Mutational Analysis of the PapB Transcriptional Regulator in Escherichia coli

REGIONS IMPORTANT FOR DNA BINDING AND OLIGOMERIZATION

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PapB is a transcriptional regulator in the control of pap operon expression in Escherichia coli. There are PapB homologous proteins encoded by many fimbrial gene systems that are involved in the regulation of fimbriae-adhesin production, and previous studies suggested that PapB binds DNA through minor groove contact. Both deletion and alanine-scanning mutagenesis were used to identify functionally important regions of the PapB protein. Mutations altering Arg$^41$ or Cys$^48$ caused deficiency in DNA binding, indicating that these residues are critical for PapB binding to DNA. Alanine substitutions at positions 35–36, 53–56, and 74–76 resulted in mutants that were impaired in oligomerization. All these amino acid residues are conserved among the PapB homologous proteins, suggesting their importance in the whole family of regulatory proteins. The transcriptional efficiency of all the mutants was clearly reduced as compared with that of wild-type PapB. Taken together, we have localized regions in the PapB protein that are involved in DNA binding and oligomerization, and our results show that both functions are required for its activity as a transcriptional regulator.

The PapB protein is a transcriptional regulator of the pap promoter; at certain levels it stimulates pap operon expression, but at higher levels it represses (1). There are several PapB homologous proteins that are involved in the regulation of the production of different fimbrial adhesins. SfaB is a positive regulator for the production of S fimbriae (2). The products of the fanA and fanB genes both show resemblance to PapB and are suggested to act as transcriptional antiterminators involved in the control of K99 fimbriae production (3). Biosynthesis of the Escherichia coli CS31A surface antigen is negatively controlled by the PapB homologous proteins encoded by many fimbrial operons. The evidence suggests that it binds to DNA through an oligomeric fashion (7). This novel DNA-binding mode could be important for PapB homologous proteins to function as transcriptional regulators of different fimbrial operons.

In the present study, both truncated derivatives and variants altered by alanine-scanning mutagenesis were used to identify residues important for DNA binding and oligomerization of PapB protein. The efficiency of such mutants in transcriptional activation of the pap operon was also tested.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—E. coli strains used in this study were JM109 (8), 71-18 lacI$^q$ (8), MC1029 (9), and JH607 (10). Cells were grown in Luria broth (LB) (11) at 37 °C. Antibiotics used in selective media were carbenicillin (Cb) (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (Cm) (10 μg/ml).

Construction of Plasmids—The plasmids used in this study are listed in Table I. The plasmid pQE30, obtained from Qiagen Inc., provides high level expression of proteins containing a 6×His affinity tag in E. coli. The DNA fragments containing wild-type (wt) and mutant papB genes obtained by PCR amplification with primers b1 (5′-GCCGCAA-TTCGGATCCATGGCGCATCATGAAGTCAT-3′) and b3 (5′-GCCCT-GCACAGAAGCTTTATATTAGCTCAATCGCGACAG-3′) were digested by BamHi-HindIII, and cloned into pQE30 to produce plasmids pYN1, pYN8, pYN9, pYN10, pYN11, pYN12, pYN13, pYN28, pYN29, pYN30, pYN31, pYN32, pYN33, pYN34, pYN42, pYN47, pYN71, pYN72, pYN74, pYN75, and pYN76. Similarly, primers b1 and b3 (5′-GCCGCGACGAGACCCATATTGGCGCAGACCTCCT-3′) were used to obtain truncated papB gene (1–80 aa) and to produce pYN2; primers b1 and b4 (5′-GCCCTGCAAGAGCGTCTTTATTACCTCTGTGTTTCTCG-CA-3′) were used to obtain truncated papB gene (1–70 aa) and to produce pYN3; primers b1 and b6 (5′-GCCGCTGAGAGCGTTTTATTACTGCACATCTCTCC-3′) were used to obtain truncated papB gene (1–66 aa) and to produce pYN4; primers b1 and b5 (5′-GCCGCTGCGA-GACCTTTATATAGCTGAGAGCGTCTCAGATAA-3′) were used to obtain truncated papB gene (1–60 aa) and to produce pYN5; primers b7 (5′-GCCCGAATTCGGATCCATGGCGCATCATGAAGTCAT-3′) were used to obtain truncated papB gene (31–104 aa) and to produce pYN14. Plasmids pMMB66EH (12), pAF01 (13), pHMG15 (6), and pBF21 and pBF22 (14) have been described before. Plasmids pYN21, pYN22, pYN23, pYN24, pYN25, pYN35, pYN36, pYN37, pYN38, pYN39, pYN40, pYN41, pYN43, pYN48, pYN44, pYN45, and pYN46 were obtained by digesting the corresponding PCR fragments containing wt, mutant, or truncated papB genes with EcoRI-HindIII and cloning them into the vector pMMB66EH. The resulting plasmids were then cut with DraI, and the Smal-HincII fragment from plasmid pAF01 containing the cat gene was inserted, which gave rise to Cm resistance. The DNA fragments containing wt or mutant papB genes obtained by PCR amplification with primers b8 (5′-GCCGAACTTTATAGCTCAGCGACGAGAGCGTCT-3′) and b9 (5′-GCCGAGATATTTATATTACCTCTGTGTTTCTCG-CA-3′) were digested with EcoRV-HindIII and then cloned into plasmid pBP21. These gave rise to plasmids pYN51, pYN52,
DNA Techniques—Plasmid isolation, gel electrophoresis, transformation, amplification of DNA by PCR, and DNA labeling were performed by standard procedures (15). Restriction endonuclease digestions and DNA ligation reactions were performed under conditions recommended by the manufacturers (Roche Molecular Biochemicals, New England Biolabs Inc.).

