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Repression and Catabolite Gene Activation in the *araBAD* Operon

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Catabolite gene activation of the *araBAD* operon was examined by using catabolite gene activator protein (CAP) site deletion mutants. A high-affinity CAP-binding site between the divergently orientated *araBAD* and *araC* operons has been previously identified by DNase I footprinting techniques. Subsequent experiments disagreed as to whether this site is directly involved in stimulating *araBAD* expression. In this paper, we present data showing that deletions generated by in vitro mutagenesis of the CAP site led to a five- to sixfold reduction in single-copy *araBAD* promoter activity in vivo. We concluded that catabolite gene activation of *araBAD* involves this CAP site. The hypothesis that CAP stimulates the *araBAD* promoter primarily by relieving repression was then tested. The upstream operator $araO_2$ was required for repression, but we observed that the magnitude of CAP stimulation was unaffected by the presence or absence of $araO_2$. We concluded that CAP plays no role in relieving repression. Other experiments showed that when CAP binds it induces a bend in the *ara* DNA; similar bending has been reported upon CAP binding to *lac* DNA. This conformational change in the DNA may be essential to the mechanism of CAP activation.

The arabinose regulon is one of many gene systems in *Escherichia coli* that are modulated by catabolite gene activator protein (CAP) complexed with cyclic AMP (cAMP) (3, 4, 15, 56, 62). Mutations in adenyl cyclase (*cya*) or CAP (*crp*) genes give rise to Ara$^-$ phenotypes (72); it is not clear whether these Ara$^-$ phenotypes are due to inactivation of one or more catabolite-sensitive promoters of the arabinose regulon. The regulon consists of four operons, *araBAD*, *araC*, *araE*, and *araFG*; these are responsible for L-arabinose catabolism, gene regulation, low-affinity transport, and high-affinity transport, respectively (7, 21, 23, 35, 37). In addition to positive control by CAP-cAMP, each operon is also regulated by AraC protein. Regulation of *ara* genes by AraC involves both positive and negative control mechanisms. AraC protein, plus the ligand L-arabinose, is required for activation of transcription at the promoters of the *araBAD*, *araE*, and *araFG* operons (35, 42). In addition, AraC protein is also a repressor of *araBAD* (22) and *araC* (12, 43, 54) transcription, functioning to further reduce the level of *araBAD* in uninduced cells and autoregulate its intracellular level under both inducing and noninducing conditions.

The CAP dependence of the arabinose regulon has been explored by using many techniques. Casadaban (12) demonstrated that a *crp* mutation reduced expression of *araC*. Kolodrubetz and Schleif (36) showed that a *crp* mutation reduced expression of the *araE*, *araFG*, and *araBAD* genes. The latter study was complicated by the fact that *araE*, *araFG*, and *araBAD* are positively regulated by AraC protein, which is itself sensitive to catabolite activation (12, 29). Mutations in the high-affinity CAP-binding site between the *araBAD* and *araC* genes (Fig. 1) allowed the CAP dependence of the *araBAD* and *araC* promoters to be tested. CAP stimulation of the *araC* promoter was shown to be mediated by this CAP site (18, 54), but its role in the stimulation of *araBAD* transcription was less clear. Miyada et al. (54) concluded that this site was not involved in the stimulation of *araBAD* transcription from experiments using a three-base deletion in the CAP consensus sequence. These results are at variance with those of Dunn and Schleif (18), who constructed deletions which extended into the CAP-binding site that resulted in a threefold decrease in enzyme activity. In the latter case, these CAP site deletions also removed a site, located 265 to 294 base pairs (bp) upstream of *araBAD* (the $araO_2$ site in Fig. 1), which is necessary for *araBAD* repression (17). To explain the discrepancy between their observed threefold reduction and the 30- to 40-fold reduction of *araBAD* activity in *cya* strains (27), they proposed that the principal mechanism of CAP stimulation of *araBAD* involves relief of $araO_2$-mediated repression. Since Miyada's experiments, which disputed the involvement of this CAP site in *araBAD* regulation (54), used strains with intact $araO_2$ regions, this explanation seems unlikely. Clearly, additional experiments are required to define the role of the CAP site located between the *araBAD* and *araC* genes.

We physically separated the *araBAD* and *araC* promoters on the chromosome so that the effect of an altered CAP site on *araBAD* promoter activity could be observed independently of its effect on the *araC* promoter. This allowed us to measure the effect of CAP on the *araBAD* promoter without affecting *araC* expression (54) or the expression of every catabolite-sensitive operon (36). Separation was accomplished by cloning the mutant promoters into lambda and integrating the recombinant phage into the *att* site of a host with wild-type *ara* promoters. The only functional *araB* gene (coding for L-ribulokinase) was linked to the mutant promoters so the kinase levels reflected the effect of each CAP site deletion. Our results indicated that the CAP site stimulated *araBAD* transcription about sixfold and significantly affected growth rates. We then tested the theory that CAP stimulation of *araBAD* is primarily through relief of $araO_2$-mediated repression and found that inclusion of the $araO_2$ site did not affect the degree of CAP activation. CAP was found to induce a conformational change in the DNA at the *ara* CAP site. A similar conformational change seen in CAP-bound *lac* DNA (67) led to the suggestion that these conformational changes are part of a general mechanism of CAP-mediated transcription stimulation.

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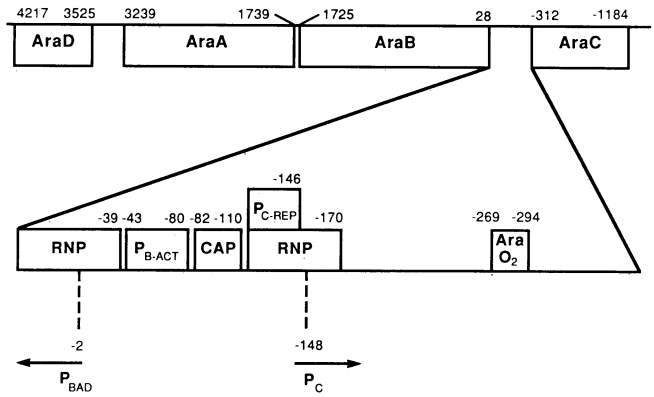

FIG. 1. Molecular organization of the *araBAD* operon. The top line indicates the translational start and end points for the *araC*, *B*, *A*, *D* genes (39, 64; N. Lee, W. Gielow, R. Martin, F. Hamilton, and A. Fowler, Gene, in press). The bottom line is a blowup of the regulatory region between *araB* and *araC*. The sites of interaction with RNA polymerase (RNP), C protein (p_{B-ACT}, p_{C-REP}, and $araO_2$), and CAP, determined by DNase I protection experiments (17, 43, 55), are indicated. The transcriptional start points at p_{BAD} and p_C are also indicated (64; N. Lee, unpublished data). Arrows indicate the direction of transcription. Nucleotides are numbered with respect to the transcriptional start point of *araBAD* (39). Recent experiments from this laboratory located the in vivo transcriptional start point of p_{BAD} at −2.

MATERIALS AND METHODS

Media. Minimal media were prepared as previously described (25) and supplemented with 0.4% carbohydrate and 0.05 mM $MnCl_2$. McConkey medium was prepared as specified by Difco Laboratories and supplemented with 1% arabinose. Kanamycin and ampicillin were used, respectively, at 10- and 30-μg/ml final concentrations. The media used in all phage λ and all phage P1 work were prepared as previously described (2, 25).

