AraC Proteins with Altered DNA Sequence Specificity Which Activate a Mutant Promoter in *Escherichia coli*

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We examined the recognition of the *araBAD* promoter by the AraC protein in the *Escherichia coli* arabinose operon. A mutant promoter, with base substitutions at positions contacted by AraC, was used to isolate suppressor mutations in *araC* by direct selection. Two hydroxylamine-induced *araC* mutations were isolated repeatedly; each contained a single amino acid substitution. When tested against a set of base substitution promoter mutants, one revertant, an Arg to His substitution at residue 250, displayed altered base specificity for a single position within the *araBAD* promoter. The other revertant, a Cys to Tyr substitution at residue 204, did not show consistent base-specific suppression. Neither demonstrated a higher affinity than the wild type protein for the mutant promoter in *vivo*. Both proteins suppress mutant sequences by a mechanism that does not appear to involve the formation of new net favorable contacts with the mutant base pairs of the promoter.

The AraC protein is a regulatory protein that exerts positive and negative effects on the various operons that make up the arabinose regulon in *Escherichia coli*. In the presence of arabinose and in conjunction with the cAMP receptor protein (CAP), it stimulates transcription of the *araBAD*, *araE*, and *araFGH* operons (1-3). These operons encode, respectively, the genes for the catabolism, low affinity transport, and high affinity transport of arabinose (1-3). The AraC protein also autoregulates its own (structural) gene (4) and represses transcription from the *araBAD* operon (5). It has been shown that AraC binds specifically to the *araBAD* (6, 7), *araE* (8), and *araC* (7, 9) promoters.

The AraC polypeptide has a length of 291 residues as predicted by the nucleotide sequence (10, 11). The *M*ₙ of the protein has been estimated to be 60 kDa by sedimentation analysis, suggesting that in solution it exists as a dimer (12). To localize the DNA-binding domains of the AraC protein, we have utilized the principle of secondary site suppression. Direct selection is used to isolate mutant proteins that have acquired amino acid substitutions that compensate for base changes in the nucleotide sequence of the promoter. This strategy was used to identify amino acids making specific DNA contacts in both the Salmonella P22 phage Mnt repressor (13) and the CAP protein (14). In the case of the CAP mutants with altered DNA binding specificity, the residue substituted was independently predicted to be involved in direct DNA contact by crystallographic modeling (15).

Transcriptional activation of the *araBAD* promoter by AraC requires the binding of the protein to the initiator site (araI) located within the promoter (see Fig. 1). Of all single base pair substitutions in *araI* that we have tested (14 total), none showed sufficient reduction in promoter activity to allow positive selection for secondary suppressors under our conditions. Consequently, a promoter with two base substitutions, which is phenotypically Ara−, was used in the selection of *araC* suppressor mutants. Both mutated positions in the promoter are contacted by AraC, based on methylation protection experiments (6, 7, 10). Two mutant alleles of *araC*, each encoding a protein with a single amino acid substitution in the carboxyl-terminal region, were obtained using our selection. The two substitutions, a Cys to Tyr substitution at residue 204 and an Arg to His substitution at residue 250, fall within regions of the protein that show considerable homology to the DNA-binding domains of other regulatory proteins. [His⁵⁵⁰]AraC showed specific suppression of one of the substituted base pairs in the promoter, whereas the suppression by [Tyr⁶⁴⁴]AraC was more complex. The new base-recognition properties of these proteins are accompanied by an overall reduction in affinity for DNA. Activation of mutant promoters by these AraC mutants must occur by a mechanism that does not require the formation of net favorable contacts with the mutated bases of the promoter.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Choosing a Parent Strain for Revertant Selection—We observed that promoter mutants containing single base changes in *araI* are phenotypically arabinose positive. On the other hand, some strains with promoters containing double base substitutions in *araI* showed much slower growth on minimal arabinose medium. Two such double mutants, 31-076 and 31-072 (Table I in Miniprint), grew slowly enough with the wild type *araC* allele on minimal arabinose medium to allow the selection of revertants. We reasoned that if such a double...
were used to select DNA-binding specificity mutants of araC, the mutant proteins would only need to suppress a single mismatch between DNA and protein, since the remaining mismatch would probably not be sufficient to inactivate the promoter. The strain 31-076 was chosen as the parent strain for araC mutant selection because the base pairs at -49 and -59 are contacted by AraC (7).

