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Effect of Mutations in the Cyclic AMP Receptor Protein-Binding Site on *araBAD* and *araC* Expression

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Maximum expression of the adjacent but divergently transcribed *araBAD* operon and *araC* gene requires the presence of cyclic AMP (cAMP) and the cAMP receptor protein (CRP). DNase I protection studies have previously revealed a high-affinity CRP-binding site in the *ara* regulatory region. Deletion mutations introduced into this site resulted in reduced expression of *araBAD* and *araC*. However, other experiments have demonstrated that spacing changes in the *ara* regulatory region may have multiple effects due to disruption of a DNA loop. Thus, the deletions could have destroyed the CRP-binding site, the ability to form a loop, or both. In the present study, substitution mutations were introduced into the CRP site in order to avoid creating spacing changes. We found that a 3-base-pair substitution resulted in a 30% reduction in *araBAD* expression, whereas a 6-base-pair substitution resulted in an 80% reduction. Both of these substitution mutations reduced *araC* expression threefold. We conclude that CRP bound to this site regulates expression in both directions. We found that a spacing change in the CRP site does not alter *araBAD* expression any more than does a substitution mutation.

Utilization of L-arabinose in *Escherichia coli* requires the expression of four transcription units (14, 24). The *araE* gene and *araFGH* operon encode proteins responsible for low-affinity and high-affinity transport of L-arabinose into the cell (4, 20, 23, 24, 37). The *araBAD* operon encodes three enzymes that are responsible for the initial reactions in the catabolism of L-arabinose (14, 24). The regulatory gene, *araC*, encodes a protein that controls expression of each of the four transcription units. In the presence of L-arabinose, AraC activates expression of *araBAD*, *araE*, and *araFGH* (4, 14, 20, 23, 24, 37). In the absence of L-arabinose, AraC represses transcription of *araBAD* (13). In the presence or absence of L-arabinose, AraC represses its own transcription (6, 18, 19), yielding a constant level of AraC protein in the cell. In addition, maximum expression of the *araBAD* operon and *araC* gene requires the presence of cyclic AMP (cAMP) and the pleiotropic regulator cAMP receptor protein (CRP) (1, 6, 27). Cells containing mutations in either the adenyl cyclase gene (*cya*) or the CRP gene (*crp*) are Ara$^-$ (45). The promoters for the *araBAD* operon and *araC* gene are adjacent to one another and are transcribed in opposite directions (43). The transcription initiation sites are separated by 147 base pairs (41). The region between the *araC* and *araB* genes is referred to as the *ara* regulatory region. Binding sites for the proteins in the *ara* regulatory region have been determined by methylation protection and DNase I protection studies (Fig. 1) (9, 25, 26, 35). Based on the location of these sites and on mutations that affect expression, models for the regulation of *araBAD* and *araC* expression have been proposed.

Several studies have focused on regulation of the *araBAD* operon and *araC* gene by the pleiotropic activator CRP. A high-affinity CRP-binding site was identified by DNase I protection studies at positions −82 to −110 (26). According to the proposed model, the cAMP-CRP complex binds to this single site in the *ara* regulatory region to activate transcription of both the *araC* gene and *araBAD* operon. In order to test this model, a 3-base-pair deletion was introduced into this site, and CRP dependence of the *araBAD* and *araC* promoters was tested (34). It was found that CRP mediates activation of *araC* through this site. The deletion reduced *araC* transcription to 30% of wild-type levels. However, this mutation in the CRP site did not have a great effect on *araBAD* expression. The effect of this deletion on *araBAD* expression differed from the effects of other deletion mutations in this site (10, 28). Larger deletions in the CRP site resulted in significantly reduced *araBAD* expression. In the studies discussed above, the mutations not only disrupted the CRP site but also introduced spacing changes into the *ara* regulatory region. Dunn et al. (9) have demonstrated that spacing changes in the *ara* region by nonintegral numbers of helix turns affect *araBAD* expression. The 3-base-pair deletion used in the previous study not only altered the CRP site but also changed the spacing by a nonintegral number of helix turns. Thus, the interpretation of the results is brought into question. In this study, the effect of alterations in the CRP site was tested by using substitution, rather than deletion, mutations in order to retain the wild-type spacing in the *ara* regulatory region.

