An operator at −280 base pairs that is required for repression of araBAD operon promoter: Addition of DNA helical turns between the operator and promoter cyclically hinders repression

(positive regulation/DNA binding/transcription/repression/DNA loops)

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ABSTRACT A site has been found that is required for repression of the Escherichia coli araBAD operon. This site was detected by the in vivo properties of deletion mutants. In vitro protection studies with DNase I and dimethylsulfate showed that araC protein can specifically bind in this area to nucleotides lying at position −265 to −294 with respect to the araBAD operon promoter (P_{BAD}) transcription start point. The previously known sites of protein binding in the ara operon lie between +20 and −160. Since the properties of deletion strains showed that all the sites required for araBAD induction lie between +20 and −110, the new site at −280 exerts its repressive action over an unusually large distance along the DNA. Insertions of −16, −8, 0, 5, 11, 15, 24, and 31 base pairs of DNA between the new site and P_{BAD} were constructed. Repression was impaired in those cases in which half-integral turns of the DNA helix were introduced, but repression was nearly normal for the insertions of 0, +11, and +31 base pairs.

The L-arabinose operon in Escherichia coli is well documented to be positively regulated by the araC protein. Additionally, the operon is negatively regulated by the same protein (1–4). Paradoxically, the negative regulation appears to involve a site lying upstream of all the sites required for induction. Initially, the site involved in this repression phenomenon appeared to be the araC-binding site, which lies from position −110 to −140 (2, 5–7) (Fig. 1). From this position, the protein could be imagined to make direct contact with the complex of cyclic AMP receptor protein araC protein–DNA polymerase, all of which are involved in induction. Since upstream repression, even from this nearby site, appeared unusual, we examined the question more carefully by using a set of deletions.

As reported here, the deletions and in vitro binding experiments revealed the existence of yet another site for araC protein binding in the ara regulatory region. This site at position −280 lies too far for any simple direct interaction to exist between the site and the complex of proteins on DNA that are required for initiation of transcription, and yet this site is required for repression of transcription. This finding of a second regulatory site located a considerable distance from the promoter is similar to the recent finding in the gal operon of a second operator site located downstream from the promoter and lying within the galE gene (8).

MATERIALS AND METHODS

Media and Strains and General Methods. Media, strains, and general methods were as described (9–12).

Construction of pTD3 and pTD4. A 440-base-pair fragment containing the araC BAD regulatory region (13) was made blunt-ended by treatment with S1 nuclease. HindIII and EcoRI linkers were ligated in 10 M excess to the blunt-ended fragment, followed by codigestion with HindIII and EcoRI. The ara fragments were then ligated to HindIII-EcoRI-cut pBR322 that had been purified from the 30-base-pair fragment (14) by A-15 agarose chromatography. Plasmids having the inserted ara fragment in the desired orientation were selected on the basis that expression of the araBAD operon promoter (P_{BAD}), and thus tetracycline resistance (15, 16), becomes arabinose dependent, while expression of the araC gene promoter (P_{C}) is not. The fragments were subsequently recloned between the HindIII and EcoRI sites of the plasmid pK01 so that the promoters could be assayed by expression of the galactokinase gene to which they were fused (17). pTD3 and pTD4 have P_{BAD} and P_{C} fused to the galk gene, respectively.

Construction of the Deletions. Either pTD3 or pTD4 was linearized with EcoRI and treated with exonuclease Bal 31 to remove 100–600 base pairs. The shortened linear plasmids were ligated to a 10 M excess of EcoRI linkers, codigested with EcoRI and HindIII, purified by electrophoresis, and recloned into EcoRI/HindIII-cut pK01 that had been purified by electrophoresis through agarose and treated with bacterial alkaline phosphatase (18). After recloning, the plasmids were transformed into TMD29, an araC^{−} galk derivative of C600 (17). Candidates were purified by rapid plasmid isolation (19) and cut with EcoRI and HindIII. The fragments were labeled with Klenow fragment and [α-32P]dATP and sized on a sequencing gel by comparison to a "G"s" dinucleotide (guamines) sequencing reaction size standard. In many cases, the deletions were later sequenced through the deletion end point and linker. With respect to +1, the start of P_{BAD} transcription, the end points of deletions extending from araC toward P_{BAD} were as follows: −385, −340, −330, −294, −263, −247, −222, −190, −174, −170, −163, −150, −143, −138, −137, −133, −132, −126, −125, −113, −111, −107, −96, −95, −84, −57, −53, −49, −48, −39, −36, −35, −30, −10; and from araB toward P_{C} they were as follows: −50, −60, −65, −84, −97, −103, −105, −110, −114, −115, −125, −126, −130, −159, −168, −190, −200, −230, −245.

