Mutants of the catabolite activator protein of *Escherichia coli* that are specifically deficient in the gene-activation function

(*lacZ* fusions/transcription *in vitro*/protein–protein interaction/RNA polymerase)

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ABSTRACT   In the presence of cyclic AMP, the catabolite activator protein (CAP) of *Escherichia coli* binds DNA and stimulates transcription at a number of promoters. We have examined a model of CAP bound at the *gal* promoter and, using directed mutagenesis, have isolated CAP mutants that are analogous to the λ repressor *positive control* (*pc*) mutants. These CAP mutants bind DNA but are defective in stimulating transcription at the *gal* P_1 promoter. These mutants are also altered in positive control at the *lac* and *malT* promoters, where CAP binds to sites further upstream from the transcription start site.

The catabolite activator protein (CAP; also known as cyclic AMP receptor protein, CRP) is both a positive and a negative regulator of gene transcription. In the presence of cyclic AMP, CAP dimers bind to 22-base-pair sites of related DNA sequence near certain promoters (1–4). The distance between the known CAP binding site and the transcription start site at different promoters varies. At the *gal* P_1 promoter, CAP stimulates transcription by binding to a site centered at about position −42 relative to the transcription start point (position +1). In contrast, at *lac* the identified CAP site is centered at about −60, and at *malT* it is centered at about −70 (Fig. 1). How does CAP stimulate transcription when bound at these various distances from the transcription start sites?

We believe that λ repressor bound to DNA activates transcription by contacting RNA polymerase and helping it to form an open complex at the adjacent promoter (5, 6). Isolation of λ repressor mutants that bind DNA normally, or nearly normally, but fail to stimulate transcription (6, 7) supports this model. These *positive control* (*pc*) mutants bear altered amino acid residues that lie on a surface of λ repressor predicted from the crystal structure, and other information, to be near polymerase at the adjacent promoter (6).

In this paper we describe the isolation of *pc* mutants of CAP. From the known crystal structure of CAP (8) and the inferred structure of the CAP–DNA complex, we predict the surface of CAP that would most likely contact polymerase at the *gal* P_1 promoter. We used site-directed mutagenesis to change residues on or near this surface of CAP and, as a control, residues on another surface that we predict would not contact polymerase. Here we report the isolation of three CAP mutants bearing changed residues on or near the designated surface. Each mutant manifests the *pc* phenotype—that is, they bind to DNA but fail to stimulate fully transcription at *gal* promoter P_1. Intriguingly, these mutants also alter positive control at promoters where CAP binds at different positions relative to the start point of transcription.

MATERIALS AND METHODS

Enzymes and Reagents. *Escherichia coli* RNA polymerase; the restriction endonucleases *Eco*RV, *Hin*dIII, *Eco*RI, *Bam*HI, and *Pvu* II; the Klenow fragment of *E. coli* DNA polymerase I; and polynucleotide kinase were purchased from New England Biolabs. [α-³²P]CTP and [α-³²P]dATP were obtained from New England Nuclear.

Bacterial Strains. The bacterial strains used were *E. coli* NI1000 (Δ*crp39*, *rpsL*, *thi*), NI1001 {Δ*crp39*, *rpsL*, *thi*, Δ*lacU169*, *relA*, *srl*::Tn*10 malT250* [I(*malT–lacZ*)542-1 (hyb)-*malt*p7]}, NI1002 (Δ*crp39*, *rpsL*, *thi*, Δ*lacU169*, *relA*, *srl*:: Tn*10 galE–lacZ*), and KC1071 (NI1000 with the *lac* promoter region converted to a *lac*UV5 promoter and the *lac* operator mutated to a symmetric CAP binding site).

Plasmids. pNI110 contains the *Hin*dIII–*Bam*HI fragment carrying *crp* (the gene encoding CAP) from the M13 phage used for mutagenesis and the *Bam*HI–*Pvu* II fragment from plasmid pBR322 cloned into *Hin*dIII/*Pvu* II-digested p*tac*12 (9). pNI55 is pNI110 carrying the Glu-55→Lys mutation. pNI191 is pNI110 carrying the Glu-191→Lys mutation. pKC111 is pHA5 (10) deleted for the *Hin*dIII fragment upstream of *crp*. pKC112 was derived from pKC111 by deletion of the *Hin*dIII fragment carrying the *crp* gene. pNI111 carries a *Hin*dIII–*Bam*HI fragment from the M13 phage used for mutagenesis encoding the Glu-171→Lys mutant *crp* cloned into *Hin*dIII/*Bam*HI-digested pKC111. pNI112 is the same as pNI111 except that it carries the Glu-171→Gln mutation. pRW75 carries the *lac* promoter. pNI10 is pRW75 with a wild-type *crp Hin*dIII–*Bam*HI fragment cloned downstream of the *lac* promoter. pNI4 is the same as pNI10 except that it carries the Gln-170→Lys mutation.