MATERIALS AND METHODS

Media. The media and antibiotics used in this study have been previously described (21). Growth medium for enzyme assays consisted of M9 (32) salts supplemented with 0.4% carbohydrate, 1 μg of thiamine per ml, and 0.05 mM $MnCl_2$. Growth medium for strains used in β-galactosidase assays was also supplemented with L-proline at 40 μg/ml and L-leucine at 40 μg/ml.

Chemicals and enzymes. [α-^{32}P]dATP (400 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. [γ-^{32}P]ATP (crude; >5,000 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. All

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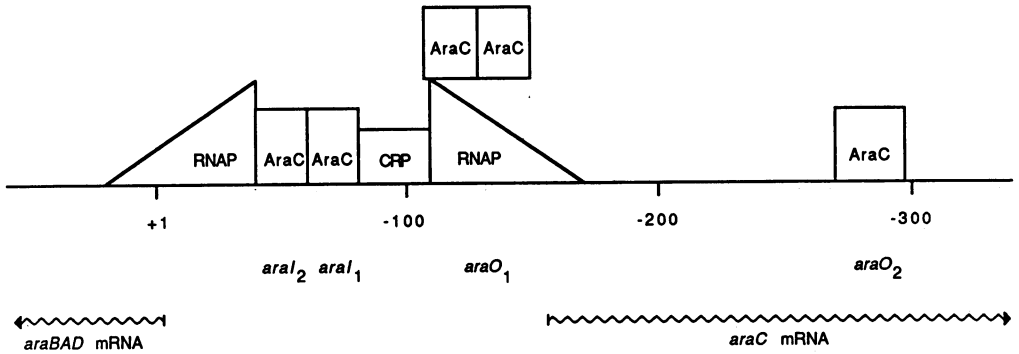

FIG. 1. Location of protein-binding sites in the *ara* regulatory region (9, 25, 26). Arrows indicate directions of transcription. Nucleotides are numbered with respect to the transcriptional start site of *araBAD*. Protein-binding sites are as follows: RNA polymerase for *araBAD*, +23 to −39; *araI2*, −38 to −54; *araI1*, −54 to −73; CRP, −82 to −110; *araO1*, −108 to −146; RNA polymerase for *araC*, −112 to −170; *araO2*, −269 to −294.

restriction enzymes and DNA-modifying enzymes were obtained from New England BioLabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as recommended by the manufacturer. The oligonucleotides were synthesized with an Applied Biosystems model 380A DNA synthesizer.

Construction of mutations. A 3- or 6-base-pair substitution was introduced into the CRP site by oligonucleotide-directed mutagenesis. The synthetic primer S3 had the sequence 5′-GCACGGCGTGGGACTTTGCTAT, and the synthetic primer S6 had the sequence 5′-TTTGCACGGTAAG GGACTTTGC (Fig. 2). These sequences were complementary to sequences in the single-stranded bacteriophage template used, M13mp2::ara1 (33). This phage contains a 2.5-kilobase (kb) *Eco*RI restriction fragment containing the entire *ara* regulatory region, part of *araA*, part of *araC*, and all of *araB*. The in vitro polymerization reaction and ligation were performed as described previously (33) and used to transform *E. coli* 71-18 (31). Phage containing the desired mutation was identified by plaque hybridization, using the radioactively labeled oligonucleotide as a probe. The DNA sequence of the *ara* regulatory region was determined by dideoxy sequencing to confirm the presence of the mutation. For clarity, the 3-base-pair substitution will be referred to as S3, and the 6-base-pair substitution will be referred to as S6. The phages containing the 3- or 6-base-pair substitution were designated M13mp2::araS3 and M13mp2::araS6, respectively.

Bacterial strains and plasmid constructions. The bacterial strains and plasmids used or constructed in this study are listed in Table 1.