Reagents. Restriction endonucleases and other DNA-modifying enzymes were obtained from New England BioLabs, Bethesda Research Laboratories, Inc., or Boehringer Mannheim Biochemicals and used as directed by the manufacturers. AraC protein was purified by a modification and expansion of the method of Wilcox et al. (66). Approximately 300 g of NL 20-164 cells was obtained from 80 liters of log phase culture in minimal-glycerol–0.5% Casamino Acids–ampicillin (10 mg/liter) at 37°C. Cells were harvested at an optical density at 600 nm of slightly over 1. All subsequent operations were carried out between 0 and 4°C. Cells were suspended in 2 volumes of BT buffer (10 mM Tris acetate [pH 7.4], 1 mM EDTA, 0.25 M potassium acetate, 0.2% L-arabinose, 0.1 mM dithiothreitol, 5% glycerol, 50 mg of phenylmethylsulfonyl fluoride dissolved in 10 ml of ethanol per liter) and disrupted at 0°C with a Branson Sonifier. The extract was clarified by centrifugation for 90 min at 65,000 × *g*. The supernatant was brought to 50% saturation with ammonium sulfate [0.29 *g* of $(NH_4)_2SO_4$ per ml] and allowed to equilibrate for 30 min. The precipitate was collected by centrifugation at 8,500 × *g* for 40 min and dissolved in BD buffer, pH 7.4 (10 mM Tris acetate [pH 7.4], 0.2% L-arabinose, 0.1 mM dithiothreitol, 5% glycerol, 50 mg of phenylmethylsulfonyl fluoride dissolved in 10 ml of ethanol per liter). BD, pH 7.4, was added till a 0.003 difference in refractive index between the resuspended pellet and the BD buffer was reached; this corresponds to 0.25 M salt. This solution was loaded onto a phosphocellulose column which had been equilibrated with BD, pH 7.4. The column volume was equal to 1.2 times the number of grams of cells. The sample was washed on with 1 column volume of BT and then eluted with a linear gradient of BD–0.3 to 0.8 M potassium acetate. A_{280} and Zubay assays (66) identified fractions enriched in AraC protein, which were pooled and dialyzed against BD (pH 5.05)–0.65 M potassium acetate. The solution was clarified by centrifugation at 13,000 × *g* for 10 min and concentrated to 10 to 20 ml by dialysis against 35% polyethylene glycol in BD (pH 5.05) (no glycerol)–0.65 M potassium acetate. After further dialysis against BD (pH 6.5)–0.65 M potassium acetate and clarification, the solution was loaded onto a Sephadex G-100 column which had been equilibrated with BD (pH 6.5)–0.65 M potassium acetate. A_{280} and Zubay assays identified active fractions, which were pooled and added to an equal volume of BD, pH 6.5. Final purification was obtained via a hydroxyapatite column equilibrated with BD, pH 6.5. The sample was washed on with BD (pH 6.5)–0.325 M potassium acetate and eluted with BD (pH 6.5)–0.8 M potassium acetate. A_{280} identified peak fractions, which were pooled and stored at −70°C in small portions. A typical preparation produced 10 mg of AraC protein, which was homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

CAP was purified from *E. coli* by a procedure essentially the same as that described by Anderson et al. (1).

Bacterial strains and plasmid constructions. All strains

TABLE 1. Strain list

Strain	Genotype	Source
NL20164	Δ*araC766 recA*(pNL4)	This laboratory
NL30183	*araA2 recA*	This laboratory
NL30269	Δ*araB716 recA*	This laboratory
NL30383	Δ(*araC-araA*)*744 supE supF metB trpR hsdR hsdM*$^+$ *fhuA21 rpsL lacU169 proC*::Tn*5*	This laboratory
HL435	Δ*araB716 recA*/λgt11::Δ*araBp905*(pMC9)	This paper
HL436	Δ*araB716 recA*/λgt11::Δ*araBp900*(pMC9)	This paper
HL437	Δ*araB716 recA*/λgt11::Δ*araBp902*(pMC9)	This paper
HL439	Δ*araB716 recA*/λgt11::*araBp*$^+$(pMC9)	This paper
HL506	Δ*araB716 recA*/λgt11::Δ*araBp*(pMC9)D3	This paper
HL507	Δ*araB716 recA*/λgt11::Δ*araBp*(pMC9)D6	This paper
HL6	*araA2 recA*/λgt11::Δ*araBp* D6 *araBp*::$^+$6 bp[a] *araO*$_2$$^+$(pMC9)	This paper
HL7	*araA2 recA*/λgt11::Δ*araBp*$^+$ *araO*$_2$(pMC9)	This paper
HL8	*araA2 recA*/λgt11::Δ*araBp* D6 *araO*$_2$$^+$(pMC9)	This paper
HL9	*araA2 recA*/λgt11::Δ*araBp* D6 *araO*$_2$(pMC9)	This paper
HL10	*araA2 recA*/λgt11::*araBp*$^+$ *araO*$_2$$^+$(pMC9)	This paper

[a] A 6-bp insertion (5′ACGTGG) between −180 and −181 (see Materials and Methods).

used in this study were derivatives of *E. coli* K-12 whose L-arabinose region had been replaced by that of *E. coli* B/r. The sources and genetic characteristics of all strains used in this study are listed in Table 1.

(i) Construction of strains carrying CAP site deletions linked to *araB*. Deletions of the CAP site were constructed by two methods. The first was by nuclease BAL 31 resection. The parent plasmid (pNL9) for construction of CAP site deletions by BAL 31 mutagenesis contained a 500-bp *Msp*I restriction fragment (−210 to +290) from the arabinose regulatory region cloned into the *Cla*I site of pBR322. pNL9 (5 μg) was linearized by nuclease *Bst*EII at position −203 in the arabinose regulatory region. The linearized plasmid was treated with 2 U of BAL 31 for 5 min at 30°C, ligated, and used to transform AraB⁻ deletion strain NL30269 to ampicillin resistance. The plasmid DNA from each transformant was isolated by a rapid procedure (49) and screened by restriction analysis. Transformants that had deletions of approximately 200 bp were sequenced by the procedure described by Maxam and Gilbert (52). From a total of 83 transformants screened in this manner, three interesting plasmids that contained the deletions *araBp900*, *araBp902*, and *araBp905* (see Fig. 4) were chosen for further study.

The second method used to make CAP site deletions was oligonucleotide-directed in vitro mutagenesis (71). The DNA template used was CF4 (constructed by C. Francklyn in our laboratory), which contains the 500-bp *Msp*I fragment from the *ara* regulatory region (−210 to +290) as an insert at the *Acc*I site of M13 mp8. The synthetic primer Δ3 had the sequence ATAGCAAAGTACGCCGTGCA, and Δ6 had the sequence ATAGCAAAGTCCGTGCAAAT. The primer extension reactions and ligations were carried out as previously described (71), and the synthesis mixtures were used to transform recipient (JM101) cells directly without further purification. Phage which contained mutant CAP sites were screened by plaque hybridization and confirmed by dideoxy sequencing. The phages containing the 3- and 6-bp CAP deletions (D3 and D6, respectively) were named mp8CF4D3 and mp8CF4D6, respectively.

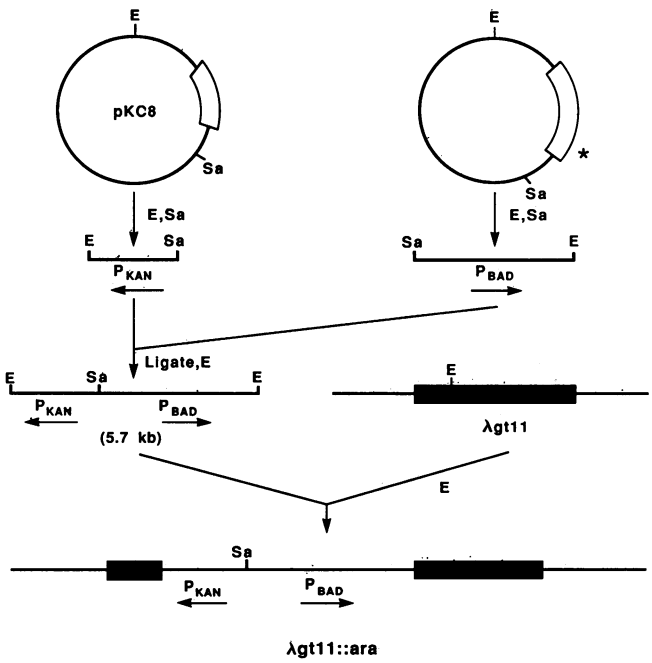

FIG. 2. Construction of CAP site deletions, linking to *araB*, and cloning into λgt11. *Eco*RI-*Sal*I (E-Sa) restriction fragments (4.2 kb) carrying the CAP deletions (*) and *araB* were isolated and ligated to a 1.5-kb *Eco*RI-*Sal*I fragment, containing the Kan^r gene, from pKC8. The 5.7-kb hybrid fragments were then cloned into the *Eco*RI site of λgt11.

