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Antirepression function in *Escherichia coli* for the cAMP–cAMP receptor protein transcriptional activator

(gene regulation/positive control/*pap* operons/protein–DNA interactions/DNA bending)

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ABSTRACT **The cAMP receptor protein (CRP) complex (cAMP–CRP) is a global regulator of gene expression. It influences transcription from a number of promoters in *Escherichia coli*, including two divergently oriented promoters in the *pap* pili-adhesin gene system. To further define the role of cAMP–CRP in *pap* regulation we monitored protein–DNA interactions *in vitro* and levels of *pap* transcription *in vivo* in wild-type and mutant *pap*-containing clones. The results showed that activation was mediated by a single cAMP–CRP-binding site centered at nucleotide positions −215.5 and −115.5 relative to the transcriptional start points. A target for the *pap*-specific regulatory protein PapB was localized adjacent to the cAMP–CRP-binding site. The long-range effects exerted from the protein-binding sites were consistent with the idea that cAMP–CRP caused a change in the local DNA conformation and that a nucleoprotein complex (involving cAMP–CRP and PapB) was formed in the region between the *pap* promotors. Moreover, transcription became independent of activation of cAMP–CRP and the PapB protein in a mutant lacking the nucleoid-associated protein H-NS. Our findings suggest that the cAMP–CRP complex mediates its positive regulatory function by alleviating transcriptional silencing and, as such, plays a role as antirepressor.**

The transcriptional activities of genes may be modulated by positive or negative control via the action of DNA-binding proteins (1, 2). In bacteria most activators bind at a distance close enough to the promoter region to allow protein–protein contact with the RNA polymerase, a contact thought important in the activation process (3, 4). The cAMP receptor protein (CRP) of *Escherichia coli* is involved in activation of many genes. Alternative mechanisms for activation by the cAMP–CRP complex have been suggested. These mechanisms involve either an interaction with RNA polymerase bound to the promoter or structural changes in the DNA from CRP-induced bending, and experimental data supporting both models exist (5–12). Recent studies with different altered promoter structures have shown that transcriptional stimulation by CRP is optimal when the center of its binding site is positioned on one side of the helix, at −41.5 or −61.5 relative to the transcriptional start point, and that stimulation decreases with longer distance (13, 14).

The cAMP–CRP complex also functions in the regulation of *pap* genes that encode digalactoside-binding pili adhesin in uropathogenic *E. coli*. The *pap* genes are not expressed in *E. coli* strains defective in formation of the cAMP–CRP complex, suggesting that the complex has a positive regulatory function in *pap* gene transcription (15–17). The *pap* genes are divergently transcribed and are organized into a major operon encoding 10 cistrons (rightward transcription, Fig. 1*A*) and a monocistronic operon (*papI*, leftward transcription; Fig. 1*A*) (refs. 15, 16, and our unpublished data). The *papB* gene product also participates in activation of *pap* expression. The *papB–papI* intercistronic region contains sites that interact with the cAMP–CRP complex and the PapB protein *in vitro* (16, 17). Furthermore, the *pap* promoters are subject to transcriptional silencing by the nucleoid-associated protein H-NS (also known as H1 or H1a) (18). A mutation (*drdX*) abolishing H-NS production also allows expression under conditions where the *pap* genes are normally repressed. Evidently this histone-like protein may cause transcriptional silencing of different operons in *E. coli* (19). Here we report experiments aimed at defining the role of cAMP–CRP in *pap* transcription.

MATERIALS AND METHODS

Media and Growth Conditions. The bacteria were cultured in L broth supplemented with medium E (20), thiamine at 1 μg/ml, and, when indicated, with 0.2% glucose. Growth occurred on plates with tryptone yeast agar (TYS, Swedish Labfab, Sollentuna, Sweden). McConkey plates containing 1% lactose, 0.4% maltose, or 0.4% arabinose were used to monitor lactose, maltose, and arabinose phenotypes. Carbenicillin or ampicillin (50 μg/ml) was used for selection and maintenance of plasmids.