Expression and Purification of Wild Type and Mutant PapB Proteins with the 6×His Affinity Tag—The plasmids containing wt and mutant papB genes were based on pQE30 and were introduced into JM109, and protein expression was induced by addition of IPTG (final concentration 1 mM) at the logarithmic phase. The His-tagged wt and mutant PapB proteins were purified as described previously (7).

Anti-PapB Antisera and Immunoblotting—Polyclonal antisera with antibodies recognizing PapB were produced by immunization of rabbits with purified PapB protein. The PapB antibody was purified as described by Taraseviciene et al. (16). For detection of PapB by immunoblotting, we used a procedure essentially as described before (17). ECL Western blot was performed according to the manufacturer’s protocol.

Analysis of Protein-DNA Interactions— Gel mobility shift assays to detect protein-DNA interaction were performed as described previously (1, 18). DNA fragments containing the PapB binding site 1 of the pap operon (1, 17) were obtained by end-labeling purified PCR products with [32P]ATP and T4 polynucleotide kinase. Oligonucleotides 740 (5′-CTTTAAACGATCTTTTAACCCACAAAAC-3′) and 1140 (5′-CAAGCTTAATCCGTTACCGCCAGCGCCT-3′) were used to produce a shortened cf, missing 96 aa residues (Leu57–Ala152) fused to wt papB gene.

