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Upstream Repression and CRP Stimulation of the *Escherichia coli* L-Arabinose Operon

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Repression of the *Escherichia coli araBAD* promoter, P_{BAD}, was studied using a mutant P_{BAD} promoter (cip-5) that is expressed in the absence of the two proteins required for P_{BAD} induction, AraC protein and the cyclic AMP receptor protein (CRP-cAMP). Like the wild type promoter, cip-5 was repressed by AraC protein, and this repression required a site well upstream of the transcriptional start site. cip-5 was used to determine whether repression results from interference with the functioning of either AraC protein at *araI* and/or CRP-cAMP. Repression of cip-5 was eliminated by a point mutation within the AraC protein binding site *araI* but was not affected in the absence of CRP-cAMP. These results suggest that repression involves an interaction between two AraC protein binding sites located over 200 nucleotides apart.

Our results also suggest that the majority of the CRP requirement for P_{BAD} is a result of P_{BAD} repression. When repression was abolished by deletion of the $araO_2$ site, the requirement for CRP-cAMP in P_{BAD} induction was greatly reduced.

1. Introduction

Transcription of the *Escherichia coli araBAD* promoter, P_{BAD}, is both positively and negatively controlled by AraC protein (Englesberg *et al.*, 1969*a*; Greenblatt & Schleif, 1971; Wilcox *et al.*, 1974). In the presence of the inducer L-arabinose, AraC protein stimulates P_{BAD} transcription, while in the absence of arabinose or in the presence of the anti-inducer D-fucose, P_{BAD} transcription is repressed. Expression of P_{BAD} also requires the cyclic AMP receptor protein, CRP-cAMP (Greenblatt & Schleif, 1971; Lis & Schleif, 1973; Wilcox *et al.*, 1974).

Both AraC protein and CRP-cAMP bind to specific sites within the P_{BAD} regulatory region (Fig. 1). AraC protein is known to bind to three sites within P_{BAD} termed *araI*, $araO_1$ and $araO_2$ (Fig. 1; Ogden *et al.*, 1980; Lee *et al.*, 1981; Dunn *et al.*, 1984). Although models based on initial footprinting experiments proposed that only the inducing form of AraC protein could bind to the *araI* site (Ogden *et al.*, 1980; Lee *et al.*, 1981; Wilcox *et al.*, 1982), further work has shown that AraC protein can bind to all three sites *in vitro* in the presence of either the inducer arabinose or the anti-inducer fucose (Ogden, 1982; Dunn *et al.*, 1984; Hendrickson & Schleif, 1984; K. Martin, unpublished results). Fine structure

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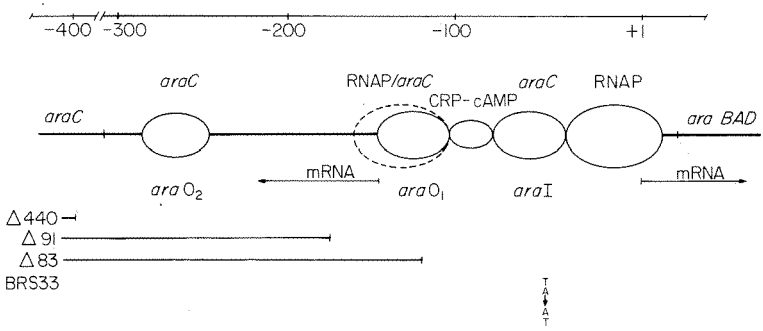

FIG. 1. The P_{BAD} regulatory region. The endpoints of the deletions used in this study relative to the start of P_{BAD} transcription are: Δ440: −404, Δ91: −176 ±1, Δ83: −126. BRS33 is T·A to A·T transversion at position −57. *araO*$_1$, *araO*$_2$ and *araI* refer to the P_{BAD} regulatory sites defined by DNase I footprinting and deletion mapping (Ogden *et al.*, 1980; Dunn *et al.*, 1984).

deletion mapping has shown that only the *araI* site is required for P_{BAD} induction (Dunn & Schleif, 1984), that is, deletion of *araO*$_1$ and all upstream sequences leaves the promoter normally inducible by arabinose.