**Isolation and Sequences of AraC Specificity Mutants—**Plasmid-encoded suppressors of 31-076 were obtained in the manner described under “Experimental Procedures,” and results are summarized in Table II. Suppressors were found in three of six pools of mutagenized DNA; a total of 20 clones carried plasmids that conferred an arabinose-plus phenotype on the parent strain, 31-076. Fragments containing the entire araC gene were isolated from these plasmids, subcloned into mp19, and the nucleotide sequences of their coding regions were determined. Our results showed that 13 carried identical GC to AT transitions, resulting in the substitution of His (CAC) for Arg (CGC) at position 250 in the protein. The other four (also GC to AT transitions) had Tyr (TAC) substituted for Cys (TGC) at position 204. As shown in Table II, the same two mutations were independently isolated twice each. In three of six pools, there were no plasmid-associated mutations, suggesting that these mutations are rare. In a separate experiment not shown here, we also attempted to obtain revertants of strain 31-072 (-49A/-71G), but no plasmid-associated mutations were found.

**Sequence Specificity of Suppression in Vivo—**We examined the sequence specificity of [Hisz5']AraC and [Tyr304]AraC by testing their activities against a set of promoter mutants that contained the original and new mutations in various combinations in single copy on the chromosome. Promoter mutant strains were transformed with plasmids containing either the wild type or a mutant araC allele. The activity of each protein-promoter pair was determined by measuring the induced levels of the araC gene product (l-arabinose isomerase) whose synthesis is regulated by the araBAD operon promoter (Table III).

[Hisz5']AraC and [Tyr304]AraC both showed maximal activities with the wild type araBAD promoter. The increased expression seen in the mutants is due primarily to decreased repression of the araBAD promoter (see below). To correct for this effect, the activity of a particular AraC protein with a mutant promoter is expressed relative to its activity with the wild type promoter (column 4, Table III). This allows us to compare the effectiveness of the mutant proteins to that of the wild type protein in the activation of the various mutant promoters (column 5, Table III).

Both mutants showed significant increases (6.7- and 3.7-fold for the [Hisz5'] and [Tyr304] mutants, respectively) on the -49A/-59A mutant promoter of strain 31-076. Other mutant promoters were used to examine the specificity of the mutant proteins for the -49 and -59 positions independently. To increase the sensitivity of these tests, a base substitution at -71 was used in conjunction with either -49 or -59.

As shown in Table III, [Hisz5']AraC was more active than wild type AraC on promoters containing the -49A mutation, i.e. -49A/-59A (6.7-fold) and -49A/-71G (4.3-fold). On all promoters lacking the -49A mutation, the activities of [Hisz5'] were near those of wild type, i.e. -59C/-71G (0.87-fold) and -59A/-71G (0.74-fold).

[Tyr304]AraC displayed a more complicated pattern of sequence specificity. Higher than wild type levels were observed for this mutant on the -49A/-59A promoter (3.7-fold), but [Tyr304]AraC was identical to wild type AraC when tested on promoters containing -49 and -59 in conjunction with -71 (1.0-fold for -49A/-71G and 1.0-fold for -59A/-71G). Unlike [Hisz5']AraC, which recognized -49 in a context-independent manner, [Tyr304]AraC showed altered specificity for -49 and -59 only when the two mutations were present on the same promoter. [Tyr304] also showed a slight relative preference for A versus C at position -59. In conclusion, this analysis of the base specificity of the mutant proteins shows that the suppression of -49A by [Hisz5'] was context-independent. The suppression by [Tyr304] reflects either context-dependent suppression or the perturbation of several contacts simultaneously.
AraC owing to a loss of autoregulatory function. This was observed with the wild type protein, corresponding to 1.5- and 1.6-fold increases, respectively. One possible cause of these increased activities is an increase in the intracellular concentration of mutant protein on the same promoter. Underlined values represent depar-mutants were more active than the wild type AraC protein on the same promoter.

PBm+ activity of the mutant protein (column 6) and sonicated to prepare cell-free extracts L-Arabinose isomerase specific activities were measured at absorbance of 440 nm in a Bio-Rad spectrophotometer. These were diluted into prewarmed DNA

In Experiment 1, a single pool of mutagenized DNA was prepared by passage through BD1528, while five independent pools were obtained in Experiment 2.