FIG. 1. Location of the protein-binding sites of the L-arabinose araBAD regulatory region, drawn to scale. Transcription of P_{BAD} is rightward from +1, and transcription of P_{C} is leftward from −148. RNAP, RNA polymerase.

Abbreviations: P_{BAD}, promoter for araBAD operon; P_{C}, promoter for araC gene; CRP, cyclic AMP receptor protein.
**GalK Assays.** Cells were grown in M10 medium/0.2% glycerol or M10 medium/0.2% glycerol/0.2% arabinose. All cells were grown for at least six generations to a density of 1–4 × 10^8 cells per ml in the presence of 20 μg of ampicillin per ml. Chloramphenicol was added to 200 μg/ml before harvesting the cells. The galactokinase assays were as described by McKenney et al. (17) except that 25 mM EDTA was added to stop the reactions. Galactokinase units are expressed as nmol of galactose phosphorylated per min per ml of cells at OD_{600} of 1.0.

**DNase I and Methylation Protection.** The DNase I protection experiments were done by the procedure of Galas and Schmitz (20) with the following modifications. DNase I reaction buffer was 20 mM Tris·HCl, pH 7.6/2.5 mM MgCl₂/0.1 mM EDTA/50 mM KCl/0.5 mM dithioerythritol/25 μg of bovine serum albumin per ml/100 mM arabinose. DNase I was dissolved just prior to use in the reaction buffer, which also contained 0.5 mM CaCl₂. araC protein was incubated with the DNA (100 μl) for 10–15 min at 37°C prior to the addition of 10 μl of freshly diluted DNase I (0.8 μg/ml) for 45 sec at 20°C. The DNase I reaction was stopped, DNA was precipitated, and the samples were resuspended in 10 mM Tris·HCl, pH 8.0/60% formamide/dyes and evaporated at 90°C prior to loading, until the formamide concentration reached 90% (30 min). The methylation protection studies were done according to the method of Johnsrud (21), with the following modifications. The buffer was as described above for the DNase I experiments, and the protein was incubated with the DNA in a 100-μl vol for 10 min at 37°C prior to the addition of 1 μl of dimethylsulfate for 45 sec at 20°C. Samples were stopped and treated according to the normal "G" reaction protocol (12).

**Spacing Mutations.** Plasmid pTD3 was cut with BstEII at −208 with respect to P_{BAD}, and digested with S1 nuclease before ligation to reclose the circles to generate the deletions of 8 and 16 base pairs. The single-stranded ends of the BstEII-cut DNA were filled in with DNA pol I before ligation to generate the +5. The filled out DNA was ligated to the Xba linker T-C-T-A-G-A to generate the +11. The +11 was cut with Xba and filled out with DNA pol I to generate the +15. The +11 was cut, partially filled out with DNA pol I, and the restriction-site mobilizing element from pUC4K (22) was added using partially filled out BamHI ends, cloned, and the kanamycin-resistance element was eliminated by digesting with Sai I and ligating, leaving a total insertion of +31 base pairs compared to wild type. The sequences of the insertions were determined by DNA sequencing. All the insertion-containing plasmids were normally inducible by arabinose.

**RESULTS**

**Construction of the Deletions.** Two plasmids, pTD3 and pTD4, were constructed by cloning the 440-base-pair ara regulatory region DNA segment (13) with EcoRI and HindIII linkers in either orientation between the EcoRI and HindIII sites of the plasmid pKO1 (17). Plasmid pTD3 has P_{BAD} fused to the galK gene of the pKO1 expression vector, and pTD4 has P_{C} fused to galK (Fig. 2). Deletions were isolated by linearizing the plasmids with EcoRI, digesting the exonuclease Bal 31, replacing the EcoRI site with a new linker, and recloning the ara fragments between the EcoRI and HindIII sites of purified pKO1. The structure of the plasmid pKO1 permits direct comparison of many different promoter activities, and reintroducing the deleted ara region into the unmodified vector ensures that the alterations in enzyme level that we measure originate from the ara DNA and not the plasmid.

