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Mechanism of *araC* autoregulation and the domains of two overlapping promoters, P_C and P_{BAD}, in the L-arabinose regulatory region of *Escherichia coli*

(DNA-protein contacts/cyclic AMP-binding protein/araC protein/transcription)

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ABSTRACT **The DNA-protein contact sites in the *ara* regulatory region, which contains the promoters for *araBAD* and *araC*, have been determined for *araC* protein, the cyclic AMP-binding protein, and RNA plymerase, by using the methylation protection and DNase I protection methods. The functional significance of binding was assessed by correlating the state of occupancy of these sites with promoter activity in transcription initiation. Our results suggest that the basis for *araC* autoregulation is that *araC* protein, in either its activator (P2) or repressor (P1) form, acts as a repressor for *araC*, by binding to the RNA polymerase attachment site at the *araC* promoter. We also found that the *araC* and *araBAD* promoters share a common site of positive control by the cyclic AMP-binding protein, located 90 bases from the *araBAD* and 60 bases from the *araC* transcriptional start points. A model for the mechanism of regulation of *araBAD* and *araC* expression by the catabolite gene-activator protein, P1, and P2 is proposed. An earlier model proposed by Ogden *et al.* [Ogden, S., Haggerty, D., Stoner, C. M., Kolodrubetz, D. & Schleif, R. (1980) *Proc. Natl. Acad. Sci. USA* 77, 3346–3350] is discussed in the light of the data presented in this paper.**

In *Escherichia coli*, enzymes required for the metabolism of L-arabinose are coded by the *araBAD* operon; their synthesis is positively controlled by the cyclic AMP (cAMP) binding protein [i.e., the catabolite gene-activator protein (CAP)] and both positively and negatively controlled by the product of the *araC* gene (1–3). The *araC* gene is located next to the *araBAD* operon and is oppositely oriented (4). The expression of *araC* is repressed by its own product and stimulated by the CAP system (5). Thus, the *araC* protein has three regulatory functions: (*i*) positive control of *araBAD*, (*ii*) negative control of *araBAD*, and (*iii*) negative control of *araC*. The purpose of this paper is to help clarify this complex role of the *araC* protein in controlling the expression of *ara* genes.

The complete DNA sequence of the *ara* regulatory region and the start points of the *araBAD* operon are known (6–9). The start points for the transcription and translation of *araC* have recently been determined (9). As shown in Fig. 1, the *araB* and *araC* genes are separated by a noncoding region of 338 base pairs. A considerable portion of this region is transcribed into the *araBAD* and *araC* leader RNAs. The nontranscribed region [147 base pairs (bp)] contains the promoters, P_{BAD} and P_C, including their sites of interaction with RNA polymerase and with their respective positive regulators; i.e., *araC* protein and CAP for P_{BAD} and CAP for P_C.

In our work, we examined the *ara* DNA region from about +26 to −322 for sites of contact with the *araC* protein, the CAP, and RNA polymerase, using the ability of bound proteins to protect DNA against methylation (10) and DNase I digestion (11). The functional significance of binding was assessed by correlating the state of occupancy of these sites with promoter activity in transcription initiation.

We show that the basis for *araC* autoregulation is that the *araC* protein, in either its activator (plus arabinose, P2) or repressor (minus arabinose, P1) form, acts as a repressor for the *araC* gene by binding to the RNA polymerase attachment site at P_C. We also show that the *araC* and *araBAD* operon share a common site of positive control by the CAP. [At the conclusion of this work, we became aware of the model of positive and negative regulation in *ara* proposed by Ogden *et al.* (12); this will be discussed.]

MATERIALS AND METHODS

DNA fragments (F1 through F7; see Fig. 1) used in the protection experiments and transcription assays were derived from the plasmid pNL4 (9). The procedures for their isolation, end labeling, and sequence analysis were from Maxam and Gilbert (13). Methylation protection and DNase I protection conditions were adopted from published procedures (10, 11), and the *in vitro* transcription assay has been described (6, 9). RNA polymerase was purified from *E. coli* strain NL20-000, according to the procedure of Burgess and Jendrisak (14); the two preparations used in this study were 73% and 64% saturated with sigma factor. The *araC* protein and the cAMP-binding protein were 98% and 81% pure, respectively.