Only 13 of these were cloned and their sequences determined.

**TABLE III**

Comparisons of wild type and mutant araC proteins in the activation of various araBAD promoters

For isomerase assays, overnight cultures were prepared in minimal media that contained 0.01 M MnCl₂, 0.02% arabinose, and 50 μg/ml ampicillin. These were diluted into prewarmed (37°C) media to an absorbance of A₆₀₀ = 0.1 and then grown until mid-log. Cells were collected by centrifugation (6000 × g) at 0°C, suspended in one-hundredth culture volume of buffer (0.01 M glycylglycine, pH 7.6), and sonicated to prepare cell-free extracts. L-Arabinose isomerase specific activities were measured at 3 and 6 min time points by the cytochrome c-hemolysis test as described previously by Park and Lee (33); protein concentrations were measured by the method of Lowry (35). Numbers represent the average values from duplicate cultures.

**TABLE IV**

Comparisons of wild type and mutant araC proteins in P₆ autoregulation

E. coli NL31-024 was transformed with the indicated plasmid and the resulting transformants were assayed for β-galactosidase activities by the procedure of Miller (36). Numbers represent the average values from duplicate cultures.

- pBR322: none (116.0 ± 9.0)
- pBM1: araC⁺ (2.2 ± 0.3)
- pRH-250: Hisz₆⁺ (2.3 ± 0.3)
- pCY-204: Tyrz₆⁺ (3.7 ± 0.1)

**[Hisz₆⁺]** and **[Tyrz₆⁺]** Are Stable in Vivo—To monitor more directly the accumulation and stability of the mutant proteins in vivo, we assayed their presence immunologically. Total cell extracts were prepared from arabinose-induced strains containing araC plasmids with the wild type or mutant alleles. Both mutant proteins accumulated to wild type levels, and proteolysis was only barely discernible (Fig. 3). The intracellular levels of AraC protein observed by immunoblot were consistent with the autoregulation results. In addition neither substitution appeared to affect the global stability of the mutant proteins, as no increased sensitivity to proteolysis was observed.

**[Hisz₆⁺]** and **[Tyrz₆⁺]** Do Not Repress the araBAD Promoter—AraC repression of the araBAD promoter requires an upstream site (araO₂) located in the araC leader sequence (24, 31). Deletion of this AraC-binding site has been shown to increase uninduced levels of araBAD promoter expression 5-fold and induced levels 2-fold (27). Decreased araBAD repression, resulting from altered specificity for araO₂, could be responsible for the increased levels of activity the mutants exhibit on the wild type araBAD promoter. To examine this possibility, we constructed araC deletion strains with (strain NL 30-391) and without (strain NL 30-387) araO₂; the mutant and wild type araC alleles were provided on plasmids where their expression was under the control of the trp-lac fusion promoter (tat). This promoter is not autoregulated by AraC; thus any differences in the araBAD transcription resulting from differences in the level of araC transcription were eliminated. The tat promoter is a stronger promoter than the araC promoter and produces toxic levels of AraC protein when de-repressed by isopropyl-β-D-thiogalactopyranoside; these experiments, therefore, were carried out in the absence of isopropyl-β-D-thiogalactopyranoside. Under these conditions, the level of expression of araC is sufficient to provide maximal induction of the araBAD promoter and produces no growth inhibition. Isomerase-specific activities were measured after growth in the presence of arabinose (Table V).

Both **[Hisz₆⁺]**AraC and **[Tyrz₆⁺]**AraC displayed substantial reductions in araO₂-mediated repression of the araBAD promoter (Table V). Repression was measured by comparing the activity of the araO₂-delet e strain with the activity of the araO₂⁺ strain. We found that the wild type strain displayed the normal level of induced repression (2-fold), whereas the
promoter, is present on the chromosome in single copy. We estimate that this loss of repression causes an approximate 1.6-fold increase in the activities of strains carrying the mutant araC alleles, accounting for their increased levels of activity on the wild type araBAD promoter.