The CAP site deletions were linked to a functional *araB* gene. The *Bam*HI fragments, which carried CAP site deletions, from plasmids pHL900, pHL902, pHL905, and pNL9 and phages mp8CF4D3 and mp8CF4D6 were isolated. Each fragment was three-way ligated in a mixture that contained *Bam*HI-linearized pBR322 and a purified *Bam*HI restriction fragment that carries the entire *araB* gene and approximately 7/8 of the *araA* gene (−44 to +3065). After ligation, the individual ligation mixtures were used to transform strain NL30269, and ampicillin-resistant colonies were selected.

Proper alignment of *araB* with the promoter was verified by restriction mapping. In addition, the *araB* promoter was required to be oppositely oriented with respect to the tetracycline promoter.

The various mutant promoter-*araB* constructions were then cloned into λgt11. The 4.2-kilobase (kb) *Eco*RI-*Sal*I restriction fragment which carries the CAP promoter mutations linked to *araB* was isolated (Fig. 2). The *Eco*RI-*Sal*I fragments were individually ligated to a 1.5-kb *Eco*RI-*Sal*I fragment (containing the Kan^r gene) from plasmid pKC8 (pKC8 was constructed in our laboratory and reverses the orientation of the *Hin*dIII-*Sma*I fragment containing the Kan^r gene in pKC7 [59; Fig. 2; obtained from G. Tschumper]). The ligation mixtures were subsequently heated at 66°C for 10 min to inactivate the ligase, ethanol precipitated, and digested with *Eco*RI, and 5.7-kb fragments were isolated. Each 5.7-kb fragment carrying the respective CAP promoter mutation plus the Kan^r gene was ligated to λgt11 that had been digested with *Eco*RI. The ligation mixture was used to transfect strain NL30-383 by the procedure described by Arber et al. (2). Clear plaques were picked from tryptone broth–X-Gal plates after 12 h. Lysates were made from individual plaques, and the lysates were used as a source in the isolation of λ DNA by the rapid method detailed by Maniatis et al. (49). Restriction analysis identified the recombinant λgt11 phage which contained the proper insert and had the *araB* promoters carried on the phage oriented oppositely with respect to the *lac* promoter in λgt11.

The recombinant phage were used to lysogenize NL30-269 (*araB recA*). These strains have the prophages integrated into the λ *att* site and are recombination deficient. The chromosomal *araB* gene contains a small deletion, so all L-ribulokinase activity is due to the *araB* gene linked to the CAP deletion promoter. Verification of mutant CAP sites was by DNA sequence analysis of the appropriate restriction fragment from recombinant λgt11 DNA. Upon sequence confirmation, single lysogens were transformed with plasmid pMC9 (obtained from G. Tschumper) to shut down the *lac* promoter in λgt11 (11). The resulting strains were HL435 (Δ*araBp905*), HL436 (Δ*araBp900*), HL437 (Δ*araBp902*), HL439 (wild type), HL506 (Δ*araBp* D3), and HL507 (Δ*araBp* D6).

(ii) Construction of strains carrying the CAP site deletion D6 linked to a functional *araA* gene. Plasmid pHL481, which carried a D6 promoter and *araB*, was digested with endonuclease *Cla*I, and the vector fragment of the plasmid was ligated to an *Asu*II-*Cla*I restriction fragment (positions 2084 to 3978) from pNL16 (Fig. 3A). Plasmid pNL16 has the entire *araCOIBAD* operon cloned as a *Cla*I-*Acc*I fragment in

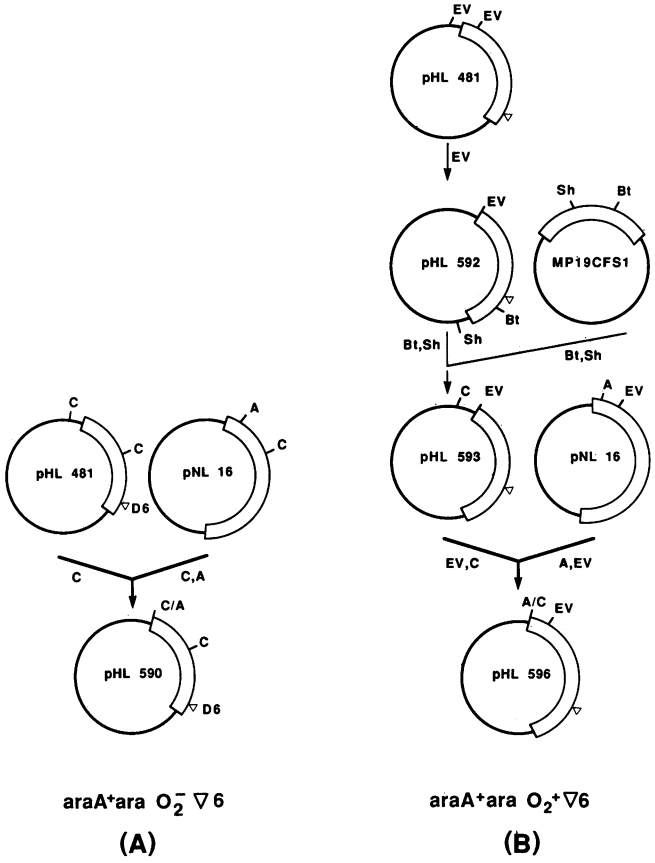

FIG. 3. Linking CAP site deletion D6 to *araA* and $araO_2$. (Part A) The plasmid pHL481, which carries D6, was cut with *Cla*I (C), and the large vector fragment was ligated to a 1.9-kb *Cla*I-*Asu*II (C-A) restriction fragment from pNL16. pNL16 has the entire *araC* and *araBAD* operons, cloned as a *Cla*I-*Acc*I fragment, in a deletion derivative of pBR322. The *Cla*I-*Asu*II (2084 to 3978) restriction fragment carries the distal part of *araA* missing in pHL481. The resulting plasmid, pHL590, is AraA$^+$. (Part B) To add the $araO_2$ site, pHL481 was digested with *Eco*RV (EV) and religated, removing a *Bst*EII site. The short *Bst*EII-*Sph*I (Bt-Sh) fragment was then removed and replaced with a *Bst*EII-*Sph*I (−203 to −860) fragment from mp19CFS1. The resulting plasmid, pHI593, carried $araO_2$. It was made AraA$^+$ by replacing on *Eco*RV-*Cla*I fragment with pBR322 sequences with an *Eco*RV-*Asu*II (1045 to 3994) fragment from pNL16. The resulting plasmid, pHL596, contained *araA* and $araO_2$.

a deletion derivative of plasmid pBR322. The ligation mixture was used to transform the AraA$^-$ strain NL30183, and Ara$^+$ Ap^r colonies were selected. The configuration of the resultant plasmid, pHL590, was verified by restriction analysis. A derivative of pHL590 carrying the wild-type *araBAD* promoter was also constructed. The construction was identical except that the starting plasmid was pHL270, which carries a wild-type promoter.

In addition to the above plasmids, three other plasmids were made which link *araA* to the *araBAD* promoter. These plasmids contain the entire *araBAD* regulatory region, including the upstream site, $araO_2$, implicated in *araBAD* repression. To add the additional upstream sequences, plasmid pHL481, which carries D6, was digested with *Eco*RV and religated, removing a *Bst*EII site (Fig. 3B). The resultant plasmid, pHL592, contains a unique *Bst*EII site at −203. *ara* DNA upstream of −203 was added to pHL592 by replacing the short *Bst*EII-*Sph*I restriction fragment with a purified *Bst*EII-*Sph*I (−03 to −806) fragment from mp19 CFS1 (Fig. 3B). mp19CFS1 (constructed by C. Franklyn in our laboratory) has an *araC* gene with an *Sph*I site at −860 cloned into the M13 phage mp19. The resulting plasmid, pHL593, was made AraA$^+$ by replacing an *Eco*RV-*Cla*I fragment with an *Eco*RV-*Asu*II (1045 to 3978) fragment from pNL16 (Fig. 3B). The final plasmid, pHL596, contains the 6-bp deletion and the entire *araBAD* regulatory region and is AraA$^+$. To make a wild-type version of this plasmid, pHL593 was digested with *Bam*HI and *Bst*EII (−44 to −203), and the small fragment was replaced by a wild-type fragment from pNL12. This plasmid, pHL597, was then made AraA$^+$, as described for pHL596, and named pHL598. Lastly, a plasmid similar to pHL596 but with a 6-bp insertion (5'ACGTGG) between −180 and −181 was constructed. As before, this was done by oligonucleotide-directed mutagenesis with a 32-mer and mp8CF4. Phage containing the appropriate 6-bp insertion were identified, and dideoxy sequencing confirmed the presence of the insertion. A *Mlu*I-*Bst*EII fragment (−150 to −203) that carried the 6-bp insertion was exchanged with the analogous wild-type *Mlu*I-*Bst*EII fragment from plasmid pHL593. The resultant plasmid, pHL599, was then made AraA$^+$ as described for the construction of plasmid pHL596.

