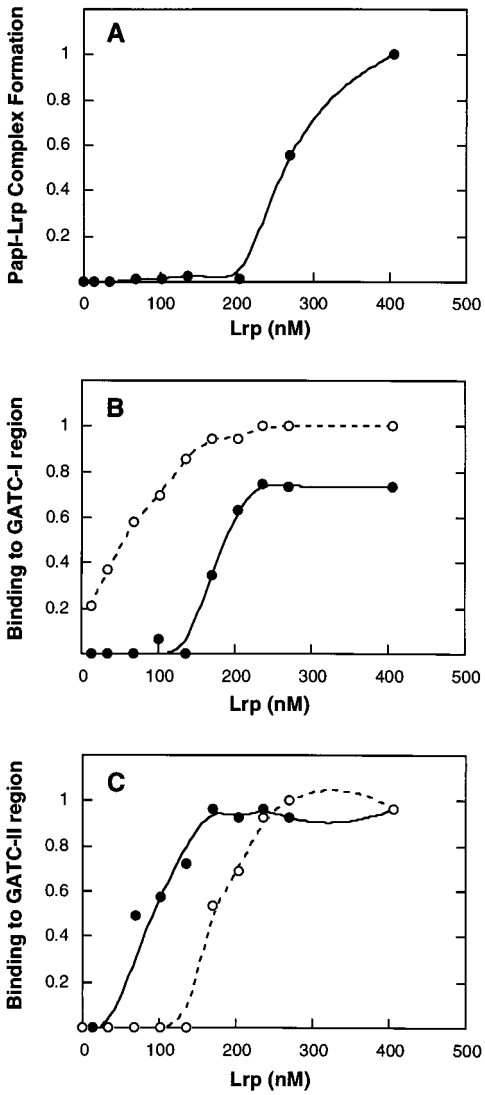

FIG. 5. Dose-response analysis of Lrp-PapI complex formation and PapI-dependent translocation of Lrp binding. (A) Amount of Lrp-PapI complexes formed as a function of Lrp concentration (see Materials and Methods). Values are relative to the maximal amount of complex formation, which was set at 1.0. (B) Binding of Lrp in the presence (open circles) and absence (closed circles) of PapI to the *pap* GATC-I region (bp 1028 to 1031). (C) Binding of Lrp in the presence (open circles) and absence (closed circles) of PapI to the *pap* GATC-II region (bp 1160 to 1163). Lrp binding in panels B and C was measured by DNase I footprint analysis (Materials and Methods) and is expressed relative to a maximal level, set at 1.0. The levels of ^{32}P-labeled and unlabeled PapI as well as binding conditions were identical in panels A, B, and C.

(Fig. 5B and C). These results suggest that stable formation of Lrp-PapI complexes may require binding of Lrp to both *pap* GATC regions. Our data support this hypothesis, since Lrp-PapI complexes were not formed with *pap* DNA lacking either the GATC-I (*pap*[1, 2, 3]) or GATC-II (*pap*[4, 5, 6, 1]) site, and mutation of the *pap* GATC sites significantly reduced Lrp-PapI-*pap* DNA complex formation (Fig. 1; Table 2).

We have incorporated our data into the following model for the translocation of Lrp between the *pap* GATC regions (Fig. 6). In the OFF phase, Lrp binds cooperatively at the GATC-II region to Lrp binding sites 1, 2, and 3, resulting in protection of GATC-II from Dam methylase and leaving GATC-I free to be methylated (Fig. 6B) (15). Under growth conditions which induce PapI expression via the cyclic AMP (cAMP)-cAMP receptor protein complex (10), PapI binds to Lrp-*pap* DNA complexes (Fig. 6C). Our data indicate that PapI decreases the affinity of Lrp for the GATC-II region and increases Lrp affinity for the GATC-I region following DNA replication (Fig. 5; see below).