Repression of P_{BAD} requires a site that lies upstream from all sites necessary for P_{BAD} induction (Englesberg *et al.*, 1969*a*). Deletion of this site results in a five- to tenfold increase in the basal level of P_{BAD}. Based on initial footprinting experiments and the extent of a deletion that abolished P_{BAD} repression, it was proposed that this site was the AraC protein binding site now termed *araO*$_1$ (Fig. 1; Ogden *et al.*, 1980; Wilcox *et al.*, 1982). However, further work has shown that P_{BAD} repression requires a region located about 280 nucleotides upstream from the transcriptional start site (Dunn *et al.*, 1984). This new site, defined by deletion mapping, corresponds to the AraC protein binding site *araO*$_2$, suggesting that repression involves the binding of AraC protein to this site. There is no direct evidence that the *araO*$_1$ site is involved in P_{BAD} repression.

In view of the complex nature of P_{BAD} regulation, we have undertaken the study of a single component of the complex P_{BAD} regulatory circuit, repression of P_{BAD}. Repression must occur by a highly unusual mechanism, since it requires a site located several hundred nucleotides upstream from the transcriptional start site. One possibility for the mechanism of repression might be interference with the functioning of either or both of the two proteins known to be required for P_{BAD} induction, AraC protein and/or CRP-cAMP. To test this mechanism, we asked whether repression could occur in the absence of either a functional *araI* site or CRP-cAMP. Since expression of the wild type promoter requires both a functional *araI* site and CRP-cAMP, a mutant P_{BAD} promoter was first isolated that lacked both these requirements for expression. This promoter is expressed in the absence of AraC protein and CRP-cAMP, and when AraC protein is added the promoter is repressed. Repression of the mutant promoter, like the wild type P_{BAD} promoter, is abolished by a deletion removing *araO*$_2$.

Repression of the mutant promoter occurred in the absence of CRP-cAMP but was abolished by a point mutation within the AraC protein binding site *araI*. This suggests that repression involves an interaction between two AraC protein

binding sites located several hundred nucleotides apart. Our results also suggest that most of the CRP requirement for P_{BAD} is a result of P_{BAD} repression. When repression was eliminated by deletion of $araO_2$, the requirement for CRP-cAMP in induction was greatly reduced.

2. Materials and Methods

(a) *Media and strains*

YT broth, minimal salts medium for plates and M10 medium were as described (Schleif & Wensink, 1981). Sugars were added to 0·2% (w/v). Thiamine was added to all media to 0·001% (w/v), amino acids when required to 0·01% (w/v), streptomycin to 200 μg/ml, and other antibiotics to 20 μg/ml. All strains are derivatives of *E. coli* K12 and are listed in Table 1.

(b) *General methods*

All general methods including P1 transduction, matings, transformation, ligation and DNA isolation were as described (Schleif & Wensink, 1981). P1 grown on strain SA1943 (GalK$^-$) was used to transduce strains to GalK$^-$ by selection on minimal glycerol, 0·2% (w/v) 2-deoxygalactose plates using the method of Alper & Ames (1975).

DNA sequencing was done as described by Maxam & Gilbert (1980). [γ-^{32}P]ATP was from ICN, and [^{14}C]galactose was from NEN. All enzymes were from either N.E. Biolabs, BRL, P-L Biochemicals or Boehringer.