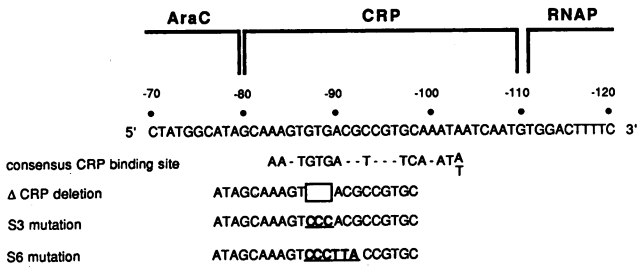

FIG. 2. CRP site mutations constructed by oligonucleotide-directed mutagenesis. The consensus CRP-binding site (11) is shown below the *ara* sequence. The deletion is indicated by an open box. The substituted bases are underlined. The binding sites for CRP, RNA polymerase (RNAP), and AraC are indicated.

(i) Construction of plasmids containing the mutations *cis* to *araC*. The 2.5-kb *Eco*RI fragments containing the mutations were isolated from the replicative form of the mutant phages and ligated into the unique *Eco*RI site in plasmid pPV33 (42). The DNA sequence in the *ara* regulatory region was determined by dideoxy sequencing to confirm the presence of the mutation on the plasmid. The plasmids containing the 3- and 6-base-pair substitutions were designated pLJS3 and pLJS6, respectively.

Plasmids were constructed in which the mutations were *cis* to an intact *araC* gene. Plasmid pTB1 (17) was digested

TABLE 1. Plasmids and strains

Plasmid or strain	Genotype	Source or reference
Plasmids		
pTB1	pBR322 Ω(*Pst*I *bla′*::*araB*$^+$ *C*$^+$)	17
pLJS3	pPV33 Ω(*Eco*RI::B/r *araA′ araB*$^+$ *araBi2003 araC′*)	This work
pLJS4	pLJS3 Ω(*Bst*EII *araC′*::B/r *araC*$^+$)	This work
pLJS6	pPV33 Ω(*Eco*RI::B/r *araA′ araB*$^+$ *araBi2006 araC′*)	This work
pLJS7	pLJS6 Ω(*Bst*EII *araC′*::B/r *araC*$^+$)	This work
Bacterial strains[a]		
71-18	F′ *lacI*^{q}Z ΔM15 *pro*$^+$ [F$^-$ Δ(*lac pro*)]	31
LA3	F$^+$/F$^-$ Δ(*araCO*)*719 lac gal pro thi hsdS*	17
UP1000	F$^-$, wild type	16
LA2000	UP1000 Δ(*araBi*)*2000*	34
LA2001	UP1000 Δ(*araBi*)*2001*	34
LA2003	UP1000 (*araBi*)*2003*	This work
LA2006	UP1000 (*araBi*)*2006*	This work
LA920	*araC*::Mu d(Ap^r *lac*) Δ*leu* Δ(*lac pro*)	34
LA922	LA920 Δ(*araCp*)*2001*	34
LA923	LA920 (*araBi*)*2003*	This work
LA926	LA920 (*araBi*)*2006*	This work

[a] All strains are derivatives of *E. coli* B/r except 71-18, which is a derivative of *E. coli* K-12, and LA3, which is a derivative of *E. coli* K-12 strain RR1, which contains the *ara* regulatory region from *E. coli* B/r.

with *Bst*EII, and the 2.3-kb fragment containing *araC* and downstream sequences was isolated. This fragment was ligated to *Bst*EII-digested pLJS3 or pLJS6. The ligation mix was used to transform the Ara$^-$ strain LA3 (17), and Ara$^+$ ampicillin-resistant colonies were selected. The constructions were verified by restriction analysis. The plasmids containing the 3- and 6-base-pair substitutions *cis* to an intact *araC* gene were designated pLJS4 and pLJS7, respectively.