Test for polylysogens by Southern hybridization. Genomic DNA was prepared as described by Gay (24) and digested with *Pvu*I. The digested DNA was prepared for hybridization and probed as described by Kidd et al. (33). The probe was a 649-bp *Xba*I-*Hin*dIII fragment (24508 to 25157) from λ DNA. It was cloned into M13 and labeled by nick translation (49). Single lysogens showed a 4.3-kb radioactive band corresponding to a *Pvu*I fragment which contained phage and chromosomal sequences. Polylysogens had an additional band, 11.3 kb, which contained only phage sequences (68).

DNase I protection of mutant CAP site promoters. The procedures for isolation of restriction fragments for DNase I protection studies were adopted from published procedures of Maxam and Gilbert (52). DNase I protection studies were performed as described by Lee et al. (43).

Gel mobility assay. The gel mobility assay described by Wu and Crothers (67) was used to map the bend position in the *ara* CAP site. Plasmid pNL9td was constructed by cloning in tandem two 500-bp *Msp*I fragments (−210 to +290) from the *araBAD* regulatory region into the *Cla*I site of pBR322. pNL9td was cut with restriction enzymes which cut only once in the sequence, and a set of circularly permutated fragments was isolated. CAP-binding reactions were carried out in 10 mM Tris (pH 7.4)–1 mM EDTA–50 mM KCl–10 mM cAMP–0.1 mg of bovine serum albumin per ml. A mixture of 3.03×10^{-8} M fragment and 3.30×10^{-7} M CAP was allowed to equilibrate for 30 min at room temperature before 10-fold dilution with 10 mM Tris (pH 7.4)–1 mM EDTA–10 μM cAMP–0.1 mg of bovine serum albumin per ml–10 μg of xylene cyanol FF per ml–5% glycerol. Samples were immediately loaded on a prerun 4% polyacrylamide gel, and electrophoresis was carried out in a vertical device modified for buffer circulation. DNA fragments in the gels were visualized by staining with ethidium bromide.

Enzyme assays. Cells were prepared for enzyme assays by growth to saturation at 30°C in minimal-arabinose (induced) or minimal-glycerol (uninduced) medium, both plus ampicillin and kanamycin. The saturated cultures were diluted, in the same media, to an A_{600} of approximately 0.1. The cells were then grown to an A_{600} of 1.0 and harvested, and

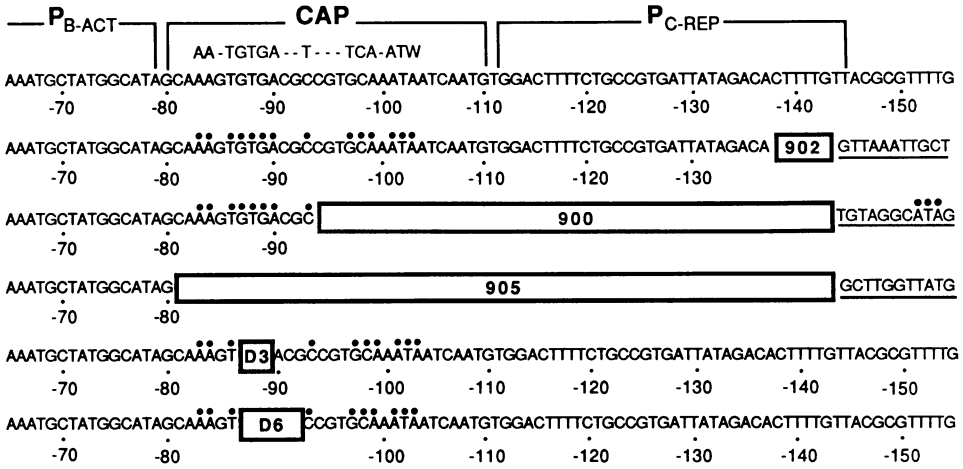

FIG. 4. CAP site deletions constructed by nuclease BAL 31 and by oligonucleotide-directed mutagenesis. The pBR322 sequences which were joined to the *ara* sequences in plasmids pHL900, pHL902, and pHL905 are underlined. Boxes correspond to bases in the *ara* region that were deleted. The numbers within the boxes designate the various mutations. The binding sites of the CAP and AraC ($p_{B\text{-}ACT}$ and $p_{C\text{-}REP}$) proteins are as indicated. Consensus bases (as defined in reference 20) in the *ara* CAP site are shown above the sequences (W = A to T). The numbers below the sequence correspond to the numbering system as shown in Fig. 1.

cell-free extracts were prepared. L-Arabinose isomerase was assayed by the method of Cribbs and Englesberg (14), in which ribulose production is detected by the cysteine-carbazole test. Under the conditions used, the assay was reliable down to 0.02 U/mg. L-Ribulokinase was assayed by a modification of the method of Lee and Englesberg (41). Each 0.25-ml reaction mixture contained 0.07 ml of 0.3 M Tris hydrochloride (pH 7.6)–0.02 ml of 0.1 M glutathione–0.01 ml of 0.1 M EDTA (pH 7.5)–0.01 ml of 1 M $MgCl_2$–0.02 ml of 0.5 M NaF–0.04 ml of 0.2 M ATP (pH 7)–0.025 ml of 0.1 M [^{14}C]ribulose (1.6×10^4 cpm/nmol)–0.055 ml of extract and water. The reaction mixture was preincubated at 37°C, and extract was added to start the reaction. Samples (0.05 ml) were taken at 0, 3, 6, and 9 min and spotted onto DE81 filter circles which were immediately immersed in absolute ethanol. The filters were washed eight times in 80% ethanol, dried, then counted in a Beckman LS-150 liquid scintillation counter. The reaction was linear with time. Protein concentrations were determined by the method of Lowry et al. (45). Specific activity represents units of activity per milligram of protein. One unit of L-arabinose isomerase activity is defined as the formation of 1 μmol of L-ribulose per h at 37°C. One unit of L-ribulokinase activity is defined as the formation of 1 μmol of L-ribulose 5-phosphate per h at 37°C.

RESULTS

Construction of CAP site deletions. Mutant CAP sites were generated by nuclease BAL31 and oligonucleotide-directed mutagenesis. A restriction fragment carrying the arabinose regulatory region was cloned into pBR322, cut internally with a restriction enzyme, and then digested with BAL31 to generate deletions (as described in Materials and Methods). Figure 4 shows a comparison of the mutant sequences with the wild type. Deletion *araBp902* removes all DNA upstream of the CAP site but does not delete any nucleotide within the CAP site. Deletion *araBp900* extends to base −94 in the *ara* CAP site, thereby deleting bases in the right half of the CAP consensus sequence (Fig. 4). Note that in *araBp900* a portion of the CAP consensus removed by BAL31 has been fortuitously restored by an upstream sequence. Deletion *araBp905* deletes the entire *ara* CAP site but does not delete any bases in the *araBAD* activator site ($p_{B\text{-}ACT}$). Smaller deletions were made by using oligonucleotide-directed in vitro mutagenesis. A restriction fragment containing the *ara* regulatory region was cloned into M13 phage, and synthetic oligonucleotides were used to construct 3- and 6-bp deletions. D3 deletes the central three bases (positions 5 to 7) in the CAP consensus sequence (Fig. 4). D6 deletes positions 5 to 10 in the CAP consensus sequence. Both of these mutations alter the highly conserved left half of the symmetric CAP site and leave the right half unchanged.