RESULTS

DNA-Protein Contact Sites in the *ara* Regulatory Region. Contact sites for the *araC* protein, the CAP, and RNA polymerase were determined by using the methylation protection and DNase I footprinting techniques. Results are summarized in Fig. 2, and most of the autoradiograms are reproduced in Fig. 3. The numbering of bases is relative to the *araBAD* transcription start point (+1).

At relatively high protein/DNA ratios, the P2 form of *araC* protein protected two regions of DNA (designated A and B sites in Figs. 2 and 3). As shown below, the B site binds only P2, whereas the A site binds both P2 and P1. No other site was protected by the *araC* protein within the DNA segment from +26 to −322.

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Abbreviations: CAP, catabolite gene-activator protein; cAMP, cyclic AMP; P1, form of the *araC* protein that represses *araBAD* expression, generally associated with the absence of inducer L-arabinose or the presence of the anti-inducer, D-fucose; P2, form of the *araC* protein that activates *araBAD* expression, acquired by the protein in the presence of L-arabinose; bp, base pair(s); P_{BAD}, promoter for the *araBAD* operon; P_C, promoter for the *araC* gene.

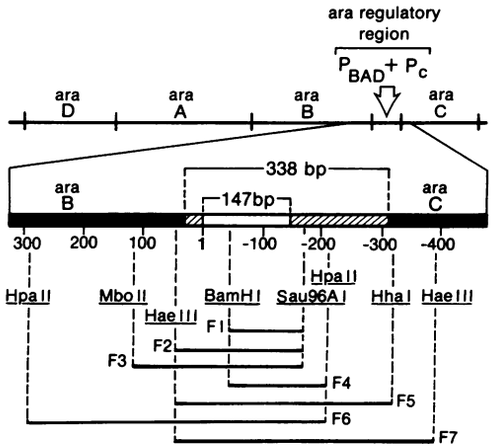

FIG. 1. *ara* regulatory region and the restriction fragments used in this work. Divergent transcription and translation of the *araBAD* operon on the left and the *araC* gene on the right originate within the regulatory region. Transcriptional start points for *araB* and *araC* are located at +1 and −148; translational start points are at +28 and −312, respectively. Numbering of bases is relative to the direction of the *araBAD* operon. Solid lines denote coding regions of DNA, and crosshatched lines denote regions that are transcribed but not translated.

The CAP without cAMP did not protect DNA. At 2.5 μM cAMP, it protected a single region located between the A and B sites (designated CAP site in Figs. 2 and 3). At 250 μM cAMP, the CAP protected an additional region, at −130 to −140, against DNase digestion (not shown in Fig. 2, but see Fig. 3*D*), which was not detectable with the methylation protection test (see Fig. 3*B*). This second CAP site lies entirely within the A site.

Polymerase binding sites at P_{BAD} and P_C were determined under conditions that activate their respective transcriptions *in vitro*. For P_{BAD}, polymerase was incubated with DNA, P2, CAP, and cAMP, and then the mixture was treated with dimethylsulfate or with DNase I. Under these conditions, we found that the A site, the CAP site, and the B site were all protected, showing the combined protection patterns of *araC* and CAP. In addition, there was an enhancement of methylation of the G residue at −15, which is associated with the presence of RNA polymerase (see Fig. 3*A*). In the DNase I protection test, we found that the polymerase protected a segment of DNA extending from −39 to at least +24 (see Fig. 5*E*).

For protection of P_C, polymerase was incubated with DNA, CAP, and cAMP before methylation or DNase I digestion. Under these conditions, in addition to the CAP protection pattern, the A residues at −114 and −137 showed reduced methylation, and the region from −105 to the end of the fragment at −170 was protected against DNase I. In the presence of polymerase, the second, lower-affinity CAP site disappeared from the protection pattern, suggesting that the polymerase prevents CAP from binding to this site.

The A Site Is a "Classical" Operator for the *araC* Gene. The congruency of the RNA polymerase binding site at P_C with the A site is evident from examination of their positions (see Fig. 2). This suggests that the A site may be the operator (15) locus for the repression of *araC* by its own product. If this were true, one might expect to find the following: (*i*) As the activity of P_C is reduced *in vivo* by introducing an *araC*+ gene, with or without L-arabinose in the medium (5), this operator may be able to bind either P1 or P2 and (*ii*) the affinity of the operator for binding *araC* protein should be strong enough to compete successfully with polymerase binding.