Mutagenesis and Sequencing. Site-directed mutagenesis of the *crp* gene was performed essentially as described by Zoller and Smith (11). The single-stranded templates used for primer extension were prepared from M13mp19 carrying the *Hin*dIII–*Eco*RV fragment encoding *crp* from pHA5. Synthetic primers containing a single mismatch were extended *in vitro* with the Klenow fragment of *E. coli* polymerase I, and the DNA was used to transform *E. coli* JM101 (from J. Messing, Waksman Institute and Rutgers University, New Brunswick, NJ). Purified plaques were screened by DNA sequencing by the method of Sanger *et al.* (12). The DNA sequence of the entire coding region of *crp* was determined. The *Hin*dIII–*Bam*HI fragment encoding CAP was cloned into expression vectors.

Gel Binding Assays. A 240-base-pair fragment containing the *lac* CAP site was obtained by digestion of pKC120 with *Pvu* II and either *Hin*dIII or *Eco*RI. Each fragment was end-labeled by use of Klenow fragment (13). Culture extracts were prepared as described by Brunelle *et al.* (14). Standard
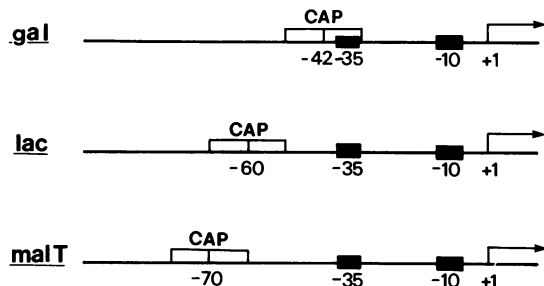
Abbreviation: CAP, catabolite activator protein.

FIG. 1. The position of CAP sites at the *gal, lac,* and *malT* promoters. The transcription start sites are indicated by +1, the −10 and −35 regions of the promoters are shown as black boxes, and the centers of the 20-base-pair CAP sites are indicated.

binding reactions were performed at 20°C for 10 min in binding buffer (20 mM Tris·HCl, pH 8/0.1 mM EDTA/0.1 mM dithiothreitol/100 mM KCl containing bovine serum albumin at 100 μg/ml) with or without 100 μM cyclic AMP. After incubation, reaction mixtures (20 μl) were supplemented with 3 μl of sample buffer [50% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.5] plus tracking dyes and immediately loaded onto a 5% polyacrylamide gel. After electrophoresis at 160 V in 10 mM Tris·HCl, pH 8.0/1 mM EDTA, the gel was fixed in 10% acetic acid/10% methanol, dried, and autoradiographed.

Transcription in Vitro. Transcription reactions *in vitro* were performed and analyzed as described in Spassky *et al.* (15). Transcripts were visualized by autoradiography and quantitated by scanning densitometry.

Protein Purification. The Glu-171→Lys mutant protein was purified from NI1000/pN171 according to methods described by Eilen *et al.* (16). Wild-type CAP was a gift from J. Krakow (Hunter College, New York).

PRELIMINARY CONSIDERATIONS

To identify the surface of CAP that would most closely approach RNA polymerase, we placed a model of CAP (17, 18) at its known binding site on a model of DNA that includes the CAP-activated *gal* P_1 promoter (19) and upstream sequences. As shown in Fig. 5 (*gal*) the locations of the phosphates contacted by the CAP and RNA polymerase