(ii) Construction of strains containing the mutations *cis* to *araBAD*. In vivo recombination was used to construct strains containing the mutations on the chromosome. Strain LA2000 (34) contains a 3-base-pair deletion, ΔACT, in the activator-binding site in the *ara* regulatory region. This strain was transformed with plasmids containing the mutations. Because the ΔACT promoter mutation allows no expression of the *araBAD* operon promoter, the transformants were Ara$^-$ on MacConkey–L-arabinose–ampicillin medium. The transformed plasmids shared homology with the strain within a part of *araA*, all of *araB*, and part of *araC*. A homogenote in which the *ara* regulatory region from the plasmid recombined onto the chromosome was detected as an Ara$^+$ papilla. Recombinants were cured of the plasmid by repeated growth of the cells in the absence of ampicillin. The Ara$^+$ ampicillin-sensitive strains containing the 3- and 6-base-pair substitutions were designated LA2003 and LA2006, respectively. Correct transfer of the mutation to the chromosome was tested by hybridization analysis. Chromosomal DNA from the mutant strains was isolated (22), digested with *Bst*EII, separated by electrophoresis, and hybridized in situ (38) to the radioactively labeled oligonucleotide used in the mutagenesis. *Bst*EII sites are located on either side of the *ara* regulatory region at positions +1816 (25a) and −203 (32a), resulting in a predicted 2.0-kb *Bst*EII fragment that contains the *ara* regulatory region. The oligonucleotide probe was expected to hybridize to the 2.0-kb *Bst*EII chromosomal restriction fragment that contained the *ara* regulatory region if that fragment contained the desired mutation.

(iii) Construction of strains containing the mutations *cis* to *araC-lacZ*. Strain LA921 contains a stabilized *araC-lacZ* operon fusion with the 3-base-pair deletion in the activator-binding site, ΔACT, in the *ara* regulatory region. This strain was transformed with plasmid pLJS4 or pLJS7, containing the 3- or 6-base-pair substitution, respectively, and all of *araC*. The resulting transformants were Ara$^-$ since the ΔACT promoter mutation does not allow expression of *araBAD*. The plasmids shared homology to the strain within part of *araA*, all of *araB*, and part of *araC*. A homogenote in which the *ara* regulatory region from the plasmid recombined onto the chromosome was detected as an Ara$^+$ papilla on MacConkey–L-arabinose–tetracycline medium. Spontaneous loss of the plasmid from these recombinants occurred following repeated growth in the absence of tetracycline. Loss of the plasmid was detected by the loss of drug resistance and by a change in the phenotype from Ara$^+$ to Ara$^-$, because no *araC* gene product was present in the cell to induce *araBAD*. The resulting strains were designated LA923 and LA926. Correct transfer of the mutation to the chromosome was tested by hybridization analysis as described above.

Hybridization analysis was performed to determine whether strains containing the *araC-lacZ* fusion contained the *araO2* site (Fig. 1). Chromosomal DNA was isolated from strain LA922 (22), digested with *Hin*fI, and separated by electrophoresis. *Hin*fI sites are located at positions +61 (25a) and −545 (32a) and result in a 606-base-pair fragment that contains the *ara* regulatory region. An oligonucleotide extending from positions −356 to −370 (within the coding region of the *araC* gene) was radioactively labeled and hybridized with the gel in situ. The oligonucleotide hybridized with a 1.2-kb *Hin*fI fragment. This result indicates that the *araC-lacZ* fusion point occurs after the sequences that hybridized to the oligonucleotide but before the *Hin*fI site. Thus, the *araO2* site is intact in the strains containing the *araC-lacZ* fusion.

TABLE 2. Effect of mutations in the CRP site on *araBAD* expression in the presence of L-arabinose[a]

Strain	L-Arabinose isomerase sp act (% wild-type activity)		% Glucose repression
	Glycerol	Glucose	
UP1000 (wild type)	26.8 ± 0.3 (100)	4.5 ± 1.0 (100)	83
LA2001 (ΔCRP)	16.9 ± 2.3 (63)	4.5 ± 0.5 (100)	73
LA2003 (S3)	18.7 ± 3.0 (70)	4.5 ± 1.5 (100)	76
LA2006 (S6)	5.1 ± 0.7 (19)	1.1 ± 0.0 (25)	78

[a] Cells were grown in supplemented M9 medium plus 0.4% glucose or 0.4% glycerol and induced with 0.4% L-arabinose. L-Arabinose isomerase was assayed. L-Arabinose isomerase specific activity is expressed as millimoles of L-ribulose formed per hour per milligram of protein at 30°C. Results are expressed as the means of three experiments ± the standard deviations.