We examined the ability of the A site to bind P1 (Fig. 4). The *araC* protein solution was exhaustively dialyzed to remove all traces of arabinose. Varying concentrations of this preparation were then incubated with a constant amount of DNA in the presence or absence of added L-arabinose. The mixtures were subjected to digestion with DNase I. The results showed that (*i*) the A site can bind either P1 or P2, whereas the B site, especially the portion designated B2, binds only P2; (*ii*) the A site has a greater affinity for P2 than for P1 (for complete protection, the protein/DNA ratio is 20 for P1 and 2 for P2); and (*iii*) the anti-inducer D-fucose, an analog of L-arabinose that competitively inhibits *araBAD* transcription, confers on the *araC* protein a conformation (P1′) that has no affinity for the B2 site and an affinity for the A site intermediate between those of P2 and P1.

To show that binding at the A site interferes with P_C activity, a set of transcription assays was set up in which the concentrations of *araC* protein and DNA paralleled those described in

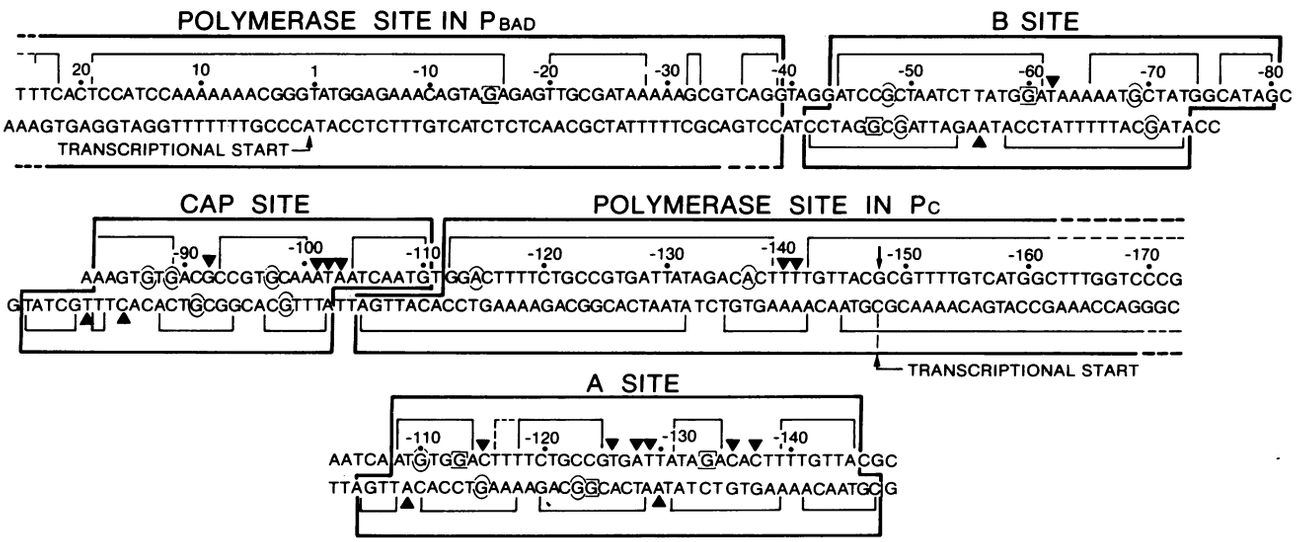

FIG. 2. DNA-protein contacts in the *ara* regulatory region. A schematic representation of the results from methylation protection and DNase I footprinting experiments (see Fig. 3). ○ and □, Purines that show decreased or increased reactivity with dimethyl sulfate, respectively; ⌐¬, DNA regions that show decreased susceptibility to DNase cleavage; ▼, enhanced cleavage by DNase at the 3′ side of the indicated base.