TABLE 1

Bacterial and phage strains

Strain	Genotype	Comments
S165	F$^-$: Δ*gal* His$^-$	McKenney *et al.* (1981)
SA1943	F$^-$: *thr1 leuB6* GalK$^-$	GalK$^-$ derivative of strain C600 from K. McKenney
NK5961	F$^-$: HsdR$^-$ HsdM$^+$ Pro$^-$ *ilv* : Tn*10 thi1*	From Nancy Kleckner
LS853	F$^-$: *trpA9065 trpR55 his85* Δ*cya283*	CGSC 5381 from *E. coli* Genetic Stock Center
SH2	F$^-$: Δ*araC-leu1022* Δ*lac74* Str^r *thi1*	Str^r derivative of RFS1367 (Schleif, 1972)
SH292	F$^-$: *leuB6* Δ*lac74* Str^r *thi1*	SH2 P1 transduced to Ara$^+$
SH314	F$^-$: *araC102 galK recA* Tn*10* : *srl dcm6 thr1 lacY1 xyl5 tonA31 rpsL31 his4 tsx78 mtl1 supE44 thi1*	GalK$^-$ derivative of GM31 (Marinus, 1973)
SH321	F$^-$: Δ*araC-leu1022* Δ*lac74 galK* Str^r *thi1*	SH2 P1 transduced to GalK$^-$ from SA1943
SH322	F$^-$: *leuB6* Δ*lac74 galK* Str^r *thi1*	SH321 P1 transduced to Ara$^+$
SH334–SH341	F$^-$: *leuB6 ara* P_{BAD}-*galK* fusion Δ*galT* Δ*galE* Δ*lac74* Str^r *thi1*	Various *ara-galK* fusions in SH292 (this work)
SH342–SH349	F$^-$: *araC102 ara* P_{BAD}-*galK* fusion Δ*galT* Δ*galE* Δ*lac74* Str^r *thi1*	Strains SH334 to SH341 P1 transduced to AraC$^-$
SH352–SH362	F$^-$: *leuB6 ara* P_{BAD}-*galK* fusion Δ*galT* Δ*galE* Δ*lac74* Δ*cya283* Str^r *thi1*	Strains SH334 to SH349 transduced to *ilv* : Tn*10* with P1 (NK5961) followed by transduction to *ilv*$^+$ Δ*cya* with P1 (LS853)
λW30	Δb2 CI$^-$	From M. Gottesman
λW248	h80 CI$^-$	From M. Gottesman
λgal8		Feiss *et al.* (1972)

(c) *Mutagenesis* in vitro *and isolation of* P_{BAD} *constitutive mutations*

Mutagenesis of the P_{BAD}-*galK* fusion vector pTD3 (Dunn *et al.*, 1984) was used to isolate constitutive P_{BAD} mutations. Using deletion analysis, promoter mutations, and *in vitro* transcription, it has been shown that galactokinase expression from pTD3 is under the sole control of P_{BAD} (Dunn *et al.*, 1984; our unpublished results). Plasmid pTD3 was mutagenized with hydroxylamine by the method of Busby *et al.* (1982). The 94 base-pair *Bam*HI-*Hin*dIII fragment containing the P_{BAD} RNA polymerase binding site was isolated and ligated into a non-mutagenized *Bam*HI-*Hin*dIII cut vector. This DNA was transformed into strain SH314 (AraC$^-$ GalK$^-$ RecA$^-$) and constitutive mutants were selected on minimal 0·08% galactose ampicillin plates. Fast-growing colonies were picked from which plasmids were isolated and characterized.

(d) *Construction of deletions and transfer of* ara-galK *fusions to single copy*

The procedure for constructing deletions ending in the P_{BAD} regulatory region is outlined in Fig. 2. *Eco*RI-*Hin*dIII fragments of pTD3 containing various deletions generated by *Bal*31 digestion (Dunn *et al.*, 1984) were ligated into the *Hin*dIII site of pKB2000 (McKenney *et al.*, 1981). The resulting plasmid is similar to pTD3 but contains about 2000 bases of DNA from a region upstream of the *gal* promoter that is necessary for eventual recombination of the *ara-galK* fusions into the chromosome. These pKB2000 derivatives were then cut with *Bam*HI and *Sma*I to remove the 112 base-pair P_{BAD} RNA polymerase binding site. The *Bam*HI-*Sma*I fragment containing the P_{BAD} cip-5 mutation was then inserted. All final constructions were verified by DNA sequencing. For construction of the BRS33-cip-5 promoter, the *Bam*HI to *Bst*EII fragment of pRFS33 containing the BRS33 mutation (Smith, 1978) was ligated into a *Bam*HI-*Bst*EII cut pTD3 cip-5 vector.