**Induction and Repression Requirements of P_{BAD}.** The deletions from the P_{C} side of the ara regulatory region, which ended before position −107, were about 100-fold inducible by arabinose, the same as the undeleted ara regulatory region. This is expected, as such deletions leave the cyclic AMP receptor protein (CRP), 1, and RNA polymerase sites of P_{BAD} intact. Similarly, deletions from the P_{BAD} side extending toward P_{C}, which ended before −80, left P_{C} with normal activity. This too is expected as these deletions leave P_{C} intact.

Surprisingly, the basal levels of P_{BAD} expression in the deletion strains reveal that, although a strain containing a deletion extending to nucleotide −294 represses P_{BAD} normally, any deletion that removes additional ara DNA expresses a 10-fold increased basal level (Fig. 3), unless the deletion extends into the CRP site. As we show in the next section, araC protein is capable of binding in vitro to a 30-base-pair sequence centered at position −280, which we term araO₂. While the above results directly demonstrate the involvement of araO₂ in the repression of P_{BAD}, these experiments do not speak to the question of whether araO₁ is also involved in the repression.

The dependence of repression of P_{BAD} on araO₂ might be due to a simple titration of araC protein made more extreme by the plasmid copy number (10–30 copies per cell). This possibility can be excluded, because deletions with and without araO₂ were transferred to the chromosome by the methods outlined by McKenney et al. (17), and in single copy they showed that loss of repression is dependent on deletion of araO₂ just as observed for the multicopy plasmids. With araO₂ present, the basal level was 0.03 units of galactokinase; without araO₂, it was 0.28 units; and when induced by arabinose, the level was 2.7 units.

**FIG. 2.** Structure of the P_{BAD} and P_{C}–galK fusion plasmids. The plasmids are identical except for the orientation of the ara fragment between the EcoRI and HindIII sites. The remainder of the plasmid is pKO1 (17). The Pvu II (destroyed in cloning)–EcoRI fragment that encodes β-lactamase (amp) is from pBR322 (14). The entire galactokinase gene resides on the Sma I–Pvu II fragment marked galK. There are stop codons in all three reading frames between Sma I and the translation start site of galK.

**FIG. 3.** Basal level araBAD promoter activities in galactokinase units from a set of deletion plasmids containing the araP_{BAD}–galK fusion in strain SH322 (F· leu lue74 galK Str− thy) under noninducing conditions (lacking arabinose). The deletions ended at −330, −294, −263, −222, −163, −126, −94, and −57. RNAP, RNA polymerase.
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Deletion of araO2 (no repression)

![Diagram of deletion of araO2](image)

**Fig. 5.** Photograph of a galactose MacConkey plate streaked with the various insertion strains as indicated. A strain with araO2 deleted was streaked across the top and strains with insertions of -16, -8, 0, +5, +11, +15, +24, and +31 base pairs were streaked as indicated and incubated at 33°C. In one experiment, these same strains grown in M10/glycerol at 34°C contained 4.1, 1.7, 1.7, 0.84, 1.9, 0.72, 0.96, 1.6, and 0.59 units of galactokinase, with the variation between duplicate assays at ±0.3 units.

31 base pairs at a nonessential site between araO2 and P_BAD. When the change in the number of helical turns was nearly an integral value, assuming ±10.5 base pairs per turn, repression was possible, but when the change generated nearly a half-integer number of turns, repression was impaired. These data are shown pictorially by the color generated on the indicating plates in which cells able to repress give white streaks and cells less able to repress possess more galactokinase and yield red streaks (Fig. 5). Direct measurement of the P_BAD-driven galactokinase levels in the strains is subject to substantial fluctuation because of its low level, but the values obtained in a typical experiment show the same trend as the indicating plate. Fortuitously the MacConkey indicating plates magnify and clearly display the 2- to 5-fold differences in enzyme levels present in the spacing mutation strains.

The spacing mutations eliminate the possibility of one artifact. Conceivably araO2 is an araC protein-dependent attenuator of transcription that originates from a site still further upstream. If so, the spacing mutations would be expected not to alter repression. Since they did, the attenuator mechanism seems unlikely.