**DNA Binding Characteristics of Revertant Proteins in Vitro**—We examined the interaction of the wild type and mutant proteins with the mutant promoter in vitro. AraC proteins were purified to homogeneity (as judged by SDS-polyacrylamide gel electrophoresis) from an araC deletion strain carrying plasmids expressing either the wild type or mutant proteins. The ability of each AraC protein to bind promoter DNA was measured by polyacrylamide gel electrophoresis of protein-DNA complexes (33). As shown in Fig. 4, neither revertant nor wild type protein was able to retard the mobility of the −49A/−59A promoter fragment at the concentrations tested (compare lanes 9–14 to lane 8). This same result was obtained when the protein-DNA ratio was increased to 40:1 (data not shown). The [TyrZo4]AraC protein bound the wild type promoter with an approximate 2–4-fold quantitative reduction in affinity; at 20 nM, an appreciable amount of the fragment remains unbound. The mobility of the [HisZ5′]AraC DNA complex is greater than those of both the wild type and [TyrZo4]AraC DNA complexes. We have not established the reasons for the diminished mobility retardation and increased smearing we observe for [HisZ5′]AraC. In conclusion, the phenotypes of the mutant proteins do not appear to involve the formation of new productive contacts between the mutant proteins and the mutant promoter DNA.

**DISCUSSION**

We have utilized the principle of secondary site suppression to isolate AraC proteins with altered DNA binding specificity. Starting with an Ara+ araBAD promoter containing base substitutions at positions −49 and −59 relative to the start of araBAD transcription, we obtained two araC mutants which restored an Ara+ phenotype. From six independently derived pools of mutagenized DNA, each mutant was isolated twice. This suggests that, of all possible hydroxylamine-induced...
mutations in araC (total 192), only these two produce the required phenotype.

The experiment we observed was not attributable to causes other than alterations in the specificity of DNA recognition. Both mutant proteins, [His²⁵⁰]AraC and [Tyr²⁴⁰]AraC, auto-regulated the araC promoter, as shown by enzyme assays (Table IV) and by immunoblot analysis (Fig. 3). These tests showed that the intracellular levels of the mutant and wild type AraC proteins were nearly equivalent. Although both mutants were defective in araC-mediated repression, the 1.6-fold increase owing to this loss (Table V) cannot account for the increase in activity observed. The suppression data have been corrected for the loss of repression (3.7–6.7, Table III, last column).

The locations of these mutations are consistent with the assignment of DNA-binding regions in AraC based on its amino acid sequence (37, 38). Two different regions within AraC show amino acid homology to the DNA-binding domains of other prokaryotic transcriptional regulatory proteins (Fig. 5). Domain I extends from residues 196–215; this region possesses the strongest resemblance to amino acid patterns within known helix-turn-helix motifs (37, 39). Domain II (residues 245–265) has been postulated to be a DNA-binding domain (37, 38), although it conforms to the pattern of amino acids in the proposed consensus only in the first 9 residues (Fig. 5). The positions of araC204CY and araC250RH are indicated in Fig. 5. The Tyr²⁴⁰ mutation is located in Domain I at the turn of the bihelical motif; it may perturb several contacts between the flanking helices and DNA, resulting in the observed pattern of suppression. The His²⁵⁰ mutation is located in Domain II. Since [His²⁵⁰]AraC recognizes -49A independently of any other mutation tested, Arg²⁵⁰ may be involved in directly contacting DNA (40, 41). We speculate that the substitution of His for Arg at position 250 may eliminate an important electrostatic contact outside the bihelical primary recognition domain.

On the basis of the in vivo results, we expected the mutant proteins to show preferential binding to the mutant promoter in vitro. This was not observed, even under conditions where the protein/DNA ratio was increased far above that required for the binding of wild type protein (data not shown). The overall reduction in the DNA binding ability of the mutant proteins was not commensurate with their in vivo activities. Both mutants were highly active on a wild type promoter in vivo, but [Tyr²⁴⁰]AraC had reduced affinity (4-fold) for the wild type araC promoter fragment, while the [His²⁵⁰]AraC complex exhibited less stability during gel electrophoresis and sometimes an unusual mobility suggestive of an altered DNA conformation. Although both substitutions clearly affect the DNA-binding functions of AraC, how they impart base-specific suppression is still unexplained. Further experiments are necessary to define the precise mechanisms.