To assess the physiological consequences of the *ara* CAP site mutations, each mutant promoter was joined to *araB* and cloned into λ phage. An *araB recA* strain (NL30269) was lysogenized with these recombinant phages. These strains were shown to be single lysogens by their sensitivity to λ *c*I90 *c*17 (60) and by Southern hybridization. Confirmed single lysogens were then transformed with plasmid pMC9 (11) to shut down any transcription originating from the *lac* promoter in λgt11.

CAP site deletions lower expression of *araBAD* in vivo. The effect of CAP site mutations on *araBAD* promoter activity

TABLE 2. L-Arabinose isomerase and L-ribulokinase activities in strains containing mutant CAP promoters

Strain (genotype)	Mean (± SEM) sp act (U/mg of protein) of:		Doubling time (min)[a]
	Ribulokinase	Isomerase	
HL439 (*ara* CAP$^+$ [control])	13.9 ± 0.4	77.4 ± 0.7	115
HL437 (Δ*araBp902* [control])	13.9 ± 0.2	66.4 ± 1.1	115
HL436 (Δ*araBp900*)	5.8 ± 0.6	82.4 ± 0.4	145
HL506 (Δ*araBp* D3)	6.1 ± 0.2	91.2 ± 3.1	155
HL435 (Δ*araBp905*)	2.7 ± 0.2	128.2 ± 5.2	235
HL507 (Δ*araBp* D6)	2.6 ± 0.2	112.3 ± 1.4	325

[a] Growth characteristics in minimal-arabinose–ampicillin–kanamycin medium.

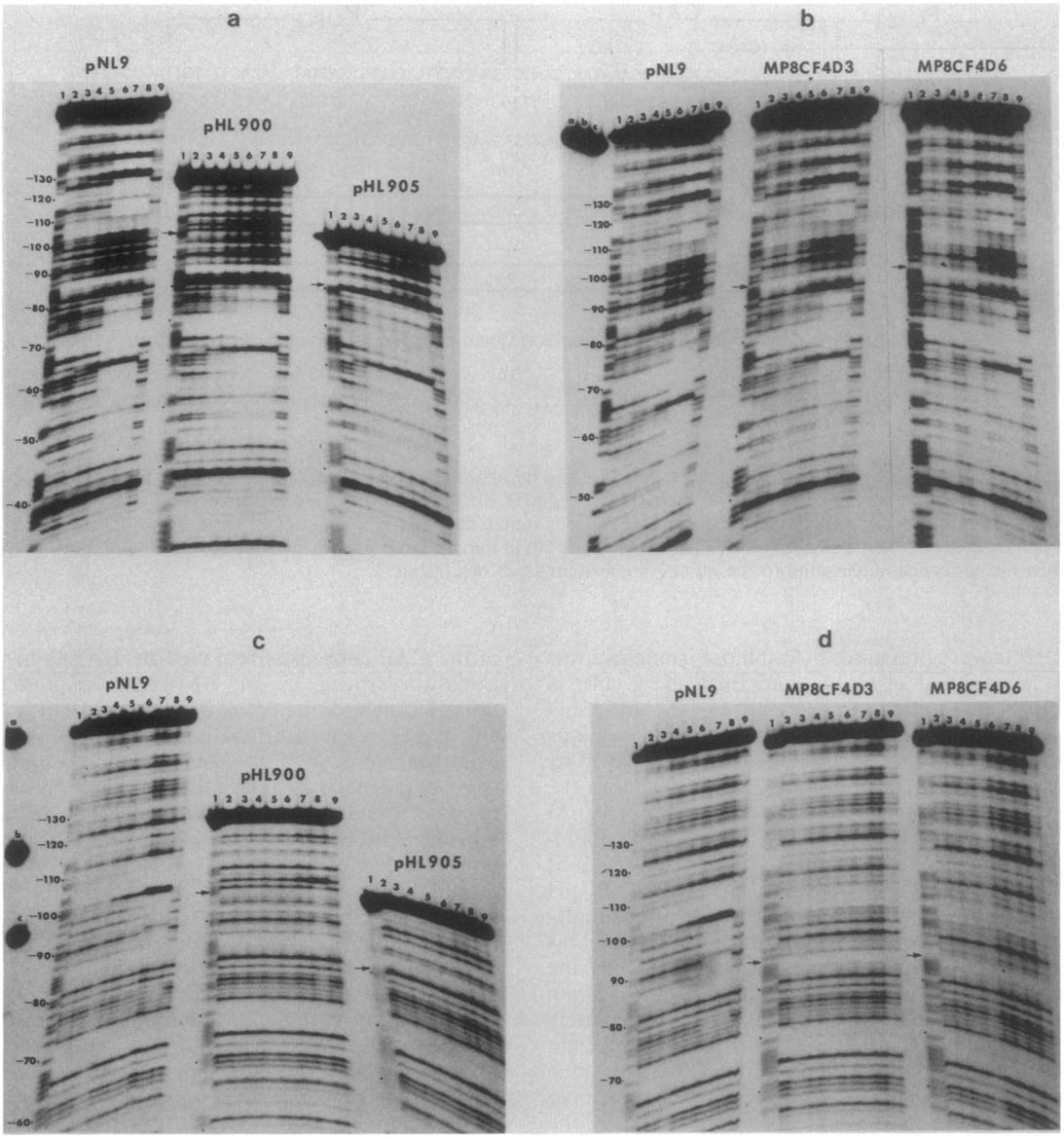

FIG. 5. DNase I protection of promoter fragment from pNL12, pHL900, pHL905, mp8CF4D3, and mp8CH4D6, with either variations in AraC protein concentration (a and b) or variations in CAP concentration (c and d). The sources of the promoter fragments are as indicated, and arrows show the positions of junctures produced by the deletions. All reactions were carried out at a DNA concentration to 10 nM. Lanes: 1, G + A reactions for position markers; 2 and 9, no protein other than DNase I; 3 through 8, AraC protein or CAP at 5, 10, 20, 40, 100, and 200 nM, respectively. Lanes a, b, and c represent fragments to which no DNase I was added.

was tested by assaying the levels of ribulokinase produced under inducing conditions. Since the chromosomal *araB* gene carries a deletion, all L-ribulokinase activity comes from the *araB* gene linked to the mutant promoters. L-Arabinose isomerase assays served as an internal control, measuring the activity of the chromosomal wild-type *araBAD* promoter in these cells.

Mutations within the *ara* CAP site directly affected the activity of the *araBAD* promoter (Table 2). When half of the *ara* CAP site was removed, a 50% decrease in ribulokinase activity was observed (Table 2, HL436). A similar decrease was seen when 3 bp of the CAP TGTGA consensus sequence were deleted (Table 2, HL506). The effect on *araBAD* promoter activity was more dramatic when the entire CAP site or 6 bp in the conserved left half of the CAP site was deleted (Table 2, HL435 and HL507); ribulokinase levels were reduced to 20%. The isomerase levels only varied slightly from strain to strain.

The ribulokinase made from λgt11 *ara* prophage complements the chromosomal ribulokinase deficiency, allowing growth in minimal-arabinose medium. Strains with lowered ribulokinase levels had decreased growth rates which paralleled the severity of the mutational defect (Table 2). This suggested that ribulokinase is the limiting enzyme in the metabolism of L-arabinose. This is not surprising, since ribulokinase has a lower molar activity than either isomerase or epimerase (38, 57), and equimolar amounts of these enzymes are synthesized (N. Lee, unpublished data).

In vitro characterization of CAP site deletions. The results presented above showed that mutations in the *ara* CAP site

directly reduced the activity of the *araBAD* promoter. We next tested the ability of our mutant CAP promoters to bind CAP by using the DNase I protection assay. By titrating against several concentrations of CAP, we hoped to identify those promoters which show reduced affinity for CAP.

Promoter fragments containing deletions D3, D6, and *araBp905* showed no detectable CAP binding (Fig. 5c and d). The promoter fragment containing deletion *araBp900* exhibited the same degree of protection at 200 nM CAP (lane 8) as did the wild-type fragment at 40 nM CAP (lane 5), indicating a fivefold reduction in its affinity for CAP. While deletion of bases within the CAP-binding site greatly diminished binding of the cAMP-CAP complex, the mutant promoter fragments showed no reduction in activator binding at the adjacent AraC-binding site ($p_{B\text{-}ACT}$) (Fig. 5a and b). The slightly stronger binding on the Δ*araBp905* and Δ*araBp900* fragments can be attributed to the absence of the autoregulatory ($p_{C\text{-}REP}$) site in these fragments; $p_{C\text{-}REP}$ competes with $p_{B\text{-}ACT}$ for AraC protein.