Enzyme assays. Cells were prepared for enzyme assays by growth in supplemented M9 salts medium at 37°C for 16 h. The cells were used to inoculate 50 ml of the same medium to a density of 3×10^7 cells per ml. After three doublings, L-arabinose was added to a final concentration of 0.4% when indicated. After one additional doubling, the cells were harvested and cell extracts were prepared. L-Arabinose isomerase was assayed as previously described (12). β-Galactosidase was measured as described by Miller (32).

RESULTS

Construction of substitutions in the CRP site. Three- and six-base-pair substitutions were introduced into the CRP site by oligonucleotide-directed mutagenesis as described in Materials and Methods. The bases that were replaced were chosen to alter the pattern of hydrogen bond donors and acceptors in the major and minor grooves of the DNA (40). The 3-base-pair substitution, S3, altered the central three bases of the CRP consensus sequence, the same three bases that were deleted in the ΔCRP mutation (Fig. 2). The 6-base-pair substitution, S6, altered the three central bases plus three additional bases (Fig. 2). This 6-base-pair substitution altered the same six bases that were deleted in the D6 mutation of Lichenstein et al. (28). Both S3 and S6 mutations altered the half of the symmetric CRP site that is the most conserved. Both S3 and S6 were substitutions, and thus the spacing was retained within the *ara* regulatory region.

Effect of the mutations on *araBAD* expression. The effect of the 3- and 6-base-pair substitutions on *araBAD* expression was determined by measuring the activity of L-arabinose isomerase, the product of the *araA* gene. All of the strains used were isogenic except for the mutations in the *ara* regulatory region. The effects of the mutations on *araBAD* expression in the presence of L-arabinose are shown in Table 2. The ΔCRP mutation removed the central 3 base pairs of the CRP consensus sequence. As shown previously, the strain containing this mutation (LA2001) exhibited a somewhat lower level of L-arabinose isomerase activity than did the wild type. In strain LA2003, the three bases deleted by the ΔCRP mutation were replaced by the bases CCC. The effect of this 3-base-pair substitution on *araBAD* expression

TABLE 3. Effect of mutations in the CRP site on β-galactosidase activity in strains carrying an *araC-lacZ* fusion[a]

Strain	β-Galactosidase sp act			% Glucose repression
	Glycerol	Glucose	Glucose + 5 mM cAMP	
LA920 (*araC-lacZ*)	1,864 ± 195	854 ± 20	1,722 ± 72	54
LA922 (ΔCRP *araC-lacZ*)	696 ± 41	532 ± 23	538 ± 29	23
LA923 (S3 *araC-lacZ*)	676 ± 35	556 ± 26	545 ± 44	18
LA926 (S6 *araC-lacZ*)	683 ± 45	555 ± 28	561 ± 13	19

[a] Cells were grown in supplemented M9 medium in the presence of either 0.4% glycerol or 0.4% glucose. β-Galactosidase activity was determined as described by Miller (32). β-Galactosidase specific activity is expressed as nanomoles of *o*-nitrophenol formed per minute per milligram of protein at 28°C. Results are expressed as the means of at least three experiments ± the standard deviations.

was similar to the effect of the 3-base-pair deletion. The effect of the 6-base-pair substitution on *araBAD* expression was more substantial. Strain LA2006 exhibited L-arabinose isomerase levels that were reduced to 19% of wild-type levels. Glucose repression was measured by comparing the L-arabinose isomerase levels in cells grown in the presence of glycerol with those in cells grown in the presence of glucose. The amount of glucose repression in the wild-type strain and in the mutant strains was similar.