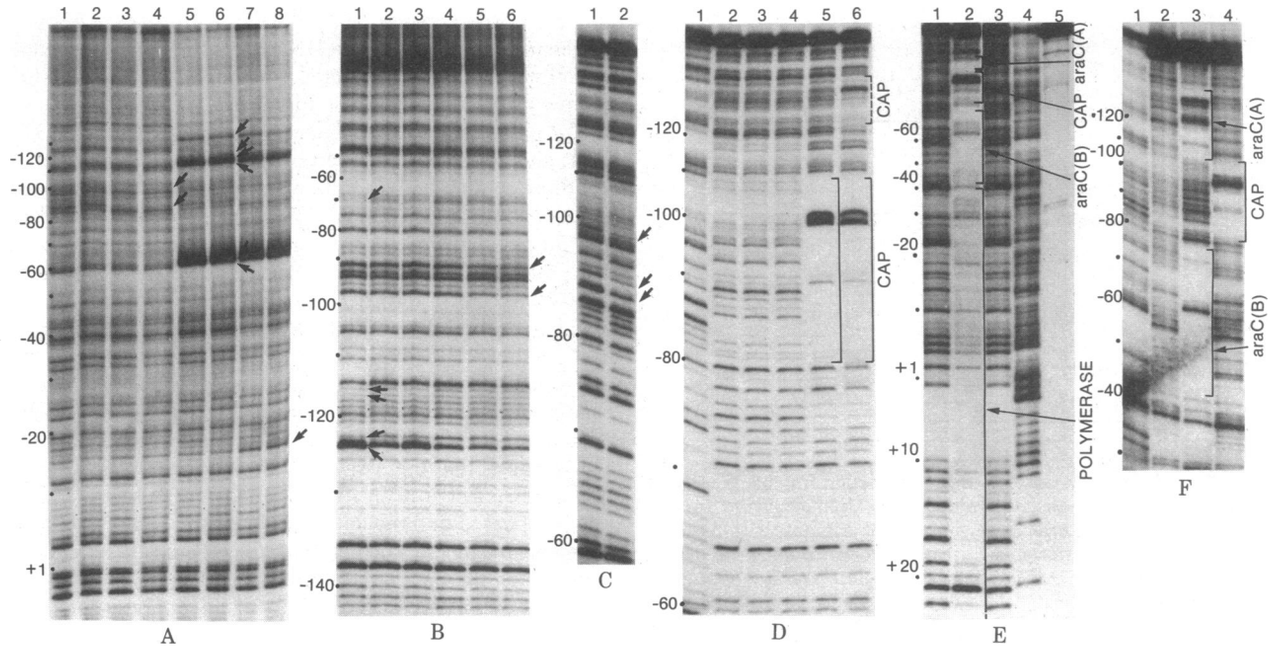

FIG. 3. DNA-protein contacts in the *ara* regulatory region. All protection experiments were conducted with the following concentrations of protein (when present) and DNA: 100 nM *araC* protein (±L-arabinose at 33 mM); 100 nM CAP (±cAMP at 250 μM); 130 nM RNA polymerase holoenzyme; and 10 nM double-stranded restriction fragment DNA, 5′ labeled at one end. Methylation protection: DNA and protein(s) were incubated 10 min at 37°C in 20 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$/90 mM KOAc, in a total vol of 100 μl. Carrier DNA (1 μl; 4 μg of sonicated salmon sperm DNA) was added, and 1 min later 1 μl of 10.7 M dimethylsulfate was added. After 1 min in the presence of dimethylsulfate, the mixture was transferred to 25 μl of a chilled solution of dimethylsulfate stop (1.5 M NaOAc, pH 7/1 M mercaptoethanol/tRNA at 100 μg/ml), and twice treated with ethanol. The precipitates were taken up in 20 μl of 10 mM sodium phosphate, pH 7/1 mM EDTA, heated to 90°C for 15 min and then treated with 2 μl of 1 M NaOH and hydrolysis at 90°C for 30 min in sealed capillaries. The hydrolysates were neutralized with 3.75 μl of 1 M HOAc, and the fragments were precipitated by addition of ethanol, dissolved in 10 μl of 80% (vol/vol) deionized formamide/50 mM Tris·borate, pH 8.3/1 mM EDTA/0.1% xylene cyanol/0.1% bromphenol blue, heated 1 min at 90°, and subjected to electrophoresis in 12% polyacrylamide/7 M urea gels (9). DNase I protection: DNA and protein(s) were incubated 16 min at 25°C in 10 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$/5 mM $CaCl_2$/100 μM dithiothreitol/90 mM KOAc. DNase I was added in the amount determined by pilot titrations as giving maximum uniformity in size distribution of fragments, and the digestions were stopped after 30 sec by the addition of 25 μl of DNase stop (3 M NH_4OAc/0.25 M EDTA/sonicated DNA at 0.15 mg/ml). The fragments were precipitated twice with ethanol and dissolved in formamide-dye solution prior to electrophoresis as above. No qualitative difference was observed in the pattern when the DNase protection experiment was performed at 37°C with a 20-sec digestion by DNase I. (*A*) Fragment F-5 labeled at the *Hae* III end and methylated in the presence of no protein (lanes 1 and 2); CAP plus cAMP (lanes 3 and 4); araC protein plus arabinose (lanes 5 and 6); CAP plus cAMP, *araC* protein plus arabinose, and RNA polymerase (lanes 7 and 8). (*B*) Fragment F-3 labeled at the *Sau*96AI end and methylated in the presence of *araC* protein plus arabinose (lane 1), *araC* protein plus no sugar (lane 2), *araC* protein plus 33 mM D-fucose (lane 3), no protein (lane 4), CAP (lane 5), CAP plus cAMP (lane 6). (*C*) Fragment F-1 labeled at the *Bam*HI end and methylated in the presence of no protein (lane 1), CAP plus cAMP (lane 2). (*D*) Fragment F-1 labeled at the *Bam*HI end, footprint. Lane 1, methylation (G > A) reaction for position markers; lane 2, no protein other than DNase I; lanes 3–6, CAP plus 0, 25 nM, 2.5 μM, and 250 μM cAMP, respectively. (*E*) Fragment F-2 labeled at the *Hae* III end, footprint. Lanes 1 and 3, no protein other than DNase I; lane 2, *araC*, arabinose, CAP, cAMP, and RNA polymerase; lane 4, G > A position marker; lane 5, no DNase. (*F*) Fragment F-2 labeled at the *Hae* III end, footprint. Lane 1, G > A; lane 2, no protein other than DNase I; lane 3, *araC* protein plus arabinose; lane 4, CAP plus cAMP.

Fig. 4. The transcription products were subjected to electrophoresis; the results are shown in Fig. 5*A*. It is clear that there is a direct correlation between A-site binding and inhibition of *araC* mRNA synthesis.

Direct competition between the *araC* protein and RNA polymerase is shown in the DNase I protection experiment (Fig. 6). It is evident that the protection pattern given by polymerase occupation at P_C (enhancement of digestion at −140 and −141) is altered by the addition of P2, so that a pattern that is characteristic of *araC* protein binding (enhancement at −136 and −138) is seen. The addition of P1 produces an intermediate response, due to the lower affinity of P1 for the A site.

The findings that the A site binds both P1 and P2 and that these interactions successfully compete with polymerase binding at P_C establish the A site as the operator locus for *araC* autoregulation.

The A Site Is Not Within P_{BAD}. The promoter P_{BAD} is under positive control by the CAP and P2. We have shown that both A and B sites bind P2 and that the A site is responsible for the autoregulation of *araC*. Does the A site also contribute to the positive control of P_{BAD}?

We tested whether the binding of *araC* to the A site is required for *araBAD* transcription. We deduced from the differences between the A and B sites in DNA sequence and in binding behavior that the B site may possess a greater affinity for P2 than the A site. We found that, at a low concentration of P2 (10 nM), *araBAD* transcription was activated in the absence of A-site binding (see Fig. 5). Note that the A site cannot bind any form of *araC* (P2, P1, or P1′) at this concentration (see Fig. 4); this is shown by the fact that, in Fig. 5*B*, lanes 7–11 showed the same number of *araC* initiations, despite the gradual replacement of P2 by P1′. On the other hand, P_{BAD} was activated with only 10 nM P2. We conclude from these results that (*i*) the A site is completely unoccupied at 10 nM P2; (*ii*) *araBAD* transcription requires binding at the B site only, not at the A site; (*iii*) the inhibition of P_{BAD} by D-fucose is due to removal of *araC* protein from the B site [because the *araC* protein–D-fucose complex, P1′, does not bind the B site (see Fig. 4)]; and (*iv*) the *araC* gene under autoregulation still produces sufficient *araC* protein to sustain *araBAD* activation.