Recent data show that PapI is required for enhancement of binding of Lrp to site 4 in the GATC-I region (Fig. 1), even under conditions in which Lrp binding to the GATC-II region is disrupted by mutation (15). These results suggest that the PapI-mediated increase in Lrp affinity for the GATC-I region (Fig. 5B) is not simply the result of disruption of Lrp binding to the GATC-II region. Instead, PapI may alter Lrp conformation, increasing its affinity for sites 4 and 5 around GATC-I. This could occur if PapI binds to Lrp-*pap* DNA, altering Lrp conformation to increase its affinity for the GATC-I region (Fig. 6C). This hypothesis is supported by recent results showing that the PapI-dependent translocation of Lrp from sites 1, 2, and 3 to sites 4 and 5 is blocked in the *pap-14* mutant (Fig. 1) lacking the GATC-I sequence (15). These results indicate that the GATC-I sequence itself may be recognized by Lrp-PapI complexes.

Methylation of GATC-II, which is required for the phase OFF to ON transition (5), could function to aid the PapI-mediated dissociation of Lrp from sites 1, 2, and 3 by inhibiting rebinding of Lrp and Lrp-PapI to the GATC-II region (Fig. 6D). This would result in a shift in Lrp binding equilibrium from the GATC-II region to the GATC-I region. In vitro analysis indicates that binding of Lrp-PapI to *pap* containing a fully methylated GATC-I site is blocked, whereas low-affinity binding of Lrp-PapI occurs to DNA containing a hemimethylated GATC-I site (16). Therefore, we hypothesize that translocation of Lrp from the GATC-II to the GATC-I region is restricted to a short time time period following DNA replication in which the GATC-I site is hemimethylated. It seems likely that following translocation of Lrp-PapI to the GATC-I region, PapI dissociates from Lrp-*pap* DNA (Fig. 5D), since stable Lrp-PapI complexes were observed only when both *pap* GATC sites were bound by Lrp-PapI (Fig. 5; Table 2). After an additional round of DNA replication, the phase ON state is generated (Fig. 6E).

On the basis of the model shown in Fig. 6, the binding of PapI to Lrp-*pap* DNA complexes that we observed (Fig. 2 and 4) may result from the stabilization of a binding transition state (Fig. 6C). At the high Lrp concentrations required for stable PapI-Lrp-*pap* DNA binding, both *pap* GATC sites were occupied by Lrp (Fig. 5B and C). The DNA used for this analysis was fully nonmethylated, in contrast to *pap* DNA from phase OFF cells, which contain a methylated GATC-I site, and phase ON cells, which contain a methylated GATC-II site (2). Thus, our in vitro assay conditions may have fostered binding of Lrp at both *pap* GATC regions, since binding of Lrp-PapI to the GATC-I and GATC-II regions would not be blocked by Dam methylation. However, it is not yet clear how binding of Lrp at both *pap* GATC regions stabilizes complex formation with PapI.

Previous results by Wang and Calvo indicated that Lrp binds to *ilvIH* DNA as a dimer (24). However, we do not know if Lrp binds to *pap* as a dimer or how many Lrp molecules are present in the phase OFF and ON DNA complexes. Moreover, the stoichiometry of PapI-Lrp binding is not known. Lrp-PapI cross-linking data (Fig. 4) indicate that the 28-kDa complex is the appropriate size to contain a single molecule of PapI (8 kDa) and Lrp (19 kDa). The 50-kDa complex could contain two molecules of Lrp and one or two PapI molecules, consistent with the possibility that Lrp binds to *pap* as a dimer.