DNA binding ability

- 60
- 66
- 70
- 80

| wt-PapB | + | 1 |
|---------|---|---|
|         | (+) | 1 |

RESULTS

Regions Involved in DNA Binding—The amino acid sequence of the PapB protein does not contain any obvious recognition motifs that are common in other DNA-binding proteins. The computer prediction of the secondary structure of PapB suggested a hydrophilic region (aa 61–70) (Fig. 1A), which, in a comparison with PapB-like proteins from other fimbrial gene systems (Fig. 2), seemed to contain several conserved residues and could be important for DNA binding. To test this prediction, different PCR fragments containing wt and truncated papB gene(s) were cloned into the QIAexpress vector PQE30 (Table I). The wt and truncated PapB proteins with a 6×His affinity tag attached to the N-terminus were purified through a Ni-NTA column (Fig. 3A), and were analyzed for specific binding to sites in the J96 pap-pili regulatory region (1). As shown in Fig. 4, addition of 6.4 μM (80 ng/ml) of the wt His-PapB could bind and shift all the labeled DNA fragments (indicated by “+”) in Fig. 1B. The gel mobility shift assay showed that the truncated His-PapB (1–80 aa) could bind DNA in a similar way, although a bit weaker (indicated by “+”) (Figs. 1B and 4A). In the case of some of the other truncated mutants: His-PapB (1–70 aa), His-PapB (1–66 aa), and His-PapB (31–104 aa). The amino acid sequence of the PapB protein does not contain any obvious recognition motifs that are common in other DNA-binding proteins. The computer prediction of the secondary structure of PapB suggested a hydrophilic region (aa 61–70) (Fig. 1A), which, in a comparison with PapB-like proteins from other fimbrial gene systems (Fig. 2), seemed to contain several conserved residues and could be important for DNA binding. To test this prediction, different PCR fragments containing wt and truncated papB gene(s) were cloned into the QIAexpress vector PQE30 (Table I). The wt and truncated PapB proteins with a 6×His affinity tag attached to the N-terminus were purified through a Ni-NTA column (Fig. 3A), and were analyzed for specific binding to sites in the J96 pap-pili regulatory region (1). As shown in Fig. 4, addition of 6.4 μM (80 ng/ml) of the wt His-PapB could bind and shift all the labeled DNA fragments (indicated by “+”) in Fig. 1B. The gel mobility shift assay showed that the truncated His-PapB (1–80 aa) could bind DNA in a similar way, although a bit weaker (indicated by “+”) (Figs. 1B and 4A). In the case of some of the other truncated mutants: His-PapB (1–70 aa), His-PapB (1–66 aa), and His-PapB (31–104 aa). The amino acid sequence of the PapB protein does not contain any obvious recognition motifs that are common in other DNA-binding proteins. The computer prediction of the secondary structure of PapB suggested a hydrophilic region (aa 61–70) (Fig. 1A), which, in a comparison with PapB-like proteins from other fimbrial gene systems (Fig. 2), seemed to contain several conserved residues and could be important for DNA binding. To test this prediction, different PCR fragments containing wt and truncated papB gene(s) were cloned into the QIAexpress vector PQE30 (Table I). The wt and truncated PapB proteins with a 6×His affinity tag attached to the N-terminus were purified through a Ni-NTA column (Fig. 3A), and were analyzed for specific binding to sites in the J96 pap-pili regulatory region (1). As shown in Fig. 4, addition of 6.4 μM (80 ng/ml) of the wt His-PapB could bind and shift all the labeled DNA fragments (indicated by “+”) in Fig. 1B. The gel mobility shift assay showed that the truncated His-PapB (1–80 aa) could bind DNA in a similar way, although a bit weaker (indicated by “+”) (Figs. 1B and 4A). In the case of some of the other truncated mutants: His-PapB (1–70 aa), His-PapB (1–66 aa), and His-PapB (31–104 aa).
The conserved aa residues are boxed. Residues that were mutated to alanine(s) are bold, and substitutions most clearly affecting DNA binding (black circle) or oligomerization (striped oval) are indicated.

**Fig. 2.** Protein sequence alignments of PapB homologous proteins. The labeled DNA fragments were slightly shifted from the position of protein-free DNA, but no distinct shifted band was observed (indicated by "(+)" in Fig. 1B). The DNA binding ability of the truncated PapB mutant composed of only the N-terminal 60 aa was almost completely abolished (indicated by "−"− in Fig. 1B). Taken together, the results suggest that the conserved hydrophilic region containing amino acids 61–70 could be important for DNA binding (Figs. 1B and 4A).

Arg<sup>61</sup> and Cys<sup>65</sup> Are Critical for PapB Binding to DNA—Alignments of the amino acid sequence of proteins in the PapB family showed some regions of strong conservation (Fig. 2). In order to further identify the critical residues, alanine-scanning mutagenesis was applied to these regions. Alanine was chosen as the replacement residue, since it is tolerated in both hydrophobic and hydrophilic environments. Importantly, this change should function to remove a small region of charge or a large hydrophobic surface.