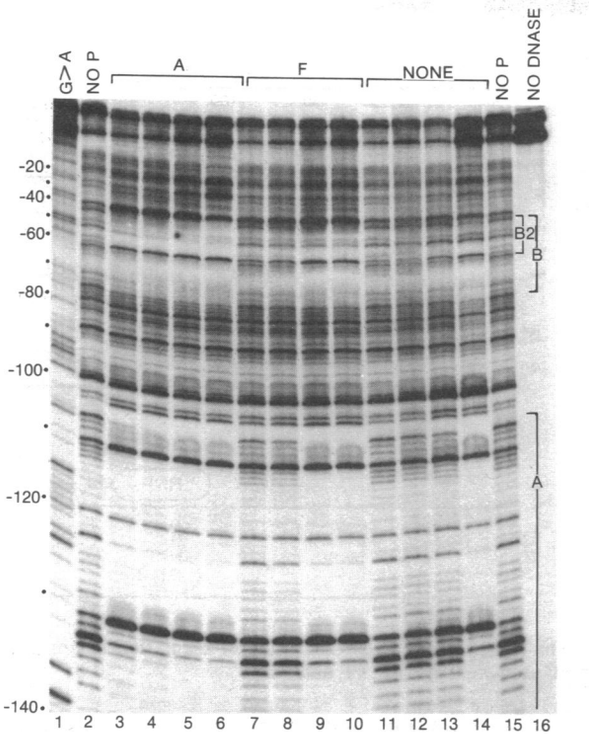

FIG. 4. Affinity of the A and B sites for the *araC* protein in its various conformational states. Fragment F-2 (10 nM) labeled at the Sau96AI end and *araC* protein concentration varied (lanes 3, 7, and 11, 20 nM; lanes 4, 8, and 12, 40 nM; lanes 5, 9, and 13, 100 nM; lanes 6, 10, and 14, 200 nM). Binding was tested for P2 (*A*, 33 mM arabinose), P1′ (*F*, 33 mM D-fucose), or P1 (none) and visualized by the footprint method. Experimental procedure was as described in Fig. 3 legend. No P, no protein other than DNase I.

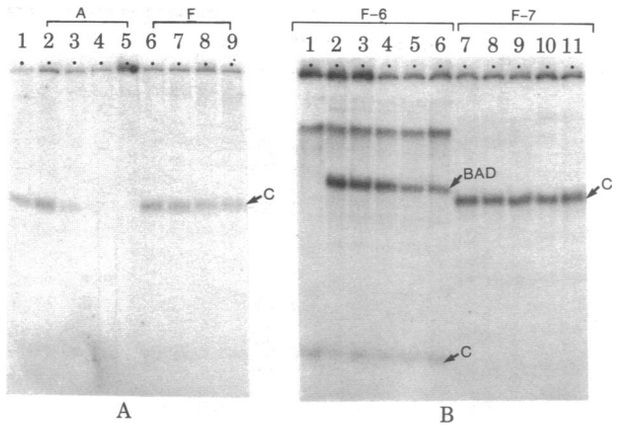

FIG. 5. Analysis of *in vitro* transcription products on 5% acrylamide/7 M urea gels. Transcription reaction was carried out as described (9), except that the reaction volume was 20 μl. (*A*) Effect of state of occupancy at A site on *araC* promoter activity. DNA template F-6 at 10 nM; CAP at 100 nM and cAMP at 250 μM in all reactions; RNA polymerase at 10 nM; and *araC* protein concentration varied (lane 1, none; lanes 2 and 6, 10 nM; lanes 3 and 7, 20 nM; lanes 4 and 8, 40 nM; lanes 5 and 9, 100 nM). DNA and proteins were preincubated for 7 min, challenged by the addition of heparin to 100 μg/ml, and followed 5 sec later by the addition of the four nucleotide triphosphates as described (9). Lanes 2–5 and 6–9 show transcription products in the presence of 33 mM L-arabinose or 33 mM D-fucose, respectively. C denotes position of the *araC* transcript. (*B*) Effect of state of occupancy at A site on *araBAD* promoter activity. DNA template F-6 for lanes 1–6 and F-7 for lanes 7–11, all at 10 nM; CAP at 100 nM, cAMP at 5 μM, and L-arabinose at 1 mM in all reactions. Lane 1, no *araC* protein; all others, *araC* protein at 10 nM. Lanes 2–6 and 7–11 represent reactions with D-fucose at 0, 0.1, 1, 10, and 100 mM, in that order. BAD and C denote positions of *araBAD* and *araC* transcripts, respectively.