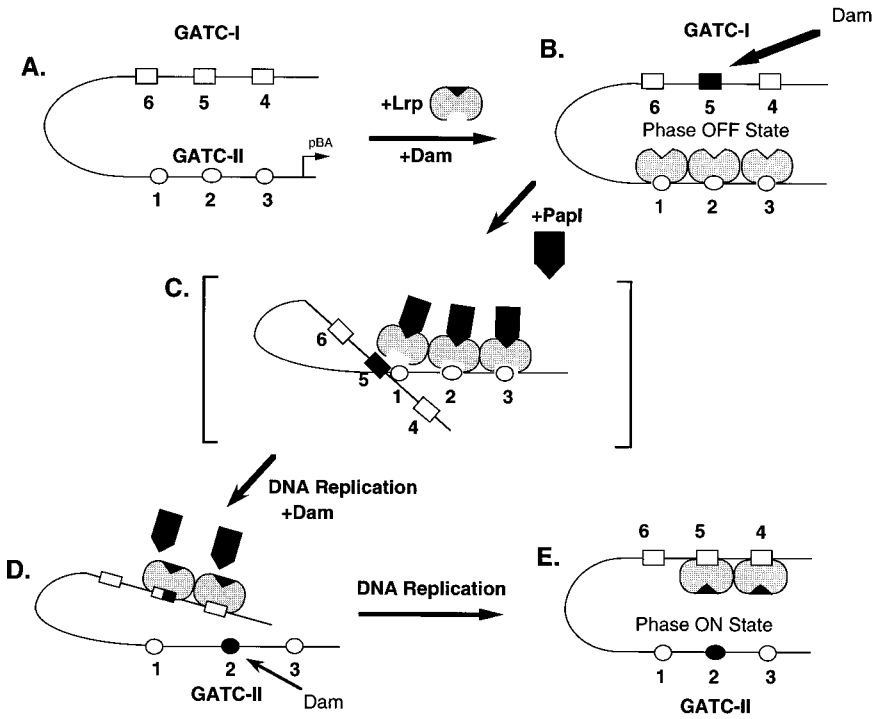

FIG. 6. Model for the PapI-dependent translocation of Lrp binding in Pap phase variation (see text for discussion). (A) The *pap* regulatory region. Lrp binding sites 2 and 5, containing the *pap* GATC-II and GATC-I sites, respectively, are shown. (B) Phase OFF *pap* DNA. The GATC-I site is fully methylated, as shown by a shaded box. (C) Formation of *pap* DNA-Lrp-PapI complexes. PapI induces an Lrp conformation change facilitating translocation of Lrp to sites 4 and 5. Brackets around panel C indicate that these steps involve an unstable transition intermediate. (D) Methylation of the GATC-II site inhibits binding of Lrp-PapI to sites 1, 2, and 3, aiding the PapI-dependent translocation of Lrp from the GATC-II to the GATC-I region. The hemimethylated GATC-I site is shown as a half-shaded box. (E) The phase ON *pap* DNA state is generated after an additional round of DNA replication.

The data shown in Fig. 5B and C indicate that binding of Lrp and Lrp-PapI to *pap* DNA sequences is cooperative, since the transition between no binding and full binding occurred over a twofold concentration range. Plotting of these data by using the Hill equation yielded high Hill coefficients (values of between 4 and 6), suggestive of cooperativity (9, 12). In support of this hypothesis, mutation of *pap* site 2 reduces the binding affinity of Lrp for both sites 2 and 3 and mutation of *pap* site 5 reduces the affinity of Lrp-PapI for both sites 5 and 4 (15). Binding of Lrp to the *ilvIH* DNA regulatory region was previously shown to be highly cooperative (24).

PapI has homologs among other fimbrial operon expression products, including SfaC, DaaF, FaeA, and PrfI in *E. coli* and PefI in *Salmonella typhimurium* (34 to 94% amino acid identity with PapI). PapI has been shown to complement SfaC$^-$ and DaaF$^-$ phenotypes (21), and PefI complements a PapI$^-$ phenotype (14). Therefore, it seems likely that these regulatory proteins contain conserved Lrp binding regions which enable them to bind to DNA-Lrp complexes. However, it is not clear if the amino acid differences between these PapI homologs (22) reflect functional differences, such as altered interactions with Lrp.

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