Bacterial Strains and Plasmids. Strain TG1 (21) was used as host in cloning and subcloning of M13- and pUC-derivatives. MC1029 and the isogenic *drdX*⁻ strain HMG5 (18, 22) were used as hosts for *lacZ* fusion plasmids. M182 and its *crp*⁻ derivative (22, 23) and CSH26 and its *cya*⁻ derivative (24, 25) have been described. Strains BSN1, BSN2, BSN3, and BSN4 were constructed by conjugating the *drdX*::*cat* allele into M182, M182crp, CSH26, and CSH26cya, respectively (ref. 16, and our unpublished data). The vector pRZ5202 (26) and its derivatives pHMG1 (27) and pHMG15 (15) have been described. Plasmid pHMG62 is a derivative of pRZ5202 containing an *Xho* I linker inserted into the *Sma* I site. An *Xho* I linker was also inserted into the *Ssp* I site at position 1148 of the *pap* sequence. To construct plasmid pHMG106 the 0.8-kilobase (kb) *Xho* I–*Hin*dIII fragment containing the *papB* promoter was inserted into the corresponding sites of pHMG62. Plasmids pPAP5 and pBR322 have been described (28, 29). Plasmid pHMG104 was constructed by insertion of 8-bp *Sal* I and *Xho* I linkers into positions 796 and 1269 [relative to the *Eco*RI site at position 1, Båga *et al.* (15)] within the *papI* and *papB* genes, respectively, and cloning of the resulting *Sal* I–*Xho* I fragment into the *Sal* I site of pUC18. An M13 clone (M13-JK8) used for site-directed mutagenesis was constructed by cloning the *Eco*RI–*Hin*dIII fragment of pHMG104 into the same sites of M13mp19 (30). For subcloning of *in vitro*-constructed mutations, we used plasmid

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Abbreviation: CRP, cAMP receptor protein.

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pHMG102, which carries an *Sph* I (position 583)–*Xho* I (position 1269) fragment of *pap* DNA inserted into the corresponding sites of pUC18.

Site-Directed Mutagenesis. Site-directed mutagenesis was done as described for the Muta-gene M13 (Bio-Rad) *in vitro* mutagenesis kit; this kit is based on a method described by Kunkel (31). A strong selection against the nonmutagenized strand was obtained by using uracil-containing M13-JK8 DNA template, extracted from a *dut, ung* mutant strain. The mutant clones obtained were subjected to nucleotide sequence analysis of a 162-bp region between the *Csp*45I and *Nsi* I sites in the *pap* DNA fragment (see Fig. 1). The *Csp*45I–*Nsi* I sites were used for subcloning of the M5 mutant DNA into the *pap*(I^+, B^+)*A–lacZ* fusion plasmid pHMG1 (27) to construct plasmid pKF21. The plasmid pKF19, which carries a 51-bp deletion that removes PapB binding site 1 (M9), was constructed in an analogous way (K.F. and B.E.U., unpublished data).

DNA Techniques. Plasmid isolation, gel electrophoresis, and transformation were done by standard procedures (32). Restriction endonuclease digestions, DNA ligation reactions, and DNA sequencing with Sequenase were done under conditions recommended by the manufacturers (New England Biolabs, Pharmacia, United States Biochemical).

Gel-Mobility-Shift Assay. Gel-mobility-shift assays were done as described (16, 17). ^{32}P-end-labeled *Csp*45I–*Hin*dIII fragments were prepared from double-stranded DNA of the M13 clones, as described (17).

β-Galactosidase Assay. To measure the β-galactosidase-specific activity we used the method described by Miller (24).