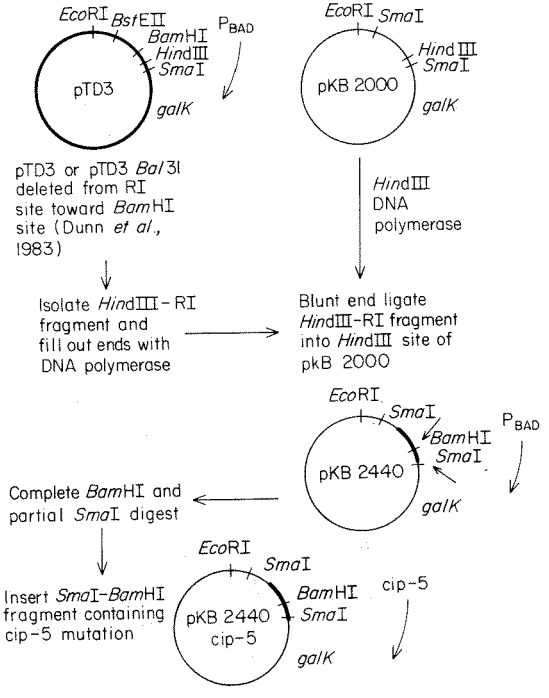

FIG. 2. Construction of deletions ending in the *ara* cip-5 regulatory region.

The *ara-galK* constructions in pKB2000 were transferred to single copy by the method of McKenney *et al.* (1981). The *λgal* transducing phage *λgal*8 was grown on strain SH2 (Gal^+) containing the recombinant plasmids. The resulting phage was used to infect strain S165 (*Δgal*) and plated on galactose tetrazolium plates. Recombinant *ara-galK* phage were identified as white plaque formers. These phage were used to infect strain SH292 (Ara^+, Gal^+). Lysogens were selected at 32°C by streaking on EMBO plates containing *λ* W30 and *λ* W248 at 10^8/plate. Finally, these lysogens were used to select recombinants that had lost both the prophage and the host *gal* operon leaving the *ara-galK* fusions in the chromosome. Lysogens were plated on MacConkey galactose at 42°C and Gal^- temperature resistant colonies were picked. These appeared at a frequency of between 0% and 80% of all temperature resistant cells, the frequency being dependent on the particular lysogen.

(e) *Galactokinase assay*

Galactokinase assays were done as described by McKenney *et al.* (1981) except that [^{14}C]galactose at a final specific activity of 12 μCi/μmol was used and the reactions were stopped with the addition of EDTA to a final concentration of 25 mM. Cells from overnight growth in YT were used to inoculate flasks containing M10, 0·2% (v/v) glycerol (Cya^+ strains) or 0·2% (w/v) glucose (Cya^- strains). Arabinose was added to 0·2% (w/v) where indicated. Cells were grown at least 5 generations to a density of about 10^8 cells/ml. Portions were delivered to tubes on ice containing chloramphenicol at a final concentration of 0·2 mg/ml. These cells were concentrated in M10 as needed for galactokinase assays such that radioactivity in the product [^{14}C]galactose phosphate was at least 4-fold above background (60 cts/min). For measurement of low galactokinase levels from the single copy *ara-galK* fusion strains, a typical 100-μl reaction contained up to 6×10^7 toluenized cells, and was incubated for 60 min at 32°C. All assays were performed in duplicate, at least two independent times. Data are presented with the standard deviation for a single experiment.

3. Results

(a) *Isolation and characterization of a mutant P_{BAD} promoter*

As a first step in the study of P_{BAD} repression, a mutation in the P_{BAD} RNA polymerase binding site was isolated that permits the promoter to be active in the absence of both AraC protein and CRP-cAMP. This phenotype is similar to the constitutive *araI*^{c} and *araI*^{c}*X*^{c} P_{BAD} mutations described previously (Englesberg *et al.*, 1969*b*; Colome *et al.*, 1977; Horwitz *et al.*, 1980). A DNA fragment containing the P_{BAD} RNA polymerase binding site was mutagenized *in vitro* and reinserted into an unmutagenized P_{BAD}-galK fusion vector pTD3 (see Materials and Methods). From this, a plasmid was obtained that expressed galactokinase in the absence of AraC protein and CRP-cAMP. The plasmid contained a single base-pair C·G to T·A transition at position −38 (Fig. 3). This mutation increases the homology of P_{BAD} to the −35 promoter consensus sequence (Hawley & McClure, 1983). From the sizing of 40-nucleotide run-off transcripts made *in vitro* from the wild type and mutant promoters, transcription of both promoters was found to initiate from the same position ±1 nucleotide (not shown).