indicate that these two proteins lie immediately adjacent to one another, in a manner analogous to the λ-repressor case (6). The polymerase phosphate closest to CAP is precisely the one which lies closest to each of three repressors (λ, 434, and P22) that activate transcription. Moreover, inspection of space-filling models reveals that the relevant surface of CAP includes its α-helix E, while the corresponding λ repressor surface includes its α-helix 2 (6). Each of these helices is part of the bihelical DNA-binding structure [helices E and F in the case of CAP; helices 2 and 3 in the case of λ repressor (20, 21)]. Helices F and 3 each are thought to lie in the major groove, making sequence-specific contacts with DNA, and helices E and 2 lie across the major groove. The λ *pc* mutations alter residues in helix 2 (and in the bend between helices 2 and 3) and, in each case, change acidic or neutral amino acids so that the net negative charge is decreased (6). We therefore mutated three acidic and one neutral residue in CAP: two in helix E (Glu-171 and Gln-170), one near helix E (Glu-55), and, as a control, a residue in helix F (Glu-191) located on a surface that could not, according to the models, touch polymerase. Five mutants were isolated: Glu-171→Lys, Gln-170→Lys, Glu-171→Gln, Glu-55→Lys, and Glu-191→Lys (Fig. 2). DNA sequencing showed that each mutant, isolated in M13 phage and subsequently cloned into expression vectors, contained only the mutation introduced by the oligonucleotide. We analyzed each mutant for its ability to stimulate transcription *in vivo* and then analyzed selected mutants by *in vitro* transcription experiments.

RESULTS

Transcription in Vivo. Plasmids bearing the wild-type CAP gene (*crp*) or one of the mutant derivatives were each introduced into three strains. These strains, all deleted for *crp*, contained a *lacZ* gene (and only this *lacZ* gene) fused to the *gal, lac,* or *malT* promoters. Synthesis of β-galactosidase in these strains can be measured directly or estimated by characteristic color of colonies on appropriate indicator plates. Table 1 shows the effect of wild-type *CAP* and each of the mutants at the *gal, malT,* and *lacZ* promoters. The two mutants altered at position 171 were defective in transcription at all three promoters. The Gln-170→Lys mutant was defective at the *gal* and *malT* promoters but behaved anomalously at the *lac* promoter, which it stimulated significantly more efficiently than did wild type. The mutant bearing a change at
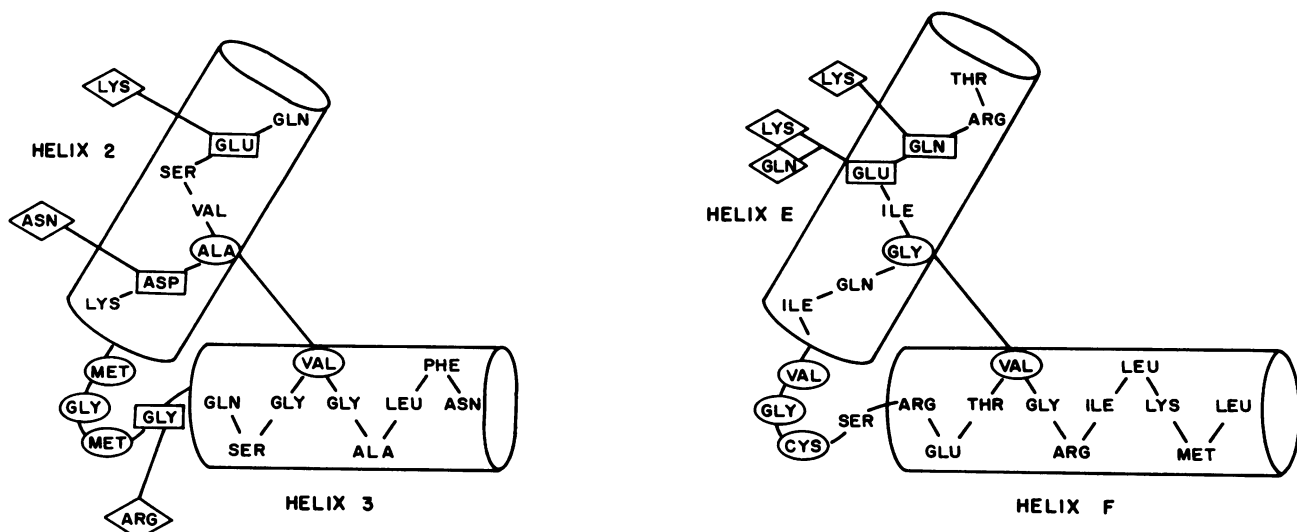

FIG. 2. Diagram of the helix-turn-helix motif of λ repressor (helices 2 and 3) and of CAP (helices E and F). The residues that are conserved among many repressors are circled. The residues in rectangles are changed in the *pc* mutants to the residues shown in the diamonds.