RNA Extraction, RNA Blot, and Primer Extension. RNA was extracted from bacterial cultures grown in LB medium at 37°C, essentially as described (33), with one modification. After phenol-extraction and ethanol-precipitation, the RNA was purified on a Qiagen tip-20, as described by the manufacturers (Qiagen). Thirteen micrograms of each RNA sample was loaded on the gel. RNA blotting, hybridization, and washing were done as described (15, 33). Purified DNA fragments used as *papI*- and *bla*-specific probes were ^{32}P-labeled (16, 34). The filter was separately hybridized with each probe, and after autoradiography the amount of mRNA was determined by densitometric scanning (Bio-Rad videoscanner, model 620). Primer-extension analysis was done as described (35) with excess oligonucleotide primer. The primer for *papA* has been described (33), and primers for *papB* and *papI* were the synthetic oligonucleotides 5′-TTTCAGACATAGAGCCGGGC-3′ and 5′-ACCGC-CAGCGCCTCCGCAATTTCTGCGG-3′, respectively.

Immunoblot. Bacteria were grown to a cell density corresponding to 4 × 10^8 cells per ml in LB medium with carbenicillin at 50 μg/ml for selection of plasmids. Bacterial proteins were separated on SDS/polyacrylamide gels (Fig. 4 *A*, 17.5%; *B*, 20%) and blotted onto nitrocellulose filters with a semi-dry Trans-Blot apparatus (Bio-Rad); procedures were essentially as described (36).

RESULTS AND DISCUSSION

A cAMP–CRP-Binding Site with Divergent Role in Transcriptional Activation. The center of the putative cAMP–CRP target sequence is located 115.5 and 215.5 nucleotides from the start points for the *papI* and *papB* operons (Fig. 1*A*), respectively. To directly investigate whether or not this binding site was important to both *pap* promoters *in vivo*, we altered the binding site by site-directed mutagenesis as shown in Fig. 1*A*. A 2-base-pair (bp) substitution (denoted M5) abolished CRP binding, as determined by gel-mobility-shift assays or by DNase I footprint analysis (Fig. 1*B* and data not shown). The effect of the M5 mutation on rightward transcription was determined by comparing the β-galactosidase specific activity of the *pap*M5 (I^+, B^+)*A–lacZ* fusion construct carrying the CRP site mutation with that of a wild-type fusion plasmid. The expression from the mutant construct in strain MC1029 was reduced to 1% of the wild-type level, which is equivalent to the background level of the *lacZ* fusion system used (Fig. 2*A*). As monitored by RNA blot analysis, the CRP site mutation also resulted in a reduced level of the leftward *papI* transcription, and only a very weak signal could be detected (Fig. 2*B*, lane 4). We conclude that CRP binding to a single site centered at position −115.5 relative to the *papI* promoter and at −215.5 relative to the *papB* promoter simultaneously mediates activation of both promoters.

The PapB protein is known also to bind to the *papI–papB* intercistronic DNA region (17). A PapB-binding site is located adjacent to the CRP-binding site proximal to the *papI* promoter (Fig. 1*A*, site 1). A 51-bp deletion that removed site 1 (Fig. 1*A*, M9, and K.F. and B.E.U., unpublished data), or a mutation in the structural gene for *papB*, caused reduced rightward *pap* transcription. Likewise, the M9 deletion or a mutation in the *papB* structural gene caused reduced *papI* transcription, as analyzed by RNA blot hybridization (Fig. 2*B*, lanes 2 and 3). PapB activation of the *papI* and *papB* operons evidently occurred in concert with cAMP–CRP activation.

Activator-Independent *pap* Transcription in Absence of a Histone-Like *E. coli* Protein. The *pap* promoters are repressed by the nucleoid-associated protein H-NS (18). One role for CRP and PapB could be to relieve the silencing caused by H-NS protein. If this were so, *pap* transcription should