In the absence of AraC protein *in vivo*, the AraC-independent promoter (cip-5) expresses galK at about 20% of the wild type induced P_{BAD} level (Table 2). When AraC protein is present, the mutant promoter is repressed two- to threefold. Surprisingly, unlike the previously described constitutive P_{BAD} mutations, cip-5 is not highly inducible by AraC-arabinose (Table 2). As shown below, these

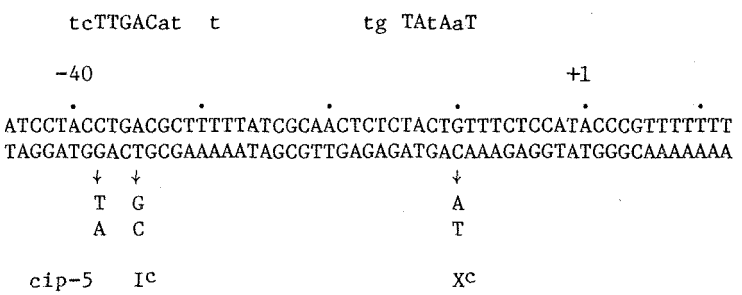

FIG. 3. Constitutive P_{BAD} mutations. Sequence of the P_{BAD} promoter with constitutive mutations indicated. The *araX*^{c} promoter consists of 2 pase-pair changes: −35 (A·T to G·C) and −10 (G·C to A·T) (Horwitz *et al.*, 1980). The proposed −35 and −10 consensus sequences (Hawley & McClure, 1983) are indicated above. Sequence hyphens have been omitted for clarity.

properties were not affected by the transfer of cip-5 to single copy on the *E. coli* chromosome. While cip-5 was not inducible, this property did not interfere with the measurement of cip-5 repression, since all these experiments were done in the absence of arabinose.

To determine whether repression of cip-5 occurs by a mechanism similar to that of the wild type P_{BAD} promoter, we tested whether repression of cip-5 required the region of P_{BAD} containing *araO*$_2$. Various amounts of the P_{BAD} regulatory region upstream of cip-5 were removed. The resulting cip-5-*galK* fusions were transferred to the *E. coli* chromosome so as to be present in single copy. In single copy, the cip-5-*galK* fusion is repressed about fourfold by AraC protein (Table 3). Deletion Δ91, which removes no known P_{BAD} regulatory sites other than *araO*$_2$ (Fig. 1) abolishes repression of cip-5. Identical behavior was seen with the longer deletion Δ83, which removes both the *araO*$_1$ and *araO*$_2$ sites. In addition to abolishing repression of cip-5, Δ91 causes a tenfold increase in the basal level of the wild type P_{BAD} promoter in the single copy *ara-galK* fusion system used here (Table 3). While we have not mapped precisely the upstream site required for repression of cip-5, the fact that repression is abolished by a deletion that removes no known regulatory sites other than *araO*$_2$, suggests that repression of cip-5, like the wild type promoter, requires *araO*$_2$.

TABLE 2

Properties of the ara *cip-5 promoter on multi-copy* ara-galK *fusion plasmids*

Plasmid	*ara* genotype	Galactokinase units
pKB2440 P_{BAD+}	*araC*$^-$	2·7±0·1
	araC$^+$	1·7±0·2
	araC$^+$ + arabinose	170±20
pKB2440 cip-5	*araC*$^-$	35±2
	araC$^+$	16±2
	araC$^+$ + arabinose	28±2

Galactokinase levels measured in strains SH321 (*araC*$^-$) or SH322 (*araC*$^+$) containing either the wild type (P_{BAD+}) or cip-5 *ara-galK* fusion plasmids.

TABLE 3

Repression of cip-5, like wild type P_{BAD}, requires an upstream site

Single copy *ara-galK* fusion	*ara* genotype	Galactokinase units
Δ440 cip-5	*araC*$^-$	1·2±0·1
	araC$^+$	0·31±0·02
	araC$^+$ + arabinose	1·2±0·1
Δ91 (ΔO_2) cip-5	*araC*$^-$	1·2±0·2
	araC$^+$	1·3±0·2
Δ83 (ΔO_1, ΔO_2) cip-5	*araC*$^-$	1·5±0·2
	araC$^+$	1·1±0·2
Δ440 P_{BAD+}	*araC*$^+$	0·03±0·01
Δ440 P_{BAD+}	*araC*$^+$ + arabinose	2·6±0·1
Δ91 (ΔO_2) P_{BAD+}	*araC*$^+$	0·28±0·01

Galactokinase activity of the various *ara-galK* fusions (Fig. 1) in single copy on the *E. coli* chromosome in otherwise isogenic *araC*$^+$ and *araC*$^-$ strains.