Biochemistry: Irwin and Ptashne

Proc. Natl. Acad. Sci. USA 84 (1987)   8317

Table 1. Stimulation of transcription by wild-type CAP and mutant derivatives, measured *in vivo*

CAP	β-Galactosidase activity		
	gal	lac	malT
Wild type	470	1740	610
Glu-171→Gln	260	810	390
Glu-171→Lys	190	50	230
Gln-170→Lys	300	3990	410
Glu-55→Lys	530	3020	640
Glu-191→Lys	480	2150	560
None	190	30	90

β-Galactosidase activities, assayed as described by Miller (22), measured stimulation of transcription by CAP at the *gal*, *lac*, and *malT* promoters. CAP was encoded on a plasmid and cells carried a *crp* deletion and a *lacZ* gene fused to the *gal*, *lac*, or *malT* promoters. Each strain was grown in minimal media containing, respectively, 1% galactose, 1% (vol/vol) glycerol plus 1 mM isopropyl β-D-thiogalactopyranoside, or 1% glycerol.

position 191 behaved like wild type. The Glu-55→Lys mutant stimulated transcription more efficiently than did wild type at all three promoters.

DNA Binding. To distinguish mutants that are defective in DNA binding from those specifically deficient in positive control, we determined the ability of each mutant to bind DNA *in vivo*. The *lac* operator site, which binds CAP only weakly, was converted into a strong CAP binding site by deleting one base pair and changing another (K. Chapman and M.P., unpublished work). CAP binds to this engineered site and represses β-galactosidase synthesis. We tested the DNA binding of each mutant by measuring repression of β-galactosidase synthesis, using plasmids that directed expression of equal amounts of wild-type and mutant CAP. The Glu-171→Gln mutant binds as well as wild type, the Gln-170→Lys and Glu-55→Lys mutants bind slightly more tightly than does wild type, and the Glu-171→Lys mutant binds DNA more weakly than does wild type (Table 2). The control mutant, Glu-191→Lys, binds as well as wild type. None of the mutants repressed β-galactosidase synthesis in a strain carrying a *cya* deletion.

We also tested our mutants *in vitro* for their ability to bind at the *lac* CAP site in gel binding assays (14). Purified Glu-171→Lys protein binds a DNA fragment carrying the *lac* CAP site with an affinity only one-third that of the wild-type protein (Table 2). Lysates of cells producing each of the other CAP mutants were assayed for DNA binding (Table 2). The results of these experiments were consistent with the results seen in the assay *in vivo* described above.

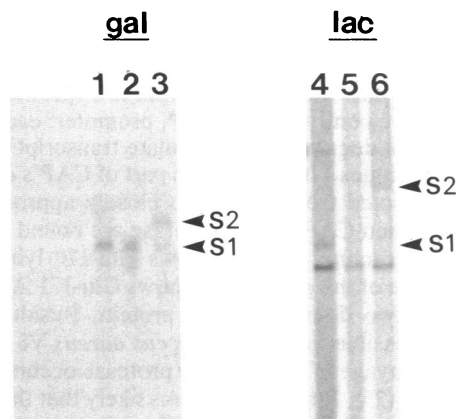**Transcription *in Vitro*.** Because the Glu-171→Lys mutant

FIG. 3. Transcription *in vitro* from the galactose and lactose promoter regions. Transcription was performed in the absence of added CAP (lanes 3 and 6), the presence of wild-type CAP (lanes 1 and 4), or the presence of the Glu-171→Lys mutant CAP (lanes 2 and 5). S1 indicates the CAP-stimulated transcript and S2 the CAP-repressed transcript.

binds at CAP sites less well than does wild type, it is not evident from experiments *in vivo* whether it is also defective in positive control. *In vitro* transcription experiments were performed using purified Glu-171→Lys protein and wild-type CAP to test directly whether the Glu-171→Lys mutant was specifically defective in positive control. Wild-type and Glu-171→Lys proteins were each prebound to a DNA fragment carrying the *gal* promoter. DNase I protection assay ("footprinting") demonstrated that the *gal* CAP site was filled identically in each case (data not shown). As seen in Fig. 3, both wild-type and mutant CAP repressed transcription from the *gal* P_2 promoter, a promoter located five base pairs upstream of the *gal* P_1 promoter (23), but wild-type CAP stimulated P_1 transcription to a level that was about 3 times that seen with the mutant. In a similar experiment performed with DNA bearing the *lac* promoter, the Glu-171→Lys CAP was about one-fourth as effective as wild-type CAP (Fig. 3).