To investigate the active form of PapB as a transcriptional regulator, a test to determine the actual capability of the PapB molecules to oligomerize in vivo was devised. Based on plasmid pBF21, which carries the bacteriophage λ cI repressor gene under the control of a tandemly repeated lacUV5 promoter, and pBF22 in which the DNA encoding the oligomerization domain of the repressor (C-terminal domain) was deleted (14), we constructed plasmid pYN51 encoding the chimeric protein consisting of the N-terminal DNA binding domain of the λ cI repressor and the wt-PapB molecule (Table I). The test relies on the capability of an oligomerization-proficient protein domain to functionally replace the natural C-terminal domain of phage λ cI repressor conferring biological activity to the N-terminal DNA binding domain of the same repressor (21) (Fig. 5A). The oligomerization proficiency of a protein can be quantified by determining the number of lytic plaques formed following infection (with phage λ of E. coli cells harboring the pBF expression vectors and producing, upon induction with IPTG, the various types of chimeric λ cI repressor molecules (14). In cells expressing the entire wt-cI repressor or the chimeric protein consisting of the N-terminal DNA binding domain of λ cI repressor and wt-PapB, the number of plaques formed was much lower (superinfection immunity) as compared with those expressing only the N-terminal domain of the repressor (superinfection sensitivity) (Fig. 5 and Table II). The constructed plasmids were also introduced by transformation of strain JH607 (= λ 1200<sub>P</sub>), which was originally described by Beckett et al. (10) as a reporter for co-operative binding by repressor to adjacent operators. The weak operator site O<s><sub>2</sub></s> overlaps the promoter P<sub>λ</sub>, while the strong operator site O<sub>1</sub> is positioned upstream of O<sub>2</sub> and does not affect transcription by P<sub>λ</sub>. Thus, co-operative binding to both operator sites increases the efficiency of repression for the downstream reporter genes <i>cet</i> and <i>lacZ</i>. Different repressor activities can be distinguished by chloramphenicol sensitivity and β-galactosidase activity. In cells expressing the entire wt-cI repressor, both of the reporters were significantly repressed as compared with those expressing only the N-terminal domain of the repressor. wt-PapB, on the other hand, could replace, to a large extent, the function of the N-terminal DNA binding domain of the same repressor (21) (Fig. 5A).
| Plasmids         | Relevant characteristics                          | References |
|-----------------|--------------------------------------------------|------------|
| pMMG1           | pap (1–70 aa) A lacZ, Cb'                        | 19         |
| pMMG15          | pap (1–70 aa) A lacZ, Cb'                        | 6          |
| pQE30           | Expression vector, Cb'                           | Qiagen manufacturer's instruction |
| pYN1            | wt papB gene cloned into pQE30, Cb'             | This study |
| pYN2            | Truncated papB gene (1–80 aa) cloned into pQE30, Cb' | This study |
| pYN3            | Truncated papB gene (1–70 aa) cloned into pQE30, Cb' | This study |
| pYN4            | Truncated papB gene (1–60 aa) cloned into pQE30, Cb' | This study |
| pYN5            | Truncated papB gene (1–60 aa) cloned into pQE30, Cb' | This study |
| pYN6            |Mutant papB gene (R61A) cloned into pQE30, Cb'    | This study |
| pYN7            | Mutant papB gene (R62A) cloned into pQE30, Cb'    | This study |
| pYN8            | Mutant papB gene (E63A) cloned into pQE30, Cb'    | This study |
| pYN10           | Mutant papB gene (E66A) cloned into pQE30, Cb'    | This study |
| pYN11           | Mutant papB gene (E68A) cloned into pQE30, Cb'    | This study |
| pYN12           | Mutant papB gene (E69A) cloned into pQE30, Cb'    | This study |
| pYN13           | Mutant papB gene (E70A) cloned into pQE30, Cb'    | This study |
| pYN28           | Mutant papB gene (F33A,F34A) cloned into pQE30, Cb' | This study |
| pYN29           | Mutant papB gene (L35A,L36A) cloned into pQE30, Cb' | This study |
| pYN30           | Mutant papB gene (I39A,S40A) cloned into pQE30, Cb' | This study |
| pYN31           | Mutant papB gene (S41A,I42A) cloned into pQE30, Cb' | This study |
| pYN32           | Mutant papB gene (H43A,S44A) cloned into pQE30, Cb' | This study |
| pYN33           | Mutant papB gene (D53A,Y54A) cloned into pQE30, Cb' | This study |
| pYN34           | Mutant papB gene (L55A,V56A) cloned into pQE30, Cb' | This study |
| pYN42           | Mutant papB gene (V47A,I48A) cloned into pQE30, Cb' | This study |
| pYN47           | Mutant papB gene (Y74A,F75A,S76A) cloned into pQE30, Cb' | This study |
| pYN71           | Mutant papB gene (L25A) cloned into pQE30, Cb'    | This study |
| pYN72           | Mutant papB gene (E62A) cloned into pQE30, Cb'    | This study |
| pYN74           | Mutant papB gene (L79A) cloned into pQE30, Cb'    | This study |
| pYN75           | Mutant papB gene (R81A) cloned into pQE30, Cb'    | This study |