(b) *Repression of cip-5 involves the* araI *site*

The mechanism of P_{BAD} repression might involve interference with the functioning of the two proteins known to be required for P_{BAD} induction, either AraC protein at *araI* and/or CRP-cAMP. If repression involves AraC protein at *araI*, repression might be abolished by a mutation within the AraC protein binding site *araI*. To test this prediction *in vivo*, a promoter was constructed containing the constitutive cip-5 mutation as well as a point mutation in the *araI* site (BRS33). The BRS33 mutation at position −57 (Fig. 1) reduces P_{BAD} expression both *in vivo* and *in vitro* (Smith, 1978; Brown, 1979). The BRS33 mutation does not significantly affect the basal level of either the wild type P_{BAD} promoter or cip-5 in the absence of AraC protein. However, BRS33 completely eliminated repression of cip-5 by AraC protein (Table 4). This result indicates that repression of cip-5 can be eliminated by altering the *araI* site.

(c) *Repression occurs in the absence of CRP-cAMP*

We also tested whether CRP-cAMP, the other known P_{BAD} regulatory protein, was involved in the mechanism of repression. If repression is generated by

TABLE 4

Repression of cip-5 requires the araI *site*

ara-galK fusion plasmid	*ara* genotype	Galactokinase units
pTD3 cip-5	*araC*$^-$	49±1
	araC$^+$	18±1
pTD3 BRS33 (*araI*$^-$) cip-5	*araC*$^-$	52±2
	araC$^+$	48±1
pTD3 BRS33 (*araI*$^-$) P_{BAD+}	*araC*$^-$	1·9±0·3
pTD3 (*araI*$^+$) P_{BAD+}	*araC*$^-$	2·7±0·1

Galactokinase levels measured in strains SH321 (*araC*$^-$) or SH322 (*araC*$^+$) containing the indicated *ara-galK* fusion plasmids.

TABLE 5

cip-5 is repressed in adenyl cyclase mutants

Single copy *ara-galK* fusion	*ara* genotype	Galactokinase units
Δ440 cip-5	*araC*$^-$	0·65±0·06
	araC$^+$	0·25±0·01
Δ91 (ΔO_2) cip-5	*araC*$^-$	0·62±0·01
	araC$^+$	0·59±0·01

Repression of the single copy *ara*(cip-5)-*galK* fusion strains in otherwise isogenic *araC*$^+$ or *araC*$^-$, Δ*cya* strains. The endpoints of the deletions are as indicated in Fig. 1.

interference with the functioning of CRP-cAMP, then repression should be severely impaired in the absence of CRP-cAMP. To test this mechanism, the cip-5-*galK* fusion strains were transduced with phage P1 to Δ*cya* (deleted for the adenyl cyclase gene). Table 5 shows that in the absence of CRP-cAMP, the cip-5 promoter is still repressed about threefold when AraC protein is present. This repression is abolished by deletion Δ91, which removes *araO*$_2$, just as occurs in the *cya*$^+$ strain. These results show that repression can occur in the absence of CRP-cAMP.

(d) *Repression and CRP stimulation of* P_{BAD}

Upon arabinose addition, the wild type P_{BAD} promoter (Δ440) is expressed poorly in cells lacking adenyl cyclase activity (Δ*cya*: Fig. 4). However, when P_{BAD} repression is eliminated by deletion of *araO*$_2$ (Δ91), P_{BAD} expression increases dramatically in Δ*cya* strains. Increasing the level of *araC* protein within the cells by means of AraC protein overproducing plasmids allows maximal expression of P_{BAD} in the cells lacking both *araO*$_2$ and adenyl cyclase. This increase in AraC protein level has no effect in strains containing a functional *araO*$_2$ site. The observation that the Δ*araO*$_2$ Δ*cya* strain required elevated AraC protein levels for full induction is likely due, in part, to the fact that AraC protein synthesis is stimulated by CRP (Casadaban, 1976) and the level of AraC protein in an *araC*$^+$ Δ*cya* strain is about threefold lower than in a wild type *cya*$^+$ background (Hahn & Schleif, 1983).