DNA Bending. Wu and Crothers (24) demonstrated that the binding of wild-type CAP to DNA causes the DNA to bend. Binding of each of the CAP *pc* mutants bends DNA to the same degree as does wild-type CAP as assessed from the mobility of CAP–DNA complexes in polyacrylamide gels. That is, migration of complexes in which mutant CAP is bound in the middle of the fragment is retarded to the same extent as is that of fragments with wild-type CAP bound (Fig. 4).

Table 2. DNA binding by wild-type and mutant CAPs measured *in vivo* and *in vitro*

CAP	β-Galactosidase activity	Relative K_D (*in vitro*)
Wild type	40	1
Glu-171→Gln	40	1
Glu-171→Lys	95	3
Gln-170→Lys	20	0.3
Glu-55→Lys	30	0.2
Glu-191→Lys	40	1
None	130	—

β-Galactosidase activities were determined as described by Miller (22), using a strain expressing CAP from a plasmid and bearing a *lac*UV5 promoter with a symmetric CAP site at the position normally occupied by the *lac* operator. When CAP binds at this site, transcription is repressed. K_D values show the relative amounts of culture extracts required to half-maximally bind a DNA fragment bearing a *lac* CAP site.
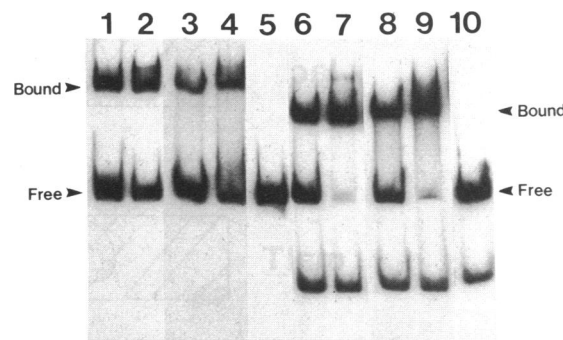

FIG. 4. Gel binding assay. In lanes 1–5, the CAP site is in the middle of a 240-base-pair DNA fragment; in lanes 6–10, the CAP site is near the end of a 240-base-pair fragment. Lanes: 1 and 6, wild-type CAP; 2 and 7, Glu-171→Gln mutant CAP; 3 and 8, Gln-170→Lys mutant CAP; 4 and 9, Glu-171→Lys mutant CAP; 5 and 10, no CAP.

DISCUSSION

We have constructed three mutants of CAP (Gln-170→Lys, Glu-171→Gln, and Glu-171→Lys) that are specifically deficient in positive control at the *gal* P_1 promoter: each mutant binds DNA but does not fully stimulate transcription at this promoter. Residues 170 and 171 are part of CAP's α-helix E, which is predicted from models to closely approach RNA polymerase when CAP and polymerase are bound at *gal* P_1. The crystal structure of CAP shows Gln-170 lying on the outside surface of the protein but shows Glu-171 in a crevice between the two domains of the protein. Residue 171 is, however, accessible to *Staphylococcus aureus* V8 protease, as the first cleavage of CAP by this protease occurs adjacent to this residue (25). It therefore seems likely that this residue would be accessible to RNA polymerase as well. The mutation Glu-191→Lys has no effect on CAP's activity. Glu-55→Lys has only slight effects. These results are consistent with the idea that at *gal* P_1, CAP stimulates transcription according to the mechanism described for gene activation by λ repressor, namely by contacting the adjacent DNA-bound RNA polymerase and helping it form an open complex. If this idea is correct, we would now have three cases (λ repressor, CAP, and 434 repressor) in which residues in corresponding parts of the bihelical motif were implicated in activator–polymerase interaction (6, 26). In a fourth case, that involving the phage P22 repressor, a different surface of the repressor is involved.

Surprisingly, the three mutants bearing the *pc* phenotype at the *gal* promoter are also altered in their abilities to stimulate transcription at the *lac* and *malT* promoters. All but one of the mutants are defective in positive control; the anomaly is the Glu-170→Lys mutant, which stimulates transcription at the *lac* promoter more efficiently than does wild-type CAP. While we have no explanation for the latter result, overall the results indicate that the same surface of CAP is involved in stimulation of transcription at all three promoters, despite the different spacings between the transcription start points and the respective known CAP binding sites.