| pYN76           | Mutant papB gene (Y96A) cloned into pQE30, Cb'    | This study |
| pHMB66EH        | Broad host range expression vector, Cb'          | 12         |
| pBF21           | cat gene of pACYC184 cloned into pBluescript, Amp', Cm' | 15         |
| pYN21           | wt papB gene and the cat gene cloned into pMMB66EH, Cm' | 12         |
| pYN22           | Mutant papB gene (R61A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN23           | Mutant papB gene (R62A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN24           | Mutant papB gene (E63A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN25           | Mutant papB gene (E64A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN35           | Mutant papB gene (F33A,F34A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN36           | Mutant papB gene (L35A,L36A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN37           | Mutant papB gene (I39A,S40A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN38           | Mutant papB gene (S41A,I42A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN39           | Mutant papB gene (H43A,S44A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN40           | Mutant papB gene (D53A,Y54A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN41           | Mutant papB gene (L55A,V56A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN43           | Mutant papB gene (V47A,I48A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN48           | Mutant papB gene (Y74A,F75A,S76A) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN44           | Truncated papB gene (1–60 aa) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN45           | Truncated papB gene (1–70 aa) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pYN46           | Truncated papB gene (1–80 aa) and the cat gene cloned into pMMB66EH, Cm' | This study |
| pBF21           | λ cl repressor under the control of lac UV5 promoter, Cb' | 14         |
| pBF22           | C-terminal domain of λ cl repressor was deleted from pBF21 | This study |
| pYN51           | wt papB gene cloned into PB21, Cb'              | This study |
| pYN69           | A derivative of pYN51, partially deleted cl repressor gene (Δ Leu57–Ala152) fused to wt papB gene | This study |
| pYN52           | Mutant papB gene (R61A) cloned into PB21, Cb'    | This study |
| pYN100          | Mutant papB gene (R62A) cloned into PB21, Cb'    | This study |
| pYN101          | Mutant papB gene (E63A) cloned into PB21, Cb'    | This study |
| pYN53           | Mutant papB gene (E65A) cloned into PB21, Cb'    | This study |
| pYN102          | Mutant papB gene (E66A) cloned into PB21, Cb'    | This study |
| pYN54           | Mutant papB gene (R67A) cloned into PB21, Cb'    | This study |
| pYN57           | Mutant papB gene (F33A,F34A) cloned into PB21, Cb' | This study |
| pYN58           | Mutant papB gene (L35A,L36A) cloned into PB21, Cb' | This study |
| pYN59           | Mutant papB gene (I39A,S40A) cloned into PB21, Cb' | This study |
| pYN60           | Mutant papB gene (S41A,I42A) cloned into PB21, Cb' | This study |
| pYN61           | Mutant papB gene (H43A,S44A) cloned into PB21, Cb' | This study |
| pYN62           | Mutant papB gene (D53A,Y54A) cloned into PB21, Cb' | This study |
| pYN63           | Mutant papB gene (L55A,V56A) cloned into PB21, Cb' | This study |
| pYN64           | Mutant papB gene (V47A,I48A) cloned into PB21, Cb' | This study |
| pYN65           | Mutant papB gene (Y74A,F75A,S76A) cloned into PB21, Cb' | This study |
| pYN66           | Mutant papB gene (1–60 aa) cloned into PB21, Cb' | This study |
| pYN67           | Mutant papB gene (1–70 aa) cloned into PB21, Cb' | This study |
| pYN68           | Mutant papB gene (1–80 aa) cloned into PB21, Cb' | This study |
| pYN77           | Mutant papB gene (L25A) cloned into PB21, Cb'    | This study |
| pYN78           | Mutant papB gene (R81A) cloned into PB21, Cb'    | This study |
| pYN80           | Mutant papB gene (Y96A) cloned into PB21, Cb'    | This study |
| pYN65           | Truncated papB gene (1–80 aa) cloned into PB21, Cb' | This study |
| pYN66           | Truncated papB gene (1–70 aa) cloned into PB21, Cb' | This study |
| pYN67           | Truncated papB gene (1–60 aa) cloned into PB21, Cb' | This study |
Pap B Mutants with Alanine Substitutions at Positions 35–36, 53–56, and 74–76 Are Impaired in Oligomerization—To test the oligomerization states of different Pap B mutants, we prepared a series of constructs (e.g. pYN52–pYN103) encoding the chimeric proteins consisting of the N-terminal DNA binding domain of λ cl repressor and mutant Pap B molecules (Table I). By using the same oligomerization test system, it was found that Pap B mutants with alanine substitutions at positions 35–36, 53–56, and 74–76 did not form function like wt-Pap B in the oligomerization of Pap B. In contrast, the DNA binding-defective Pap B mutants did not function like wt-Pap B to replace the C-terminal domain of the cl repressor and repress the expression of the reporter genes (Table II) and prevent the lytic development of phage λ (Fig. 5).