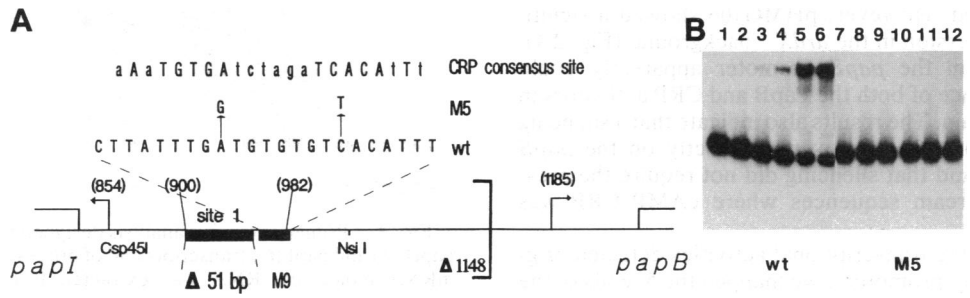

FIG. 1. (*A*) Physical map of the *papI–papB* intercistronic region. The transcriptional start point for the major *pap* operon (*papB*) and the *papI* operon are indicated by arrows (numbering is as in ref. 15, relative to an *Eco*RI site at position 1). The binding sites for CRP and PapB (denoted site 1 as in ref. 17) are indicated by filled boxes. Mutation M5 was constructed by making base-pair substitutions in two of the most invariant positions of the CRP consensus-binding site (most invariant bases shown as uppercase letters) (37), as indicated above map. A mutation (M9) deleting the 51-bp-long PapB site 1 is indicated below map. A bracket shows the endpoint of the 1148 deletion, which removed all *pap* DNA upstream of the *papB* operon (rightward transcription) promoter. (*B*) Analysis of CRP binding to wild type (wt) (lanes 1–6) and M5 mutant (lanes 7–12) DNA by gel-mobility-shift assay. Purified CRP protein (38) was added to final concentrations of 0 (lanes 1 and 7), 2.2 (lane 2), 4.4 (lane 3), 8.9 (lanes 4 and 8), 18 (lanes 5 and 9), 36 (lanes 6 and 10), 71 (lane 11), and 220 (lane 12) nM in the presence of 20 mM cAMP.

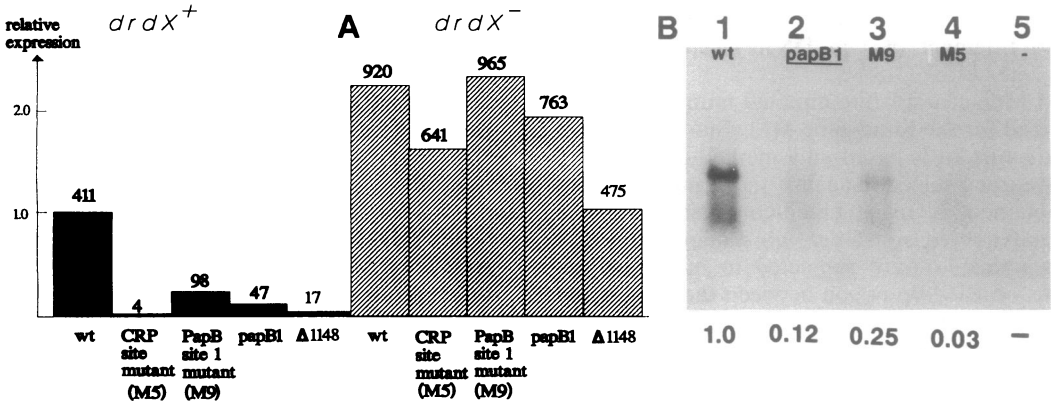

FIG. 2. Effects of binding-site mutations on transcription of divergently oriented *pap* operons. (*A*) Analysis of β-galactosidase activity expressed from various *pap–lacZ* operon fusions in *drdX*$^+$ (MC1029) and *drdX*$^-$ (HMG5) strains (18). Expression levels are given both as β-galactosidase-specific activity in units (shown above columns) and as relative values with the level of MC1029/pHMG1 set to 1.0. Values represent the mean of at least three separate measurements. Expression from the *pap*(*I*$^+$,*B*$^+$) *A–lacZ* fusion plasmid pHMG1 and its derivatives pMHG15 (*papB1*), pFK19 (M9), pKF21 (M5), and pHMG106 (Δ1148) results from initiation at the *papB* promoter (refs. 15 and 33, Fig. 3, and data not shown). Vector pRZ5202 (26) gave 4 and 16 units in *drdX*$^+$ and *drdX*$^-$ strains, respectively. Expression levels of the *PalaS–lacZ* (39) and *P1*(pBR322)–*lacZ* (ref. 40, and our unpublished data) control plasmids were 460 and 98 units in a *drdX*$^+$ strain and were 980 and 190 units in a *drdX*$^-$ strain, respectively. (*B*) RNA blot analysis of *papI* transcription from *pap–lacZ* fusion plasmids in strain MC1029. Lanes: 1, pHMG1 (wild type; wt); 2, pHMG15 (*papB1*); 3, pKF19 (M9); 4, pKF21 (M5); 5, pRZ5202 (vector control). Relative levels of papI-specific mRNA, as indicated below, were calculated using *bla* gene expression as internal reference.