The Magnitude of CAP stimulation is not altered by the presence or absence of *araO*$_2$ sequence. While this work was in process, Dunn et al. (17) identified an AraC protein-binding site (*araO*$_2$) located 265 to 294 bp upstream of the *araBAD* promoter as the site responsible for repression of *araBAD*. Hahn et al. (27) proposed that *araBAD* repression occurs when AraC protein bound at *araO*$_2$ interacts via a DNA loop with AraC protein bound at $p_{B\text{-}ACT}$, rendering the promoter inactive. Since the CAP site is located within the loop, it was postulated that the primary mechanism of CAP stimulation of *araBAD* transcription involves its ability to dissolve the loop structure and thereby relieve repression (27). Direct stimulation of the *araBAD* promoter by CAP was judged to be quantitatively insignificant (18, 27).

Strains were constructed that allowed us to test the above model of CAP action. The activities of mutant D6 and wild-type CAP promoters, with and without *araO*$_2$, were compared. We also constructed a strain (HL6) in which the bases deleted by the D6 mutation were scrambled and reinserted between *araO*$_2$ and $p_{B\text{-}ACT}$ to conserve the number of helical turns between these sites. In these constructions, the promoters were linked to araA (Fig. 3), incorporated into λgt11, and introduced as prophages into the *araA recA* host NL30183. The constructs were verified by DNA sequence analysis, and the presence of single prophages was ascertained by Southern hybridizations. After transformation with plasmid pMC9, the strains were assayed for their isomerase specific activities under inducing (Table 3) and noninducing (Table 4) conditions.

If the above model of CAP action were correct, one would expect the presence of *araO*$_2$ to elicit appreciable magnification of the degree of CAP stimulation. Examination of the results showed that this is not the case. The D6 mutation reduced *araBAD* expression approximately sixfold when *araO*$_2$ was absent (Table 3, HL7 versus HL9). When *araO*$_2$ was present, D6 reduced *araBAD* expression by approximately fivefold (Table 3, HL8 versus HL10). Clearly, there was no change in the magnitude of CAP stimulation in the presence of *araO*$_2$.

TABLE 3. Effect of *araO*$_2$ on the activities of mutant CAP promoters under L-arabinose induction

Strain (genotype)	Mean (± SEM) isomerase sp act (U/mg of protein)	Doubling time (min)[a]
HL6 (Δ*araBp* D6 +6[b] *araO*$_2^+$)	46.5 ± 2.5	177
HL7 (*ara* CAP$^+$ *araO*$_2$)	329.4 ± 4.5	150
HL8 (Δ*araBp* D6 *araO*$_2^+$)	25.9 ± 0.9	187
HL9 (Δ*araBp* D6 *araO*$_2$)	54.2 ± 0.8	149
HL10 (*ara* CAP$^+$ *araO*$_2^+$)	136.2 ± 0.6	152

[a] Growth characteristics in minimal-arabinose–ampicillin–kanamycin medium.
[b] See the footnote to Table 1.

TABLE 4. Effect of *araO*$_2$ on the activities of mutant CAP promoters in the absence of L-arabinose

Strain (genotype)	Mean (± SEM) isomerase sp act (U/mg of protein)
HL6 (Δ*araBp* D6 +6[a] *araO*$_2^+$)	0.08 ± 0.02
HL7 (*ara* CAP$^+$ *araO*$_2$)	1.49 ± 0.02
HL8 (Δ*araBp* D6 *araO*$_2^+$)	0.07 ± 0.01
HL9 (Δ*araBp* D6 *araO*$_2$)	0.13 ± 0.02
HL10 (*ara* CAP$^+$ *araO*$_2^+$)	0.12 ± 0.01

[a] See the footnote to table 1.

It was surprising that *araO*$_2$-mediated repression of the *araBAD* promoter operated even when the cells were fully induced, since activation and repression by AraC have previously been regarded as mutually exclusive conditions. The presence of *araO*$_2$ consistently lowered *araBAD* expression twofold in induced cells, both in CAP$^+$ (Table 3, HL7 versus HL10) and in CAP$^-$ (Table 3, HL9 versus HL8) strains. Restoration of the spacing between *araO*$_2$ *and* $p_{B\text{-}ACT}$ did not affect repression (Table 3, HL6 versus HL8).

Under noninducing conditions, a 12-fold increase in *araBAD* expression was seen when the *araO*$_2$ site was deleted (Table 4, HL7 versus HL10). It is this very pronounced effect which first disclosed the negative control of the *araBAD* promoter by AraC (22). Under noninducing conditions, the 12-fold increase in *araBAD* promoter activity was entirely CAP dependent, for removal of the *araO*$_2$ site in the absence of CAP was not accompanied by a 12-fold increase in *araBAD* activity (Table 4, HL8 and HL9).

Growth experiments on these strains indicated that growth in minimal-arabinose medium was retarded when isomerase levels fell below the induced activity see in HL9 (Table 3). Apparently, isomerase levels do not limit cell growth as readily as do ribulokinase levels. (Compare with Table 2).

CAP induces a DNA conformational change at the *ara* CAP site. Recently it was shown that DNA can assume a bent conformation (26, 49) which is important in gene regulation (6). CAP binding to *lac* operon DNA causes it to assume a bent conformation (67). Bent DNA fragments have been shown to migrate considerably slower than other DNA fragments of equal length (50), and gel electrophoresis theories (46) predict that the retardation is governed by the distance of the bend position from one end of the fragment.

We constructed a plasmid that allowed us to test whether CAP bends the DNA at the *ara* CAP site. Plasmid pNL9td contains two identical *ara* CAP site fragments (500 bp, from −210 to +290) that were cloned in tandem in plasmid pBR322. By digesting pNL9td with a variety of restriction endonucleases which cleave only once in each tandemly cloned *ara* fragment, we obtained a set of 500-bp fragments with circularly permuted sequences wherein the distances between the CAP sites and the end of the fragments differed. Each fragment was preincubated with a purified CAP preparation, and the mixture was analyzed by electrophoresis in a polyacrylamide gel (Fig. 6A).

Figure 6B shows the distance of migration in the gel matrix as a function of the location of the CAP-binding site on the