Pap B DNA Binding and Oligomerization Regions

To further assess the different oligomerization states of wt and mutant Pap B proteins, we performed in vitro cross-linking studies in the presence of cross-linker dimethyl suberimidate. Such cross-linking experiments showed that Pap B indeed could form a kind of dimer in solution, together with a strong band on the top of the gel which might be the aggregates (Fig. 6).

The DNA binding ability of these oligomerization-defective mutants (according to the in vitro test) with alanine substitutions at positions 35–36, 53–56, and 74–76 did not form dimers in the presence of dimethyl suberimidate, although most of the proteins aggregated (Fig. 6). The results of the in vitro co-operative binding and the in vitro cross-linking tests were consistent with each other, pointed to the importance of these aa residues in the oligomerization of Pap B.

Both the DNA Binding and Oligomerization Capabilities Are Required for Pap B to Function as a Transcriptional Activator—To test the in vivo activity of the different Pap B mutants, the DNA fragments containing all the mutant pap B genes were cloned into plasmid pMMB66EH in which expression is controlled by the IPTG-inducible tac promoter (12). The constructs were then introduced into the strain MC1029 together with the plasmid pCM15 containing the pap (I, B1) A: lacZ operon fusion (6). The relative efficiencies as transcriptional activators were then measured (Table II).
of the different PapB mutants were monitored by their effects on expression of β-galactosidase activity. The expression level of PapB from the tac promoter vector in absence of inducer complemented the deficiency due to the papB1 mutation, and activated production of β-galactosidase from the papA: lacZ fusion. The β-galactosidase activity thereby obtained in the case of wt-PapB was set to 1.0 (Fig. 7). Induction of higher levels of PapB expression gave a reduced activation due to its autoregulatory properties (1). However, it was of interest in the present study to test if any of the mutant PapB proteins could cause activation if present at higher cellular levels. The test therefore was performed both in absence and in presence of the inducer IPTG. The results indicated that the transcriptional activities of PapB mutants defective in either the DNA binding or the oligomerization ability were clearly reduced as compared with wt-PapB (Fig. 7). In some cases (e.g. H43A,S44A, V47A, I48A, and K62A), overproduction of mutant protein by IPTG induction led to partially or fully restored capability to activate papA: lacZ expression. The E66A mutant, which was only partially defective in DNA binding, retained activating and repressing properties that were rather similar to that of wt-PapB. Taken together, we conclude from these studies that both the DNA binding and the oligomerization capabilities are required for PapB to fully function as an active transcriptional regulator.

**DISCUSSION**

In the present study, we investigated the regions essential for DNA binding and oligomerization of PapB by using both deletion and alanine-scanning mutagenesis. The gel mobility shift assays with different truncated PapB mutants showed that the predicted hydrophilic region (aa 61–70) is important for DNA binding (Figs. 1B and 4A). The alanine-scanning mutagenesis indicated that the conserved Arg61 and Cys65 are the two most critical residues required for DNA binding (Figs. 4B and 7), and this is consistent with the fact that the iodoacetamide-modified PapB showed defective DNA binding.

The in vivo oligomerization studies (Fig. 5 and Table II) suggested that wt-PapB could function as an oligomer in vivo. This is also in keeping with our previous findings that the PapB protein interacts as an oligomer of 8–10 subunits at J96 site 1 (7). There are other examples of DNA binding proteins that interact as oligomers at repeated DNA sequence motifs. For example, the E. coli oxidized OxyR protein recognizes the two-fold dyad symmetry sequence ATAGXXXYaXCTATXX- XXXXTATAGXXXYaXCTAT, and binds to four adjacent major grooves of the DNA helix as a tetramer (27, 28). In the case of PapB, we propose that it binds to DNA by recognizing 9-base pair repeats with T/A triplets at conserved positions and by contacting two adjacent minor grooves in a tetrmeric fashion. As reviewed by Werner et al. (29), the interaction of minor groove intercalating proteins with DNA is primarily hydrophobic at the site of insertion. For TATA box-binding protein TBP (30–32), the high mobility group domain proteins SRY (33) and LEF-1 (34), and for the E. coli purine repressor protein PurR (35, 36), these nonpolar interactions extend over most of the interaction surface between the protein and the DNA. Base recognition may occur through van der Waals interactions between β- or γ-branched amino acids (Val, Ile, Leu) or the faces of aromatic amino acids (Phe or Tyr) and the minor groove edges and faces of the DNA bases. Formation of salt bridges or hydrogen bonds occurs between DNA phosphates and Lys or Arg residues of the proteins. In the above mentioned cases, relatively few hydrogen bonds between protein side chains and the DNA bases are present at the interface, with Ser, Thr, Asn, or Tyr forming base–specific hydrogen bonds (29). The integration host factor of E. coli contacts the DNA exclusively via the phosphodiester backbone and the minor groove (37). The crystal structure of an integration host factor-DNA complex indicates that several arginines are involved in minor groove con-
tact by making salt bridges or hydrogen bonds to conserved bases (38). However, the interaction of viral histone-like protein p6 with DNA is proposed to be mainly electrostatic, and Arg6 is essential for the activity (39). Considering our present results, we suggest that Arg 61 and Cys65 are involved in the minor groove contact of PapB to DNA. However, we can not determine what kind of interaction it is at the present time.