DISCUSSION

We have concluded that there are five DNA-protein contact regions in the 147-base-pair DNA segment lying between the transcriptional start points of *araBAD* and *araC*. These are (in their order from left to right in Figs. 2 and 7) (*i*) the site of polymerase binding for *araBAD* transcription; (*ii*) the B site, responsible for the positive control of the *araBAD* operon by P2; (*iii*) the CAP site, which positively controls the expression of *araBAD* on the left and *araC* on the right; (*iv*) the site of polymerase binding for *araC* transcription; and (*v*) the A site, which is located within site *iv* and identified as an operator locus for the *araC* gene. These five DNA-protein contact regions make up the promoters of *araBAD* and *araC*.

We have presented evidence to show that the *araBAD* promoter is comprised of the first three sites described above. The interaction of the CAP–cAMP complex at the CAP site and the interaction of P2 at the B site are both necessary for the attachment of RNA polymerase to this promoter. On the other hand, we have shown that the binding of P2 to the A site is not required for transcription of *araBAD* in the experiment described in Fig. 5*B*. Thus, the domain of the *araBAD* promoter, previously defined by the segment from −1 to −143, which corresponds to the DNA that is retained in the deletion mutant *araC719* (8), has now been defined as the segment from −1 to about −110.

The *araC* promoter is comprised of the sites *iii* and *iv* above. We believe that the low-affinity CAP site located between −130 and −140 probably does not constitute the site for CAP-stimulation of the *araC* promoter, not only because its position (in relation to the polymerase binding site and transcriptional start point) is at variance with other CAP sites (16, 17), but also because the *in vitro* transcription of *araC* is fully stimulated by a 2.5 μM cAMP (data not shown), which does not produce any binding of the CAP at the low-affinity site (see Fig. 3*D*). Thus, the *araC* promoter shares with the *araBAD* promoter the site of interaction with CAP.

Fig. 7 also summarizes our idea concerning the relationship between the DNA-protein contact sites and promoter activity at P_C and P_{BAD}. At P_C, competition between P2 and RNA polymerase in the induced cell (see Fig. 7*A*) and between P1 and RNA polymerase in the uninduced cell (see Fig. 7 *B* and *C*) maintains the intracellular concentration of *araC* protein at a low level. Because of the high affinity of the B site for P2, this low level of *araC* protein is sufficient to maintain activation of P_{BAD}. In the absence of L-arabinose, P1 can bind to the A site and thereby continue to regulate its own synthesis. The binding of the CAP alone, in the absence of P2, activates P_C but not P_{BAD}.

At the conclusion of this work, we learned of the model for *araC* and *araBAD* regulation proposed by Ogden *et al.* (12), who located two sites on *ara* DNA for the binding of CAP and two sites for the binding of *araC* protein. The CAP-binding sites, designated CRP_{BAD} and CRP_C, correspond to the high- and low-affinity CAP-binding sites described in this paper. The *araC* protein binding sites, designated *araO* and *araI*, correspond, by their locations, to the A and B sites, respectively. Based on these four sites, the known start point for *araBAD* transcription at +1 (6), and an estimated start point for *araC* transcription near −170, Ogden *et al.* proposed (*i*) that *araBAD* and *araC* are stimulated by the binding of CAP to distinct regions on the DNA—CRP_{BAD} and CRP_C, respectively, and (*ii*) that the *araO*, when bound to *araC* protein, prevents the binding of CAP to either CRP_C or CRP_{BAD}, and this in turn de-