Our failure to observe preferential binding of the mutant proteins to the mutant promoter in vitro may have several causes. It is possible that the mutant proteins make productive contacts with the mutant promoter only in the presence of other macromolecules; consequently, binding is not observed in vitro. During the activation of transcription, AraC binds to araC, placing it in close proximity to both RNA polymerase and CAP. Multiple protein-protein interactions may act to stabilize the complex. The mutant proteins may be more dependent on these interactions for promoter activation than wild type AraC. Another factor which could affect the binding is the conformation of the DNA; linear DNA fragments lack supercoiling and other structural features which might enhance the binding of proteins, particularly the mutant proteins.

![Helix 2](image1.png)
![Helix 3](image2.png)

**Fig. 5.** Sequences in AraC with homology to the DNA-binding helix-turn-helix motif. The two sequences in AraC showing the greatest homology to the α2,α3 sequences of λ cl and CAP eE,eF have been aligned for best match, adapted from Pabo and Sauer (39). Boxed areas indicate hydrophobic residues important in packing interactions between the two recognition helices. The locations of the different sequences in the proteins are as follows: AraC I, 196–215; AraC II, 245–264; Gal repressor, 4–23; λ repressor, 33–52; CAP, 169–188; Lac repressor, 6–25; Trp repressor, 66–85. The positions of the DNA binding specificity mutations in AraC described in this study are indicated above the lines for AraC I and AraC II.
Activation of the araBAD promoter requires the cooperative binding of AraC to two regions of araI, araI1, and araI2 (42). In cases where multiple DNA-binding sites are cooperatively bound, the establishment of a single favorable contact or the loss of an unfavorable one could be offset by an equal or greater number of contacts lost in the neighboring site. This would explain the reduction in overall affinity for araI.

Finally, suppression may be achieved without restoration of direct contact with the mutant base pairs. The activation of the araBAD promoter may involve the contact of RNA polymerase by AraC at the -35 region of the promoter (42). In the wild type promoter, this requires occupancy of araI by AraC; the -49A mutation is located within this region. An intriguing possibility is that a mutant protein may form stronger protein-protein contacts with RNA polymerase and thereby circumvent the requirement for araI occupancy. Studies are underway to explore these possibilities.

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AruC Proteins with Altered Sequence Specificity

Supplemental Material

**AruC Proteins with Altered Nucleic Sequence Specificity**

**Activating a Mutant Protein in Bacillus**

By

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Natural and chemical - Restriction enzymes, ligases, and other DNA-modifying enzymes were purchased from New England BioLabs and Boehringer Mannheim. (EcoR1) and (EcoR1) substrates for sequencing and analysis were purchased from New England BioLabs. (EcoR1) restriction enzyme digestion was performed on PCR products obtained from R.-phage. [77] Protein A was purchased from DPN.

Strain Construction and Plasmids - Four bacterial strains used in the construction of the mutant AruC proteins were derivatives of the strain B. 16-000, and the strain was transformed by electroporation as described above. 

**Figure 2. Construction of the plasmid for expression of wild type and mutant AruC proteins**. The plasmid pUC19-NH was constructed by inserting the wild type DNA sequence corresponding to the wild type strain B. 16-000. The plasmid pUC19-NH was transformed into the E. coli strain DH5α. The plasmid pUC19-NH was digested with EcoR1 and HindIII and then ligated with the wild type DNA sequence corresponding to the wild type strain B. 16-000.
### Table 1
Nucleotide Sequences of the Promoter Mutations used in this study

| Strain   | Mutation | Sequence       |
|----------|----------|----------------|
| 20-272   | wild type| TATAATCTCTAGTGATTTGTCCTTCT |
| 25-016   | -49A-746 | TATAATCTCTAGTGATTTGTCCTTCT |
| 25-071   | -48A-716 | TATAATCTCTAGTGATTTGTCCTTCT |
| 31-022   | -59C-716 | TATAATCTCTAGTGATTTGTCCTTCT |
| 31-221   | -59G-716 | TATAATCTCTAGTGATTTGTCCTTCT |

*In the text, promoter mutants are either referred to by the designation in this column or by their strain number.

*In addition to these mutations, all strains have the araC57 deletion, in which the distal two-thirds of the araC gene coding sequence has been deleted.*