On the basis of preliminary measurements, Wilcox *et al.* (1982) have oppositely concluded that elevated amounts of AraC protein can eliminate the CRP requirement for induction of the wild type P_{BAD} promoter. However, their conclusion was based on the properties of a strain containing a deletion that removes the upstream repression site, *araO*$_2$. Therefore, their data are consistent with our conclusions.

As detailed in the Discussion, one interpretation of these results is that P_{BAD} expression is reduced in the absence of CRP-cAMP because the promoter remains repressed, even upon addition of arabinose. That is, in the absence of CRP-cAMP, repression prevents AraC-arabinose from functioning at *araI* to induce the wild type P_{BAD} promoter.

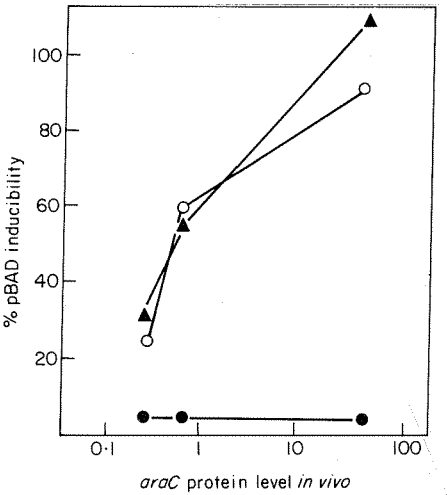

FIG. 4. Deletion of $araO_2$ eliminates the requirement for CRP-cAMP in induction of P_{BAD}. P_{BAD} inducibility relative to wild type measured in single copy P_{BAD+}-*galK* fusion Δ*cya* $araC^+$ strains containing either: no plasmid, 2-fold, or 60-fold AraC protein overproducing plasmids (Steffen & Schleif, 1977; Hahn & Schleif, 1983). Since both the chromosomal *araC* promoter and the plasmid-borne *araC* promoters are CRP stimulated (Hahn & Schleif, 1983; Arditti *et al.*, 1973), the level of AraC protein in these strains is approximately $\frac{1}{3}$, $\frac{2}{3}$ and 50 times the wild type level. Shown are the results for (●) Δ440 ($araO_1^+$, $araO_2^+$), (○) Δ91 (Δ$araO_2$), and (▲) Δ83 (Δ$araO_1$, Δ$araO_2$) P_{BAD}-*galK* fusions. The endpoints of the deletions are as indicated in Fig. 1. Cells were grown in 0·2% glucose, 0·2% arabinose, 0·2% Casamino acids. Upon addition of 4 mM-cAMP, the strains lacking adenyl cyclase were induced to the wild type level.

4. Discussion

In this paper, we describe a study of P_{BAD} repression *in vivo*. Repression of P_{BAD} is highly unusual in view of the fact that it requires an AraC protein binding site located several hundred nucleotides upstream from the transcriptional start site. These studies were undertaken to determine whether repression of transcription is controlled solely by the distant site, $araO_2$, or whether it involved an interaction between $araO_2$ and other known protein binding sites located closer to the start of transcription.

One possibility for the mechanism of repression might be interference with the functioning of the two proteins required for induction, AraC protein at *araI* and/or CRP-cAMP. To test this mechanism, we asked whether repression could occur in the absence of either a functional *araI* site or CRP-cAMP. Since expression of the wild type promoter requires both a functional *araI* site and CRP-cAMP, a mutant P_{BAD} promoter (cip-5) was first isolated that lacked both these requirements for expression.

Although we have used a mutant promoter for the study of repression, our results suggest that repression of both cip-5 and the wild type P_{BAD} promoter occurs by a similar mechanism. We note that the transcription start site of the mutant was the same as the wild type promoter, and that cip-5 was repressed by AraC protein, like the wild type and other constitutive P_{BAD} promoters

described before. Finally, repression of cip-5 required a site well upstream of the transcriptional start site. A deletion that removed no known P_{BAD} regulatory sites other than $araO_2$ eliminated repression of cip-5, just as it does for the wild type P_{BAD} promoter.