**DISCUSSION**

The properties of deletions extending toward P_BAD led to the identification of a site involved in P_BAD repression. Remarkably, this site is centered at position -280, far from the sites that are required for the induction of P_BAD, which span bases -110 to approximately +10. The newly discovered site is termed araO2, because it functions as an operator in being required for repression of P_BAD, and it is the second operator discovered in the P_C−P_BAD regulatory region. The first operator to have been discovered is now termed araO1, and its role, if any, in the repression of P_BAD has not been established by our experiments. However, if araO1 is involved in repression of P_BAD, its involvement is complex, because increased levels of araC protein can generate almost complete repression of P_C, presumably by fully occupying araO1, while P_BAD remains fully inducible (25).

The operator araO2 was shown to be capable of binding araC protein by DNase I and dimethylsulfate protection studies in vitro. araO2 lies in the transcribed but untranslated leader region of the araC operon.

The involvement of araO2 in repression of P_BAD is consistent with published deletion data. The Engelsberg deletion Δ719, which originally revealed the phenomenon of repres-
sion from an upstream site, removes araO2 in addition to entering the site we now name araO2*, whereas the shorter deletion Δ766, which did not interfere with repression, does not enter the ara regulatory region (2, 7).

Three general mechanisms appear possible for repression generated by the binding of a protein at a substantial distance from the transcription start point. An alteration in the structure of the DNA could be propagated between the repression site and the transcription site. Alternatively, the protein could polymerize from the repression site along the DNA and interfere with transcription. The third possibility is the generation of a loop in the DNA, which brings the protein bound at the distant repression site near the DNA or proteins on the DNA at the transcription start site. For example, the DNA could bend so that araC protein bound at araO2 could bind to araC protein bound at araI. This could leave araC protein in a state incapable of activating transcription from PBad and would therefore mean that binding at araI is not tantamount to stimulating transcription.

The spacing mutations that alter the distance between araO2 and the induction region of the araBAD also alter the number of DNA helical turns between the induction region and araO2. Introduction of 11 and 31 base pairs, both near integral multiples of the 10.5 base pairs per turn of DNA in vitro (26), permit repression, but alterations in the number of turns by half-integral values interfered with repression of araBAD. These data are most easily compatible with a DNA-looping mechanism. Repression could be hindered in the strains with insertions generating half-integral turns because araC protein then is on the wrong side of the DNA to form the loop required for repression (Fig. 6).

If a loop is to form when araC protein is on the wrong side of the DNA, the DNA between PBad and araO2 must be twisted by half a turn. In vitro, =4 kcal/mol (1 cal = 4.184 J) are required to twist linear DNA of 200 base pairs by half a turn (27, 28). Although the supercoiled state of DNA in vivo as well as the presence of other DNA-binding proteins undoubtedly will alter this value, this order of energy is likely to be involved. Typical protein–DNA dissociation constants indicate that 10–15 kcal per mol of energy are available from binding. Therefore, the consumption of several kcal/mol in twisting the DNA up to half a turn could substantially alter a delicately balanced interaction required for repression.

Upon locating the binding sites of the proteins involved in regulation of the arabinose operon by DNase I protection, we proposed a model that accounted for much of the operon’s known behavior (6). Part of this model was that under inducing conditions araC protein selectively bound at the araBAD site to stimulate PBad and under repressing conditions it did not bind to araI, but instead, it selectively bound to araO2 to repress PBad as well as PC. A second model, also derived from DNase I protection data, for ara regulation also incorporated the simple mechanism of selective binding (29). DNase I protection is not a good method for measuring binding constants of a tight-binding protein, and repeated experiments (23) failed to reproduce the tendency of selective binding shown by araC in the initial experiments (6). Similarly, the data of Lee et al. (29) show no clear selectivity in binding.

More definitive experiments by the gel electrophoresis DNA binding assay clearly show that the affinities of araC protein for araO2 and araI vary in parallel (24). That is, there is no apparent selectivity in binding by araC protein, at least not in vitro on linear DNA.

In light of the absence of selective binding and the findings presented in this paper, we now modify the model for regulation of the arabinose operon. In the absence of arabinose, araC protein bound at araO2 could interact via a DNA loop with a component of the induction complex, CRP, araC protein, or RNA polymerase, and the loop’s formation could prevent induction by holding one of the components in an inactive state. However, in inducing conditions, presence of arabinose and functional CRP, the loop could open and RNA polymerase would be permitted to initiate transcription from PBad. Until a mutational analysis is completed, we must leave open any role for araO2 in repression of PBad.

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