To identify the regions important for oligomerization, the conserved amino acid residues were mutated by alanine substitutions. The DNA binding-defective mutants R61A and C65A showed similar oligomerization ability as that of the wt-PapB (Figs. 5 and 6 and Table II). Alanine substitutions at positions 35–36, 53–56, and 74–76 resulted in mutants that were impaired in oligomerization (Figs. 5 and 6 and Table II). All these oligomerization-defective mutants only retained weak DNA binding ability (Figs. 4C and 7), which suggested that the protein interaction is important for PapB to bind cooperatively to the DNA. The transcriptional activation efficiency of either the DNA-binding or the oligomerization-defective mutants was much weaker than that of wt-PapB (Fig. 7).

The \textit{E. coli} H-NS is another relatively small protein (136 amino acids) known to bind DNA in an oligomeric fashion. It may act as a negative regulator of transcription (40, 41), functioning by “transcriptional silencing” (42) and “repression via DNA topology” (41, 43) through oligomeric binding to DNA.

### Table II

| Plasmid | Protein* | Superinfection immunityb | Cm sensitivityc | β-Galactosidase activityd |
|---------|----------|--------------------------|----------------|--------------------------|
| pBF22 cI (1–159) | Sens. | 150 | 1061.2 | 1.0 |
| pBF21 cI (wt) | Imm. | 10 | 76.5 | 0.07 |
| pYN69 cI (Δ57–152)-PapB(wt) | Sens. | 150 | 1062.7 | 1.0 |
| pYN51 cI (1–159)-PapB(wt) | Imm. | 25 | 300.4 | 0.28 |
| pYN52 cI (1–159)-PapB(R61A) | Imm. | 50 | 342.7 | 0.32 |
| pYN100 cI (1–159)-PapB(K62A) | Imm. | 50 | 479.0 | 0.45 |
| pYN101 cI (1–159)-PapB(E63A) | Imm. | 25 | 314.0 | 0.30 |
| pYN53 cI (1–159)-PapB(C65A) | Imm. | 50 | 428.9 | 0.40 |
| pYN102 cI (1–159)-PapB(E66A) | Imm. | 25 | 280.4 | 0.26 |
| pYN103 cI (1–159)-PapB(K67A) | Imm. | 25 | 305.9 | 0.29 |
| pYN57 cI (1–159)-PapB(F33A,F34A) | Imm. | 50 | 649.7 | 0.61 |
| pYN58 cI (1–159)-PapB(L55A,L56A) | Sens. | 150 | 1095.2 | 1.03 |
| pYN59 cI (1–159)-PapB(I39A,S40A) | Imm. | 50 | 382.6 | 0.36 |
| pYN60 cI (1–159)-PapB(S41A,I42A) | Imm. | 100 | 581.2 | 0.55 |
| pYN61 cI (1–159)-PapB(H43A,S44A) | Imm. | 50 | 403.4 | 0.38 |
| pYN62 cI (1–159)-PapB(D53A,Y54A) | Sens. | 150 | 1034.1 | 0.97 |
| pYN63 cI (1–159)-PapB(L55A,V56A) | Sens. | 150 | 1025.5 | 0.97 |
| pYN64 cI (1–159)-PapB(V47A,I48A) | Imm. | 50 | 434.3 | 0.41 |
| pYN68 cI (1–159)-PapB(Y74A,F75A,S76A) | Sens. | 150 | 1072.0 | 1.01 |
| pYN77 cI (1–159)-PapB(L23A) | Imm. | 50 | 410.2 | 0.39 |
| pYN78 cI (1–159)-PapB(E30A) | Imm. | 50 | 368.9 | 0.35 |
| pYN80 cI (1–159)-PapB(I39A,S40A) | Imm. | 50 | 552.5 | 0.52 |
| pYN81 cI (1–159)-PapB(R81A) | Imm. | 50 | 352.8 | 0.33 |
| pYN82 cI (1–159)-PapB(Y96A) | Imm. | 50 | 551.3 | 0.52 |
| pYN65 cI (1–159)-PapB(1–80) | Sens. | 150 | 1126.5 | 1.06 |
| pYN66 cI (1–159)-PapB(1–70) | Sens. | 150 | 1147.7 | 1.08 |
| pYN67 cI (1–159)-PapB(1–60) | Sens. | 150 | 1253.9 | 1.18 |