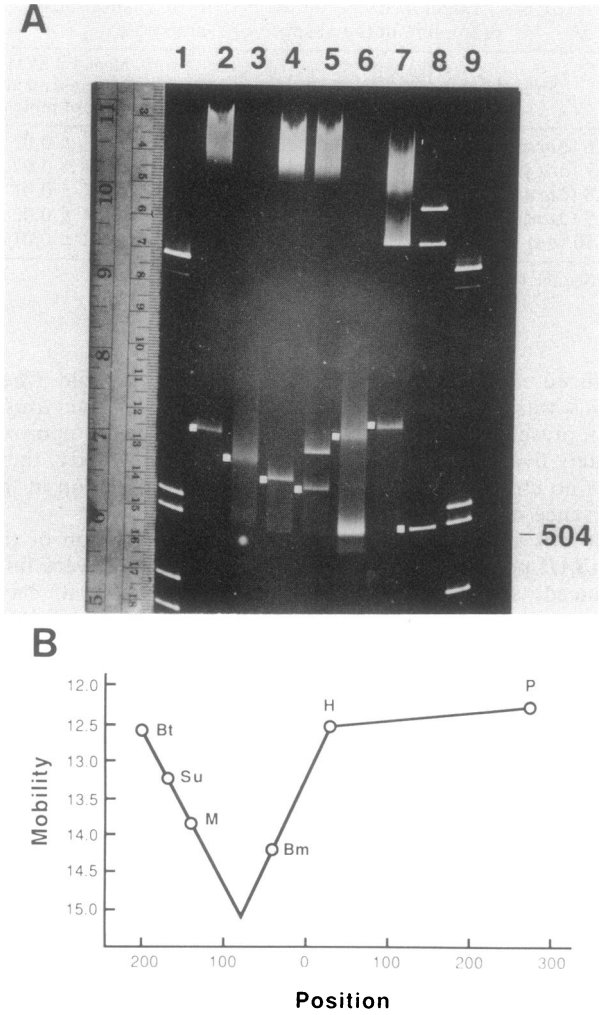

FIG. 6. (A) A 4% polyacrylamide gel showing the presence of a CAP-induced bend locus in the *araBAD* operon promoter. Plasmid pNL9td, a pBR322 derivative carrying two tandem insertions of a 500-bp fragment containing the *araBAD* promoter, was digested with various restriction enzymes to generate a set of 500-bp overlapping fragments with circularly permuted sequences. Two micrograms of the digests or 0.2 μg of purified fragments (in the cases of *Sau*96 and *Hae*III digests) was incubated with CAP (1) and analyzed by electrophoresis in polyacrylamide gel as described by Wu and Crothers (67). The molar ratio of CAP/DNA was 11:1 in 20-μl reaction volumes. Lanes: 1 and 9, size markers produced by *Hin*fI digestion of pBR322 plasmid DNA, showing fragments of 1,631, 517, 506, 396, and 344 bp; 2 through 7, CAP incubated with pNL9td digested with *Bst*EII (lane 2), *Sau*96 (lane 3), *Mlu*I (lane 4), *Bam*HI (lane 5), *Hae*III (lane 6), and *Pvu*II (lane 7). Lane 8 was identical to lane 7 except that CAP had been omitted. In each lane, the bands corresponding to the 500-bp fragment containing the *ara* CAP site is indicated. In Lane 6, the faster-migrating band is a 500-bp contaminant which copurified with the *Hae*III fragment that contained the CAP-binding site. (B) Mapping the CAP-induced bend locus in the *araBAD* operon. The graph is a plot of the distance of migration shown in A as a function of the position of the restriction sites used to generate the fragments.

fragment. This treatment of the data permits mapping of the bend locus on the fragment (67), here shown to be at approximately −84. The 21-bp consensus sequence in the *ara* CAP site is located at −83 to −103 (Fig. 2) with its center by dyad symmetry at −93. Thus, as in *lac*, the bend locus is positioned within the consensus sequence and located several bases away from its center of dyad symmetry.

DISCUSSION

We found that the high-affinity CAP site located at −82 to −110 in the *araBAD* operon is essential for maximal transcription from this operon. We tested the effect of four CAP site deletion mutations on *araBAD* promoter activity. The resemblance of the *ara* CAP site to the CAP consensus sequence (15, 19) is shown in Fig. 4. The consensus is composed of a highly conserved TGTGA sequence, a block of 6 bp with little sequence preference, and a second less well conserved sequence which contains an inverted repeat of the TGTGA sequence.

Deletion of the less well conserved right half of the *ara* CAP site resulted in a greater than twofold decrease in *araBAD* activity (Table 2) and a fivefold reduction in CAP-cAMP binding by DNase I protection (Fig. 5). In other catabolite-sensitive operons, the sequences in this region are required for efficient CAP binding. Removal of these sequences from the galactose operon blocks CAP action in vivo (10) and in vitro (34). The effect on *araBAD* was less dramatic, but three of the consensus base pairs were restored by upstream pBR322 sequences (Fig. 4). The *araBAD* operon appears to be less CAP dependent than the *gal* operon.

Deletion of the entire CAP site resulted in no detectable CAP binding (Fig. 5) and a 5.8-fold drop in *araBAD* activity (Table 2). A slightly reduced drop (threefold) was observed by Dunn and Schleif (18), who used similar deletions on plasmids carrying p_{BAD}-*galK* fusions.

Our results showed that a 3- or 6-bp deletion in the conserved left half of the CAP consensus leads to either partial or complete loss of CAP stimulation. Miyada et al., using a 3-bp deletion in this sequence, first showed that the deletion prevented induction of *araBAD* mRNA from a plasmid (53), but later this deletion was transferred to the chromosome, and enzyme assays showed that it caused only a slight (1.3-fold) reduction in *araBAD* expression (54). We tested Miyada's deletion (D3) and a 6-bp deletion (D6) (Fig. 4). We found that D3 did not completely abolish CAP stimulation. There is, however, consistently a twofold decrease associated with the Miyada deletion (Table 2). One difference between our experiment and Miyada's is that his cultures were induced for only one generation, whereas our cells were grown for multiple generations in the presence of arabinose. The CAP mutation phenotype may not be as pronounced in partially induced strains. D6 caused a greater-than-fivefold decrease in kinase levels; these levels were indistinguishable from those seen after deletion of the entire CAP site (Table 2). Deletion of 6 bp from this highly conserved region eliminated all CAP stimulation from this site. Both D3 and D6 showed no detectable CAP binding at concentrations of CAP up to 200 nM (CAP/DNA at a 20:1 molar ratio) (Fig. 5). Miyada et al. (54) did observe some protection of their 3-bp deletion CAP site in the presence of polymerase. It is conceivable that the D3 promoter in vivo is aided by the presence of other factors.

Catabolite gene activation is weaker in *araBAD* and is less sequence dependent than in other operons. In the *gal* and *lac* operons, point mutations and deletions in the TGTGA sequence abolish CAP stimulation (8, 9, 16, 47, 69). We note, however, that in the *gal* operon CAP-cAMP activates transcription as a result of two simultaneous but separable

events. It indirectly activates the principal promoter (*galP1*) by preventing RNA polymerase binding at a secondary overlapping promoter (*galP2*), and it also directly stimulates the primary promoter (5). Two promoters are also present in the *lac* operon, but there is some controversy as to how they are involved in CAP activation (48, 70). The absence of a second overlapping promoter in the *araBAD* operon suggests that the stringency of binding is less important when promoter blocking is not involved in activation. Furthermore, unlike the *lac* and *gal* operons, activation of *araBAD* requires a second positive regulator, the AraC-arabinose complex (42). If one adopts the view that CAP and AraC are similar in their mechanisms of action, then the upper limit of their combined activity may be defined not by the sum of their individual activities but by the limit set by the RNA polymerase. Consistent with this view is the finding reported in the *malPQ* operon, which has a CAP-binding site but can be rendered fully constitutive in the absence of cAMP-CAP by overproduction of the activator MalT (13). In fact, in a hybrid *malP-lacZ* promoter (*mac*), the MalT protein is able to force RNA polymerase to start transcription, even in the absence of a recognizable Pribnow box (63). On the other hand, if the quantity of AraC activator is considerably below that present in the induced cell, then one might expect the cAMP-CAP complex to play a larger role in *araBAD* promoter stimulation, as it does in the case of uninduced $araO_2$ cells (about 12-fold [Table 4]).

In the experiments shown in Table 2, L-arabinose isomerase (*araA*) assays were also done on the CAP site deletion strains. The only functional *araA* gene in these cells is linked to the wild-type *araBAD* promoter, so these assays allowed us to monitor the activity of the unmutagenized promoter. The isomerase levels were about the same from strain to strain, in contrast to the kinase levels. The slight variation in isomerase levels was inversely correlated with the kinase levels. One possible explanation for this small but consistent observation is that "self catabolite repression" (32, 40) operates in strains with higher kinase levels (which metabolize arabinose more effectively, as shown by their growth rates in minimal-arabinose medium [Table 2]), reducing the activity of the wild-type promoter that governs production of isomerase. We cannot rule out, however, the possibility that an enzymatically inactive isomerase gene (*araA*) distal to the *araB* gene and the mutant promoter present on the prophage results in production of inactive heteropolymers of isomerase (58).

Another aspect of CAP regulation we examined was its relationship to repression of the *araBAD* operon. Hahn et al. (27) suggested that *araBAD* repression involves an interaction between AraC protein bound at $araO_2$ and *araI* ($p_{B\text{-}ACT}$). They proposed that CAP stimulates the *araBAD* promoter by interfering with this interaction to relieve repression and allow induction. Our CAP site deletion strains do not have $araO_2$ linked to the mutant promoter, so repression should not occur. If CAP works primarily through relieving repression, our CAP site deletions should have a more pronounced effect on *araBAD* expression when $araO_2$ is present.