depend less on the cAMP–CRP complex and PapB in a *drdX* mutant (H-NS protein-deficient) strain.

To address this question we used the *drdX* mutant strain HMG5, which is deleted for the H-NS structural gene (18). Fig. 2*A* shows that the effect of mutations in the binding sequences for either cAMP–CRP (M5) or PapB (M9) was suppressed in the *drdX*$^-$ strain. The β-galactosidase-specific activities expressed from the M5 and M9 mutants were restored to levels similar to that of the wild type. In addition to the specific suppression of *pap* expression, the H-NS protein deficiency seemed to cause a gene-dosage effect that contributed a little to the quantitative result. Both the wild-type *pap–lacZ* fusion plasmid and two unrelated fusions (*PalaS–lacZ* and P1 (pBR322)–*lacZ*; see also Fig. 2 legend) showed an increased expression (≈2-fold) in the *drdX*$^-$ strain. However, the increases for the M5 and M9 mutants were 10- to 100-fold. We, therefore, conclude that *pap* transcription specifically was restored and became virtually independent of the cAMP–CRP complex and PapB in the absence of H-NS protein. We also tested a deletion mutant (pHMG106) lacking *pap* DNA sequences upstream of the *papB* promoter (from position 1148). The deletion had its endpoint within a DNA linker that had been inserted 1 bp upstream of the −35 hexameric promoter sequence. There was virtually no expression of this fusion construct in a *drdX*$^+$ background. However, pHMG106 showed a significant level of expression in the *drdX*$^-$ background (Fig. 2*A*). Transcription from the *papB* promoter apparently could occur in the absence of both the PapB and CRP activators in a *drdX* mutant strain. The results also indicate that a silencing effect of H-NS protein was exerted directly on the *papB* promoter region and that silencing did not require the presence of the upstream sequences where cAMP–CRP was found to bind.

To ensure that the transcriptional activities detected originated from the *pap* promoters, we mapped the 5′ ends of the *pap* transcripts by primer-extension analysis. Fig. 3*A* shows that the 5′ ends of papB mRNA from pHMG1 were identical in *drdX*$^-$ and *drdX*$^+$ strains. The M9 and M5 mutants showed the same start site, and the position was identical to the known start site of the *papB* promoter (16). The 5′ ends of *papA*-encoding mRNA molecules known to be processing products from endonucleolytic cleavage of the primary papB–A transcript (33) were also identical in *drdX*$^+$ and *drdX*$^-$ strains. The 5′ ends of the papA and papB mRNAs produced by the Δ1148 deletion construct (pHMG106) were also mapped to the above-mentioned positions (data not shown).

Similarly, the papI mRNA start point was unchanged either by the *drdX*$^-$ allele or by the M5 mutation (Fig. 3*C*). For the M9 deletion mutant (the PapB-binding site mutant) an additional transcript appeared with the start point 4 bp further upstream. In all cases we detected primer-extension products that presumably appeared due to secondary structures in the RNA. The level of papI mRNA expressed from all constructs in the *drdX*$^-$ strain appeared the same. These results support