The effect of the mutations on *araBAD* expression was also measured in the absence of L-arabinose. Since the ΔCRP mutation alters the spacing in the *ara* regulatory region by a nonintegral number of DNA helix turns, the effects of the ΔCRP mutation and the 3-base-pair substitution were compared to determine whether the ΔCRP mutation relieved *araBAD* repression. L-Arabinose isomerase levels in strains UP1000, LA2001, LA2003, and LA2006 ranged between 0.3 and 0.5 U when the cells were grown in the presence of glycerol and between 0.1 and 0.6 U when the cells were grown in the presence of glucose (data not shown). L-Arabinose isomerase levels are very low in the absence of L-arabinose, and the experimental error is significant at such low levels. Relief of *araBAD* repression due to a deletion of the *araO2* site resulted in a 10-fold increase in *araBAD* expression in the absence of L-arabinose (10). If the 3-base-pair deletion had resulted in a 10-fold increase in *araBAD* expression, this increase would have been detected by our assay. However, no such increase was detected.

Effect of the mutations on *araC* expression. The *araC-lacZ* fusions have been used to study *araC* regulation because of the difficulty in assaying the *araC* gene product directly (6). The effects of the 3- and 6-base-pair substitutions on *araC* expression were determined by measuring the effects of the mutations on an *araC-lacZ* fusion. All of the strains were isogenic except for the mutations in the *ara* regulatory region. The cells were grown in the presence of glycerol, glucose, or glucose plus 5 mM cAMP, and the level of β-galactosidase was measured (Table 3). As shown previously (34), when the cells were grown in glycerol, β-galactosidase activity in the strain containing the ΔCRP mutation was only 30% of that measured in the wild-type strain. In strain LA923, the three bases deleted by ΔCRP were substituted. The effect of the 3-base-pair-substitution on *araC-lacZ* expression was almost identical to the effect of the 3-base-pair deletion. The effect of the 6-base-pair substitution on *araC-lacZ* expression was almost identical to that of the other two mutations. As shown previously, β-galactosidase levels in strain LA920 were repressed by growth in glucose (34). This repression was relieved by supplementing the medium with 5 mM cAMP. The strain containing ΔCRP *cis* to the *araC-lacZ* fusion (LA922) exhibited a slightly reduced β-galactosidase level when grown in glucose. This slight reduction was not relieved by supplementing the medium with 5 mM cAMP. Strains containing the 3- and 6-base-pair substitutions in the CRP site (LA923 and LA926) also exhibited a slightly reduced β-galactosidase level when grown in glucose. This slight reduction was not relieved by the addition of cAMP to the medium.

DISCUSSION

The results described in this report support the conclusion that a CRP site at −82 to −110 in the *ara* regulatory region is involved in the activation of both *araC* and *araBAD*. Three- and six-base-pair substitution mutations and the previously constructed ΔCRP mutation all resulted in similar reductions in *araC* transcription, thus supporting the hypothesis that this CRP site is a part of the *araC* promoter (34). A slight reduction in *araBAD* expression was observed in the mutant containing the 3-base-pair substitution, while a large reduction was seen in the mutant containing the 6-base-pair substitution, supporting the conclusion that the CRP site is a part of the *araBAD* promoter (28).

One could argue that the effects of the mutations on *araBAD* expression were mediated indirectly through the effects on *araC* expression. The *araC* gene is regulated by CRP activation of transcription and by autoregulation. Miyada et al. (34) previously demonstrated that because *araC* autoregulation is dominant, the level of *araC* transcription in the cell varies only slightly in the wild-type and ΔCRP-containing strains. We showed that ΔCRP and the two substitution mutations affected *araC* transcription to the same extent. It is unlikely that the substitution mutations would affect *araC* translation differently than would the ΔCRP mutation. Therefore, we expect the levels of AraC to be approximately the same in the mutants containing the 3- and the 6-base-pair substitutions.