To explore the above possibility, we constructed a second set of strains. D6 was introduced into plasmids carrying the *ara* sequence either from −860 to +3978 ($araO_2^+$) or from −210 to +3978 ($araO_2$) (Fig. 1). The *ara* DNA fragments were cloned into λgt11, and a *recA araA* strain were lysogenized. In these strains, *araA* gene activity, L-arabinose isomerase, reflects the activity of the mutant promoter. Isomerase assays on induced strains showed two distinct effects. First, the CAP site deletion caused a five- to sixfold decrease in enzyme levels (Table 3). This effect was seen in the presence or absence of $araO_2$. Second, removal of the $araO_2$ site (and repression) resulted in a twofold increase in enzyme activity (Table 3). This increase was seen in the presence or absence of the CAP site deletion. These two effects were clearly independent. We concluded that CAP plays no role in relief of repression of the *araBAD* operon under inducing conditions.

In uninduced strains, the data against CAP relieving repression was even more dramatic. No repression was possible in $araO_2$ strains, yet we saw a large CAP-stimulatory effect on the *araBAD* promoter. An 11-fold decrease in uninduced *araBAD* expression was seen when the CAP site was deleted (Table 4, HL7 versus HL9). A smaller effect was observed when the CAP site was deleted from $araO_2^+$ strains (Table 4, HL6 and HL8 versus HL10), probably because further reduction in *araBAD* expression was masked by the basal level of the operon. The basal (or absolute minimum) expression from the *araBAD* operon is its activity in the absence of AraC protein. Studies on *araC*-deleted strains showed that *araBAD* levels were twofold lower than wild-type levels in the absence of arabinose (E. Hamilton, unpublished data). Similar minimum levels were reached by deleting the CAP site of $araO_2^+$ strains.

Catabolite gene activation is stronger in the *araBAD* operon under uninduced conditions. The concentration of the activator form of AraC protein is one major difference between induced and uninduced cells. Wilcox et al. (65) reported that overproduction of AraC reduces the dependence of *araBAD* expression on CAP. A similar effect has been seen in the maltose regulon; overproduction of the positive regulator, MalT, results in CAP independence or decreased CAP dependence for the operons it regulates (13). Since increased activator concentrations result in reduced CAP dependence, one might expect that greater CAP dependence would occur in uninduced cells, where the concentration of AraC activator is exceedingly low. The data shown in Table 4 are consistent with this view.

Previous in vivo data, which led to the suggestion that CAP is involved in relieving AraC repression of the *araBAD* promoter, were obtained from strains lacking the cAMP-CAP system (Δ*cya*) (27). The researchers pointed out that their data could be entirely explained if the internal arabinose concentration were affected by the Δ*cya* mutation but discarded this possibility on the basis that there remains a "strong-fold" effect of the *cya* mutation on *araBAD* expression when the external concentration of L-arabinose is raised. Examination of the data cited shows that the very low *araBAD* activity exhibited by Δ*cya* and Δ*crp* strains was substantially increased by raising the external concentration of L-arabinose. Comparing wild-type and Δ*cya crp* strains, Heffernan et al. (30) saw a 73-fold cAMP stimulation of *araBAD* reduced to 10-fold upon raising the external L-arabinose concentration from 0.4 to 2.0%. Similar observations were reported by Wilcox et al. (65). Clearly, the magnitude of cAMP stimulation of the *araBAD* operon, when measured by *cya crp* mutations, is strongly subject to the effect of these mutations on the internalization of inducer. The fact that the in vivo experiment which led to the model of Hahn et al. (27) was conducted at a 0.2% arabinose concentration casts in doubt any interpretation which discounts the effect of *cya* on arabinose transport. The catabolite sensitivity of the arabinose transport operons is well documented (36, 61).

Other evidence cited in support of the model included the magnitude of cAMP stimulation in a DNA-dependent in vitro

protein synthesis and the magnitude of cAMP stimulation of *araA* protein synthesis in a *cya* mutant permeablized with EDTA (44). In both instances, however, it remains to be shown that the cAMP is exclusively directed toward transcriptional activation of the *araBAD* promoter.

In vitro transcription data supporting the above model used transcription conditions deliberately manipulated to give this result (28). Additional experiments in the same study, using different conditions, showed a threefold stimulation by CAP which was independent of the presence of $araO_2$. Actually, the latter results are in closer agreement with the in vivo results reported in this paper and with the data previously observed in vitro (31, 42). In conclusion, our in vivo data clearly showed that the model is incorrect and that relief of repression must involve some other mechanism.

It is not known how CAP stimulates transcription, but DNA bending may play an important role. Wu and Crothers (67) found that when CAP binds to the concensus sequence in the *lac* operon it induces a bend in the DNA. We found that DNA bending occurred upon CAP binding in the arabinose operon also (Fig. 6). As in *lac*, this bend lies in the CAP-binding site. Wu and Crothers suggested that polymerase may recognize the bend conformation directly, sliding from that point to its normal interaction site. Alternately, the bend may potentiate the transient conversion of adjacent promoter DNA to a different conformation which allows polymerase to bind more efficiently. They also proposed that the DNA banding may allow CAP-polymerase interactions not sterically possible in a linear promoter. In any event, it is of interest that all of these models are compatible with the noted lack of directionality in the orientation of the asymmetric CAP consensus sequence with respect to the direction of transcription (20). The CAP site in *ara* furnishes the first example in which the bidirectional effect of CAP stimulation has been quantitated (18, 54; this paper). Further experiments will be necessary to elucidate the role of DNA bending in transcriptional activation.

Our examination of catabolite gene activation of the *araBAD* promoter also involved an investigation of AraC-mediated repression of this promoter. The recently proposed model in which the major physiological role of repression is to provide a mechanism for CAP stimulation (27) was tested and disproved. Our data clearly differentiated CAP stimulation and AraC repression of *araBAD* transcription as separate regulatory mechanisms.

The site on *ara* DNA involved in negative regulation of the *araBAD* promoter by AraC is covered by a deletion which removes 971 bp upstream of the *araBAD* transcription start point beginning at −144 (22). The precise location of the operator has only recently been identified at −265 to −294; this AraC protein-binding site, named $araO_2$, is required for repression (17). Repression of *araBAD* was abolished by deletions which remove sequences upstream of −210 (Table 4, HL7 and HL9). The 12-fold derepression that we observed is in good agreement with those reported previously (17, 22, 27). It was not known, however, whether the *araBAD* promoter could be repressed under inducing conditions. Our results (Table 3) clearly showed that greater-than-twofold repression could be demonstrated in induced strains. Interestingly, recent experiments with in vivo methylation by Martin et al. (51) showed a marked alteration in the pattern of DNA methylation in the $araO_2$ region associated with the presence of an *araC* gene in the cell. This altered methylation pattern was observed in cells grown in either the presence or absence of L-arabinose. If the altered methylation pattern at $araO_2$ is indicative of repression as proposed by Martin et al. (51), then repression during L-arabinose induction is predicted from their data. In addition, we found that the magnitude of repression is induced cells was five times less than that in uninduced cells, a fact which correlates well with the twofold reduction in $araO_2$ occupancy in induced cells (51). Thus, the *araBAD* operon can be modulated to one of four different levels of expression by the action of AraC protein. The lowest regulated level is that of an uninduced but repressed cell (1×). If repression is eliminated by deletion of the $araO_2$ operator, then *araBAD* operon expression increases 12-fold (12×). In wild-type cells growing on arabinose, both induction and repression are operative, with a 100-fold increase in *araBAD* expression (1,200×). The highest level is attained in induced cells deleted of the $araO_2$ operator (2,400×). Cells without an $araO_2$ operator therefore exhibit an induction ratio of only 200 (2,400/12), whereas cells equipped with both negative and positive control mechanisms exhibit an induction ratio of 1,200. Catabolite gene activation adds a further level of control to this system. Five- to sixfold stimulation of *araBAD* expression can be attributed to the action of cAMP-CAP in induced cells. Thus, the predominant factor in expression of the *araBAD* operon is the presence of the AraC–L-arabinose activator complex, whereas AraC-mediated repression and catabolite gene activation play less prominent roles in the regulation of this operon in a direct manner. However, the operon may be more sensitive to catabolite regulation than is indicated by these studies since the L-arabinose permease operons are catabolite activated (36, 61). Under conditions in which catabolite gene activation is inoperative, decreased permease levels may lower the intracellular concentration of L-arabinose enough to affect *araBAD* activation.

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