The results of abortive initiation experiments, performed by M. Schwartz and W. R. McClure (personal communication), using two of our mutants (Glu-171→Lys and Glu-

171→Gln) support our findings. In each case the rate of open complex formation at the *lac* promoter in the presence of the mutant CAPs was about one-third that measured with wild-type CAP.

We imagine three ways that the same surface of CAP might mediate positive control via contact with RNA polymerase at the three promoters discussed in this paper. According to the first two of these models, we imagine that a single CAP dimer is bound at each promoter at the known CAP binding site. The first of these ideas is that the DNA contorts so that the RNA polymerase at the *lac* and *malT* transcription starts could contact CAP as it evidently does, without DNA contortion, at *gal* P_1. It might be relevant, in this regard, that our *pc* mutant CAPs bend DNA to the same extent (as measured in gels) as does wild-type CAP. The second idea is that polymerase contacts DNA-bound CAP and then moves to the transcription start site. The hypothesized ability of polymerase to move would have to be shared by CAP: just as CAP helps polymerase bind at the transcription start site, so does a bound polymerase help CAP bind to the CAP site (unpublished observation). These models are further contradicted by the fact that a single base-pair insertion at the *lac* promoter between the CAP site and the transcription start site abolishes CAP action (27).

According to the third model, there is more than one CAP bound to each of these promoters (Fig. 5). It is known that at the *gal* promoter, in the presence of RNA polymerase, two CAP dimers bind; the second one adds immediately upstream of the CAP bound at the identified site when polymerase binds (31). At *lac* the identified CAP site is located at the same position as the upstream CAP site at *gal* (32). If a second CAP dimer binds at *lac* between the known CAP site and bound RNA polymerase, then the location of CAP dimers at each of these promoters would be identical. The known CAP site at *malT* is located 10 base pairs, or about one helical turn of the DNA, further upstream of the promoter than is the CAP site at *lac*. Perhaps a second CAP dimer at *malT* binds in a position analogous to the identified CAP bound at *gal* (see Fig. 5). In this case, we assume that the two CAPs interact across a major groove; perhaps CAP's ability to bend DNA permits this interaction. Further work will be required to confirm or contradict this suggestion.
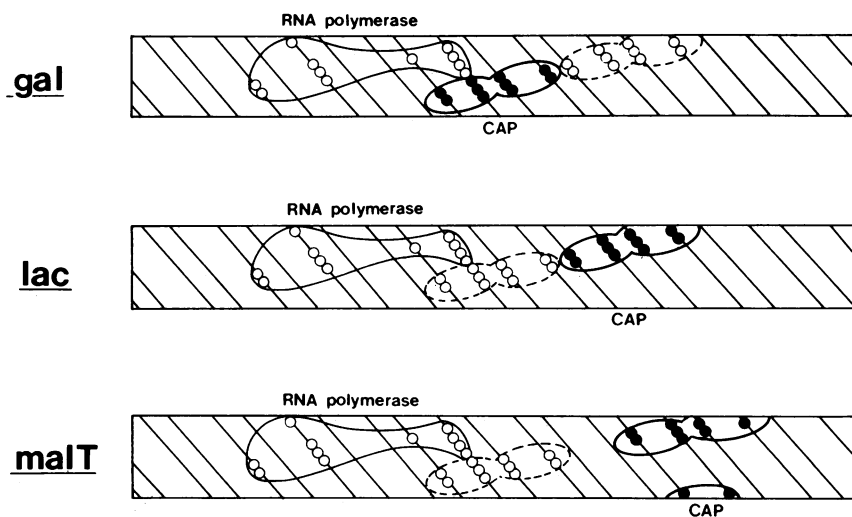

FIG. 5. Diagram of positions of proteins at the *gal*, *lac*, and *malT* promoters, including proteins at known sites and those predicted by the third model. Phosphates believed to be in close contact with CAP at identified sites are marked with ●, phosphates thought to be in close contact with RNA polymerase by analogy to those determined for the *lac*UV5 and T7 A3 promoters (28) are marked with ○, and phosphates predicted to be in close contact with a second CAP dimer bound only in the presence of RNA polymerase are marked with ○ and are encircled by a dashed line on an "unwrapped" DNA helix. Phosphates contacted by CAP at primary sites in the *gal* and *lac* promoters have been determined directly (29, 30).

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