We can compare the effects of deletion and substitution mutations in the CRP site. The original mutation in the CRP site, ΔCRP, deleted 3 base pairs and thus changed the spacing between *araI* and *araO2* by a nonintegral number of helix turns. Spacing changes by nonintegral numbers of helix turns have been shown to affect *araBAD* expression in the absence of L-arabinose (9). Since loop formation has been proposed to exist in the presence of L-arabinose (28), spacing changes may also affect *araBAD* expression in the presence of L-arabinose. For comparison, a 3-base-pair substitution was introduced into the CRP site; the 3 base pairs that were deleted by ΔCRP were replaced with 3 different base pairs. We found that the effect of the 3-base-pair substitution was very similar to the effect of the 3-base-pair deletion on *araBAD* expression in the presence of L-arabinose. In both instances, the level of expression was approximately 60 to 70% of the wild-type level. Thus, the 3-base-pair spacing change did not greatly influence *araBAD* expression. A 6-base-pair substitution was introduced into the CRP site; the 6 base pairs deleted by Lichenstein et al. (28) were replaced with 6 different base pairs. The 6-base-pair deletion resulted in a reduction of *araBAD* expression to 19% of wild-type levels. We found that the 6-base-pair substitution also resulted in a reduction of *araBAD* expression to 19% of

wild-type levels. Although different cell growth and assay conditions were used in the two studies, the similarity of the results indicates that the 6-base-pair spacing change itself did not greatly, if at all, influence *araBAD* expression. Therefore, a comparison of the effects of 3- and 6-base-pair substitution and deletion mutations demonstrates that spacing changes in the CRP site did not affect *araBAD* expression to any greater extent than did substitution mutations in the presence of L-arabinose. This result is consistent with the DNA loop model proposed by Hamilton and Lee (19). They have proposed that the DNA loop formed in the presence of L-arabinose involves AraC bound to the *araO1* and *araO2* sites. The CRP site thus lies outside of the proposed DNA loop. Spacing changes in the CRP site would thus not be predicted to directly alter loop formation in the presence of L-arabinose.

Dunn et al. (9) have concluded that, in the absence of L-arabinose, repression of *araBAD* is relieved by deletion of the *araO2* site or by introduction of spacing changes by nonintegral numbers of helix turns. In that study, the spacing changes were introduced into the *Bst*EII site at −203, and *araBAD* promoter expression was measured under noninducing conditions. We compared the effects of the 3-base-pair deletion and 3-base-pair substitution in the CRP site to determine whether the deletion relieved *araBAD* repression in the absence of L-arabinose. We found that the level of *araBAD* expression was approximately the same. Why do spacing changes at −203 but not spacing changes in the CRP site affect *araBAD* expression? One possible explanation is that the CRP site is not within the DNA loop and thus spacing changes in this site do not of themselves have an effect. However, this explanation is inconsistent with the current DNA loop models (19, 30). The DNA loop models propose that in the absence of L-arabinose a loop is formed between AraC bound at the *araI* site and the *araO2* site. The existence of mutations in *araI* and *araO2* that relieve repression supports this model (30). In such a model, the CRP site lies within the intervening DNA loop. The effect of spacing changes on *araBAD* repression may depend on the location at which the spacing changes are introduced, on the presence of L-arabinose, and on the binding of proteins within the intervening DNA.

The effect of ΔCRP on *araBAD* expression has been determined by Miyada et al. (34) and Lichenstein et al. (28), as well as in this study. Lichenstein et al. report that this mutation results in a reduction of *araBAD* expression to 44% of the wild-type level, whereas we observed a somewhat greater level of expression (60 or 80%). To determine whether the source of this discrepancy was the different induction times used, we measured L-arabinose isomerase activity in cells grown for many generations in minimal L-arabinose medium. We found that although the levels of L-arabinose isomerase activity were slightly higher, the ratio between the mutant and wild-type levels remained approximately the same (data not shown). The difference between the effect of the ΔCRP mutation reported by Lichenstein et al. and that reported by us was most likely due to a difference in the construction of the strains in which *araBAD* expression was measured. The strain used by Lichenstein et al. contained the ΔCRP mutation *cis* to the *araB* gene on a λ lysogen. The *araC* gene with a wild-type promoter was unlinked in this construction. The strain constructed by Miyada et al. (34) contained the ΔCRP mutation within the wild-type *ara* regulatory region, *cis* to both the *araBAD* operon and *araC* gene. The location of the *araC* gene adjacent to *araBAD* in the construction of Miyada et al. and separated from the *araBAD* promoter in the construction of Lichenstein et al. possibly resulted in the small difference in the effect of the mutation on *araBAD* expression.