The experiments presented here suggest that repression involves an interaction between the two AraC protein binding sites $araO_2$ and *araI*. Repression occurred in the absence of CRP-cAMP, but was eliminated by a point mutation within *araI*. This mutation at position −57 has been shown to affect the binding of AraC protein at *araI*, increasing the dissociation rate of AraC protein from *araI* by greater than tenfold (K. Martin, unpublished results). A mechanism in which repression involves AraC protein at *araI* seems reasonable, given the order of the binding sites at P_{BAD}. AraC protein bound at this site lies directly adjacent to the RNA polymerase binding site. In the repressed state, the interaction between AraC protein at both $araO_2$ and *araI* could hold AraC protein bound at *araI* in a state that prevents transcription from the constitutive cip-5 promoter or the wild type P_{BAD} promoter.

Since $araO_2$ and *araI* are separated by over 200 nucleotides, one mechanism of repression is that AraC protein bound at these distant sites interact directly *via* a loop in the intervening DNA. This mechanism is consistent with the finding that insertions or deletions between $araO_2$ and *araI* that are not multiples of about ten nucleotides interfere with repression while insertions of 11 and 31 nucleotides have no effect on repression (Dunn *et al.*, 1984). Such a mechanism may also explain repression of the *E. coli gal* operon. Like P_{BAD}, repression of *gal* has been shown to require two *gal* repressor binding sites separated by about 110 nucleotides (Irani *et al.*, 1983). Our results, however, do not rule out more complicated mechanisms for P_{BAD} repression. For example, we have not tested whether the $araO_1$ site is also involved in repression.

Finally, our results suggest that the majority of the 30 to 40-fold CRP requirement of P_{BAD} is a result of repression of P_{BAD}. In the absence of CRP-cAMP, P_{BAD} expression increases dramatically when repression is eliminated by deletion of $araO_2$. Increasing the level of AraC protein allowed full induction in the cells lacking both $araO_2$ and adenyl cyclase. The increased levels of AraC protein had no effect on cells carrying an intact $araO_2$ site. These results are consistent with those presented in the accompanying letter (Dunn & Schleif, 1984). In cya^+ strains deleted for $araO_2$, deletion of the CRP-cAMP binding site reduces the induced level of P_{BAD} expression only two- to threefold below the wild type level (Dunn & Schleif, 1984).

One mechanism consistent with these results is that in the absence of CRP-cAMP, insufficient arabinose can enter the cell to convert all AraC protein from the repressing to the inducing state. Since the repressing form of AraC protein is dominant to the inducing form, no induction is observed. However, when repression is abolished by deletion of $araO_2$, the fraction of AraC protein that is in the inducing state is now free to induce P_{BAD}. One prediction of this mechanism is that if the internal arabinose concentration were not limiting in the Δ*cya* strain, P_{BAD} expression would increase to the same level as in the Δ*cya* Δ$araO_2$ strain since, under these conditions, all AraC protein would be in the inducing state.

We disfavor this mechanism, however, for two reasons. Increasing the extracellular arabinose concentration tenfold above the levels used in these experiments does not overcome the strong-fold CRP requirement for P_{BAD} (Heffernan *et al.*, 1976; our unpublished results). Second, under conditions where arabinose concentrations are not limiting in induction, P_{BAD} expression is still greatly stimulated by cAMP. A 20 to 40-fold cAMP stimulation is observed both in cell-free extracts and in Δ*cya* strains made permeable by treatment with EDTA (Lis & Schleif, 1973).

The mechanism that we favor is that in the absence of CRP-cAMP, AraC-arabinose is still capable of repressing P_{BAD} induction through interaction of AraC protein bound at $araO_2$ and *araI*. When repression is abolished either by deletion of $araO_2$ or by addition of CRP-cAMP, AraC-arabinose at *araI* can then induce P_{BAD}. This mechanism is supported by direct experiments using *in vitro* transcription (S. Hahn, W. Hendrickson & R. Schleif, unpublished results). AraC-arabinose stimulates P_{BAD} poorly on DNA templates with an intact $araO_2$ site, while either deletion of $araO_2$ or addition of CRP-cAMP results in a tenfold stimulation of P_{BAD}.

The results presented above indicate that when P_{BAD} repression is eliminated, the requirement for CRP-cAMP in induction is greatly reduced. Since the only other known function of repression is a rather small reduction in the basal level of P_{BAD}, it may be that the major physiological role of P_{BAD} repression is to provide a mechanism for CRP stimulation of this promoter.

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