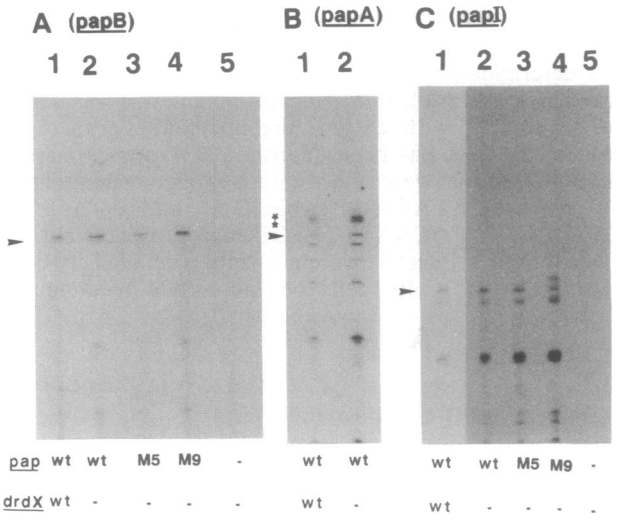

FIG. 3. Primer-extension analysis designed to detect 5′ regions of papB (*A*) and papI (*C*) transcripts and of processed papA-specific (*B*) mRNA molecules. RNA was extracted from the *drdX*$^+$ strain MC1029 (*A*, *B*, and *C*, lanes 1) and the *drdX*$^-$ strain HMG5 (*A*, lanes 2–5; *B*, lane 2; and *C*, lanes 2–5) containing plasmids pHMG1 (*A*, *B*, and *C*, lanes 1 and 2), pKF21 (*A* and *C*, lanes 3), pKF19 (*A* and *C*, lanes 4), and pRZ5202 (*A* and *C*, lanes 5). Relevant genotypes are shown at bottom. Transcriptional start points for *papB* and *papI* were determined to be at positions 1185 and 854, respectively. The major *papA* processing product, as determined in ref. 33 (position 1676), is indicated by arrowheads, and minor products (positions 1670 and 1671) are indicated by stars. wt, Wild type.

the conclusion that both *pap* promoters may become activator-independent in the absence of H-NS protein.

As an alternative approach to investigate the cAMP-CRP-independent *pap* expression in an H-NS-deficient background, we used strains containing the entire *pap* pili-adhesin gene system. A *drdX* knock-out allele (*drdX*::*cat*) was used to construct *crp*$^-$,*drdX*::*cat* and *cya*$^-$,*drdX*::*cat* double-mutant derivatives. The effects on Pap pili subunit expression were determined by immunoblot analysis using a polyclonal antiserum directed against Pap pili. Fig. 4*A* shows that the levels of pili protein expressed in H-NS protein-deficient strains (*drdX*$^-$ and *drdX*$^-$, *crp*$^-$) were similar to that of the wild-type strain, whereas no pili could be detected in the H-NS protein-proficient *crp*$^-$ strain.

With respect to the CRP regulon we obtained results indicating that the H-NS protein deficiency did not cause a general effect on cAMP–CRP-mediated regulation. The use of arabinose and maltose by *E. coli* illustrates two well-characterized cases where the cAMP–CRP complex is involved in transcriptional regulation and binds to DNA upstream of the promoters (1). By monitoring phenotypes of the *crp*/*drdX*::*cat* mutant strains, we tested whether or not those systems were also affected by H-NS protein deficiency. Table 1 shows that expression of both properties depended on a functional *crp* gene, irrespective of the status of H-NS protein.

That cAMP–CRP might affect *pap* transcription indirectly via regulatory effects on H-NS protein expression was also considered and tested with the strains constructed. However, the amount of *drdX* gene product (H-NS protein) expressed was not affected by mutations in the *crp* or *cya* loci, as determined by immunoblot analysis of the H-NS protein (Fig. 4*B*). We propose that the antagonistic roles of cAMP–CRP and H-NS protein in *pap* transcription are exerted by virtue of their separate interactions with the *pap* DNA sequences.