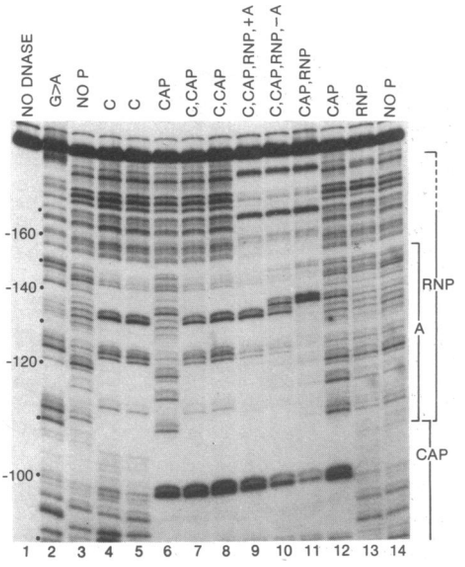

FIG. 6. Competition between *araC* protein and RNA polymerase for the same binding site at *araC* promoter. DNA fragment F-4 labeled at the *Bam*HI end at 10 nM and additions as indicated. A, arabinose; No P, no protein other than DNase I; RNP, RNA polymerase. Experimental procedure as described in Fig. 3 legend. Binding of polymerase near the *Hpa* II end of the F-4 fragment is not due to a true binding site; no binding at this region is observed in a longer fragment (obtained by cutting with *Mbo* II at −274 instead of with *Hpa* II at −209). End-binding to *Hpa* II fragments such as F-6 also gives full-length transcripts (see Fig. 5*B*).

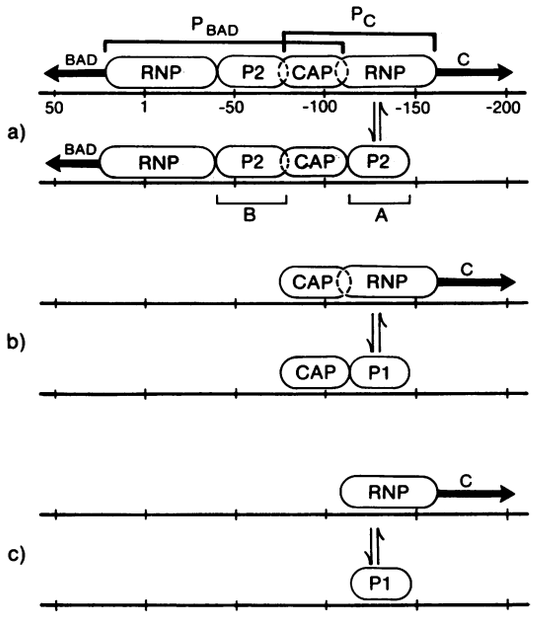

FIG. 7. Schematic representation of regulation of *araBAD* and *araC* promoters by CAP, P1, and P2.

creases transcription of *araC* and *araBAD*.

We believe that the above model is untenable for the following reasons.

1. The start point for *araC* transcription has been located at −148 (9), and to initiate *araC* transcription, RNA polymerase attaches to P_C and protects a 65-bp region from −105 to −170. The low-affinity CAP site, therefore, lies entirely within the polymerase attachment site, and it does not appear likely that the binding of CAP to CRP_C would enhance polymerase binding to P_C.

2. The high-affinity CAP site, located between −77 and −110, has been shown to be the site of CAP stimulation of both P_C and P_{BAD}.

3. Direct competition between *araC* protein and RNA polymerase for the −105 to −147 region has been shown to result in repression of P_C.

4. Our results show that there is no mutual interference in the binding of *araC* protein to the A site ("*araO*") and the binding of CAP to the high-affinity CAP site ("CRP_{BAD}").

We agree with Ogden *et al.* (12) that the sites of positive control in P_{BAD} are the high-affinity CAP site at −77 to −110 (which should not be termed "CRP_{BAD}," because it is shared by P_{BAD} and P_C) and the B site for *araC* protein binding at −42 to −80. [The designation of this region as "*araI*" departs from the original sense of "*araI*," which is synonymous with P_{BAD} (1); the mutational changes that accompany *araI* mutations so far sequenced actually all lie outside this region (18).]

We have provided models for the manner of positive control of P_{BAD} by P2 and CAP, of positive control of P_C by CAP, and of negative control of P_C by P1 and P2. At present, there is no evidence to support the idea that the A site, the operator locus for the *araC* gene, is also the site for negative control of *araBAD*.

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