The effect of mutations in the CRP site demonstrates that the sequence of the CRP-binding site in the *araBAD* operon is more flexible than that in the *lac* and *gal* operons. Each of these CRP sites contains the highly conserved half of the consensus CRP sequence and, separated by 6 base pairs, a less conserved inverted repeat. In the *lac* and *gal* systems, single base-pair changes in the CRP site may result in greatly decreased activation by CRP (5, 8). In the *lac* system, a change in the second G in the consensus TGTGA results in a 50-fold decrease in *lacZ* expression (8). However, in the *ara* system, a deletion of the central GTG in the ΔCRP mutation results in 80% of wild-type *araBAD* activity, and a 6-base-pair substitution in the CRP site results in 19% of wild-type activity. This difference in sequence specificity may be due to different roles for CRP in the different systems. In the *gal* operon, CRP plays a role in excluding RNA polymerase from a second promoter, as well as in activation of transcription from the primary promoter (2, 5). The *lac* operon also contains two promoters, although the role of the second promoter in vivo is not clear (44). There is no evidence for a second promoter in the *araBAD* operon. The lack of sequence dependence for the CRP site in the *ara* region may also be because the *araBAD* operon, unlike the *gal* and *lac* operons, depends not just on CRP activation but also on activation by a specific activator, AraC. Even when the CRP-binding-site sequence is not optimal for CRP binding alone, other factors, such as AraC binding, may stabilize CRP binding in vivo.

Expression of both the *araBAD* and *araC* genes is subject to repression by growth in glucose. The connection between glucose repression and the intracellular level of cAMP is complex and poorly understood (15, 36, 39). For the *araC* gene, disruption of the CRP-binding site resulted in an *araC* promoter with decreased sensitivity to glucose repression. This suggests that glucose repression is mediated by cAMP-CRP bound to this site. However, expression of *araBAD* remained sensitive to repression by glucose in the strain containing the 6-base-pair substitution in the CRP site. This suggests that glucose repression of *araBAD* is not mediated by cAMP-CRP bound to this site. Is glucose repression mediated by inducer exclusion as in some other operons (29, 39)? To test this possibility, glucose repression is measured in strains that synthesize the enzymes constitutively in the absence of the inducer. In the *ara* system, the *araI*(Con) and *araC*(Con) mutations result in cells that express *araBAD* in the absence of L-arabinose (12). Isomerase levels are repressed by glucose approximately 75% in the wild-type strain and approximately 60% in the mutants. Thus, inducer exclusion most likely plays only a small role, if any, in glucose repression of *araBAD*. The mechanism of glucose repression of *araBAD* expression is not yet clear.

Although the single CRP site is involved in activation of both *araBAD* expression and *araC* expression, different mutations in the site did not affect expression to the same extent. A 3-base-pair substitution reduced *araC* expression to 40% of the wild-type level. However, this same mutation affected *araBAD* expression only slightly. A 6-base-pair substitution led to a much greater reduction in *araBAD* expression, but it did not further reduce *araC* expression. If the cAMP-CRP complex had bound to the CRP site to activate *araBAD* in mutants containing either the 3- or the 6-base-pair substitution, then the binding was below the level necessary to activate *araC* transcription. This differ-

ence in the activation by CRP of *araBAD* and *araC* reflects differences observed at other CRP-stimulated operons (7). Although the nucleotide sequences of different CRP-binding sites are similar, several differences are evident. (i) The distances between the CRP site and the transcription start site vary widely. (ii) The consensus CRP sequence may be found on either the template or the nontemplate strand. (iii) The highly conserved sequence TGTGA can be located either proximal or distal to the start site of transcription. All of these differences are present in the CRP site in the *ara* regulatory region, since the CRP site activates transcription in both directions. The differential effects of the 3- and 6-base-pair substitutions on expression in either direction indicate that different mechanisms of activation may be at work. In the *araC* promoter, the CRP site is adjacent to the RNA polymerase site, whereas in the *araBAD* promoter, another protein-binding site exists between the CRP and RNA polymerase sites. This bidirectional function of the cAMP-CRP complex is unique among the CRP sites studied.

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