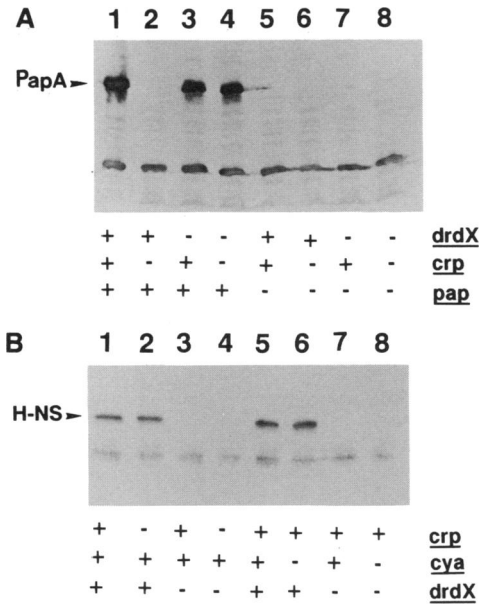

FIG. 4. (*A*) Effect of *crp* and *drdX* mutations on expression of Pap pili subunits. Immunoblot analysis of strains harboring the *papA–K* plasmid pPAP5 or the vector pBR322 (29), using a polyclonal serum against Pap pili. Lanes: 1, M182/pPAP5; 2, M182 *crp*$^-$/pPAP5; 3, BSN1 (*drdX*::*cat*)/pPAP5; 4, BSN2 (*crp*$^-$, *drdX*::*cat*)/pPAP5; 5, M182/pBR322; 6, M182 *crp*$^-$/pBR322; 7, BSN1 (*drdX*::*cat*)/pBR322; 8, BSN2 (*crp*$^-$, *drdX*::*cat*)/pBR322. (*B*) Immunoblot analysis, using a polyclonal antiserum against H-NS protein. Lanes: 1, M182; 2, M182 *crp*$^-$; 3, BSN1 (*drdX*::*cat*); 4, BSN2 (*crp*$^-$, *drdX*::*cat*); 5, CSH26; 6, CSH26 *cya*; 7, BSN3 (*drdX*::*cat*); 8, BSN4 (*cya*, *drdX*::*cat*).

Table 1. cAMP–CRP-dependent use of arabinose and maltose in H-NS protein-deficient *E. coli*

Strain	Phenotype* McC + arabinose	Phenotype* McC + maltose
M182	Red	Red
M182 *crp*	White	White
BSN1 *drdX*::*cat*	Red	Red
BSN2 *crp*, *drdX*::*cat*	White	White

McC, McConkey plate.
*Bacterial phenotype was scored after growth on plates at 37°C overnight.

Our studies of *pap* transcription in *E. coli* suggest that the *pap* promoters can be repressed by the histone-like protein H-NS and that the cAMP–CRP complex may relieve such repression in certain cases. We, therefore, propose that cAMP–CRP here has a role as antirepressor. This is an unusual role to assign to the well-recognized cAMP–CRP regulator, and it will be important to determine whether activation of transcription by antirepression may also occur in other bacterial operons. Antirepression by the cAMP–CRP complex evidently required relatively long-range (100–200 nucleotides) action mediated between the protein-binding site and the *pap* promoters. According to results from *in vitro* studies, the DNA of the *papI–papB* intercistronic region showed evidence of pronounced intrinsic bending (our unpublished data). The *papI–papB* intercistronic region is A+T rich (≈70%) and contains several A- or T-tracts of the type known to contribute to bending of the DNA (41). In addition, binding of the cAMP–CRP complex could be expected to induce DNA bending (12). Using the circular permutation assay (42), we found that cAMP–CRP interaction with the intercistronic *pap* DNA induced additional bending (unpublished data). It seems likely that the *papI–papB* intercistronic DNA could form a nucleoprotein complex with the CRP and PapB proteins, involving both intrinsic and protein-induced bending in this DNA region. Such local alterations of the DNA conformation may constitute part of an antirepression mechanism. The proposed antirepression of the cAMP–CRP complex bears intriguing resemblance to the recently described antirepression carried out by some eukaryotic transcription factors that are proposed to counteract histone-mediated repression (43, 44). It will be interesting to learn whether the molecular mechanisms of antirepression have features in common among prokaryotes and eukaryotes.

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