*Proteins that were tested included wild-type (wt) and the N-terminal DNA binding domain of a cI repressor, chimeric proteins composed of the DNA binding domain of cI and wt or mutant PapB.

bSuperinfection immunity (Imm.) means the number of plaques formed after infection was low (less than $10^5$ pfu/ml), while superinfection sensitivity (Sens.) means the number of plaques was high (up to $10^{10}$ pfu/ml).

cChloramphenicol sensitivity was tested as described. Numbers indicate the Cm concentration at which the plating efficiency of the cells fell below 50%.

dβ-Galactosidase activity was tested as described under “Experimental Procedures.” The value of strain JH607/pBF22 which produced the N-terminal DNA binding domain of the cI repressor was set as 1.0 for comparison.

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**FIG. 6.** Chemical cross-linking with dimethyl suberimidate of wt and mutant PapB. Protein bands were detected by immunoblotting with purified polyclonal anti-PapB antiserum. Presumed positions of PapB derivatives on the gel are indicated along the left-hand side, together with molecular size markers along the right-hand side.
PapB DNA Binding and Oligomerization Regions

H-NS (44): the N-terminal domain involved in transcriptional repression, the central region involved in DNA binding, and the C-terminal domain involved in DNA binding. The possibility that H-NS oligomerization and DNA binding functions can be coupled has been highlighted recently (14, 44). For a small protein like PapB (104 amino acids), it may be difficult to identify certain separate domains. Based on the results we have obtained, we conclude that we have identified the primary regions in the PapB protein involved in DNA binding and oligomerization. Functionally the two properties may be related to each other, and they are both required for it to function as an active transcriptional regulator. These functionally defined amino acid residues are conserved among all other PapB homologous proteins (Fig. 2), suggesting their importance in the structure and function of the whole PapB-family of proteins.

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Table 1: Protein DNA binding activity Oligomerization capability Transcriptional efficiency

| Protein | DNA binding ability | Oligomerization capability | Transcriptional efficiency |
|---------|---------------------|---------------------------|---------------------------|
| Vector control | NA | NA | -IPTG | +IPTG |
| wt-PapB | ++ | + | 1.0 | 0.39 |
| L23A | + | + | NT | NT |
| E30A | + | + | NT | NT |
| F33A, F34A | (+) | + | 0.31 | 0.31 |
| L54A, L56A | - | - | 0.29 | 0.21 |
| E94A, S40A | (+) | + | 0.33 | 0.21 |
| S41A, H42A | (+) | + | 0.33 | 0.21 |
| H43A, S44A | (+) | + | 0.30 | 0.36 |
| V47A, H8A | (+) | + | 0.33 | 0.48 |

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FIG. 7. Summary of DNA binding ability (cf. Fig. 4), oligomerization capability (cf. Fig. 5 and Table II), and transcriptional efficiency for wild-type and mutant PapB (the positions of amino acid residues substituted by alanine(s) are indicated). In the test of how efficient PapB protein derivatives could activate transcription, we monitored expression of β-galactosidase from the pap (I–, B1) A: lacZ operon fusion construct pHMG15. The experiment was performed both in absence and in presence of IPTG and the expression level obtained with wt-PapB in absence of IPTG was set to 1.0. NA, not applicable; NT, not tested.
Mutational Analysis of the PapB Transcriptional Regulator in *Escherichia coli*: REGIONS IMPORTANT FOR DNA BINDING AND OLIGOMERIZATION
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