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Activation of *ara* operons by a truncated AraC protein does not require inducer

(arabinose/positive control/DNA-recognition domain/repression/*in vivo* methylation)

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ABSTRACT **The *araC* gene of *Escherichia coli* encodes a protein that binds the inducer L-arabinose to activate the transcription of three *ara* operons. In a study to determine the functional domains within the AraC protein, we have generated a set of overlapping deletions from the proximal end of the *araC* gene. We found that the removal of up to nearly 60% of the coding sequence of this protein still allows transcriptional activation of the *ara* operons *in vivo*, up to 27% that of the wild type. These truncated proteins, however, no longer require arabinose for induction. The ligand-induced conformational change apparently either releases or unmasks an existing functional domain within AraC, rather than generating a new conformation that is required for activation of the promoter of *araBAD*. Since the truncated protein of the mutant C154 (which lacks 153 amino acid residues from the N terminus) retains DNA binding specificity, the DNA-recognition domain is localized in the C-terminal half of the AraC protein. Truncated proteins were unable to repress *araBAD* or *araC in vivo*, even though they were able to bind all *ara* operators. We propose that the N-terminal half of AraC is essential for the formation of the DNA loops that are responsible for repression of *araBAD* and for autoregulation of *araC*.**

Proteins that activate multiple transcription units are widely distributed among biological systems—procaryotic (1), eucaryotic (2), and viral (3). This arrangement may reflect an advantage to the cellular economy in being able to control the synthesis of many genes by a single regulatory protein (1). In addition, when the transcriptional activator undergoes a change in function by binding a small molecule, the cell has an economical way of controlling the expression of a large number of genes in response to an effector molecule in the environment.

The *araC* gene of *Escherichia coli* encodes a protein that binds the inducer L-arabinose to activate the transcription of three widely separated *ara* operons. Two of these encode proteins involved in arabinose transport, and the third, the *araBAD* operon, encodes enzymes of arabinose catabolism (4–6). All three *ara* operons are preceded by common DNA sequences that are tandem repeats of a 17-base-pair (bp) consensus, TATGGAN$_7$GCTA (ref. 7 and W. Hendrickson, personal communication). The binding of AraC protein to one such sequence ($araI_2$) within the *araBAD* operon promoter (P_{BAD}) is inducer-dependent and occupancy of this site is required for P_{BAD} transcription (Fig. 1) (7). In this manner, the regulatory protein AraC signals the presence of the metabolizable substrate in the medium and initiates the synthesis of proteins required for its utilization.

The AraC protein is biologically active in both ligand-bound and ligand-free forms. In the absence of the inducer, AraC is capable of activating P_{BAD} to about 1% that of the induced level. This 1% activation by AraC without inducer is repressed by the binding of AraC to a far upstream low-affinity operator ($araO_2$) (4, 8). Furthermore, it has been shown that repression of P_{BAD} in the wild type requires not only $araO_2$ but also the high-affinity $araI_1$ site; it was proposed that repression requires an association between AraC molecules bound at $araO_2$ and $araI_1$ that loops out 20 turns of intervening DNA (9). This interaction also produces negative autoregulation of the *araC* promoter (P_C) (10). With inducer, AraC binds $araI_1$ and $araI_2$, activating the P_{BAD}; the repression of P_C now results from the occupancy of $araO_2$ and $araO_1$ (10, 11), which are separated by 15 turns of the DNA helix. It thus appears that the activities of the *ara* promoters are governed by the state of occupancy of AraC cognate sites on the DNA.

To fully understand how promoter occupancy can lead to transcriptional activation or repression and how the binding of ligand can dictate occupancy, we need to better understand the structure and function of the AraC protein. In this paper, we report that removal of nearly 60% of this protein still allows transcriptional activation of the *araBAD* operon. The truncated protein, however, no longer requires inducer for activation. This observation suggests that the ligand-induced conformational change either releases or unmasks an existing activation domain within AraC.

The mutant proteins bind specifically to *ara* promoter DNA, indicating that the DNA recognition domain resides within the C-terminal 40% of the protein. This finding agrees with conclusions from less-direct evidence (12, 13). A representative truncated AraC protein was unable to repress *araBAD* or autoregulate *araC* expression *in vivo* despite its ability to bind all *ara* operators, suggesting that the N-terminal 60% of this protein is essential to its negative regulatory functions—i.e., repression of *araBAD* and autoregulation of *araC* (9, 10). Since both of these functions involve the formation of DNA loops by the interaction of AraC molecules bound to widely separated sites (9, 10), we speculate that element(s) required for these interprotein interactions reside in this region.

MATERIALS AND METHODS

Media. MacConkey arabinose medium (Difco MacConkey agar base with 1% L-arabinose) was supplemented with ampicillin at 100 μg/ml. Bacto-peptone broth, with or without 0.4% L-arabinose, contained per liter: 8.5 g of Bacto-peptone, 1.5 g of Proteose-peptone, 2.5 g of NaCl, 8 mg of thiamine, and 100 mg of ampicillin. $MnCl_2$ was added to a final concentration of 20 μM.

Bacterial Strains. Three bacterial strains were used in the characterization of the AraC mutants obtained in this work. Strains 30-391 and 30-387 are derivatives of JM101Δ(lac pro) *thi rpsL endA sbcB15 supE* F'*traD36 proAB lacI^{q}ZΔM15* (14). In 30-391 the wild-type *araC* gene has been replaced by

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Abbreviation: nt, nucleotide(s).

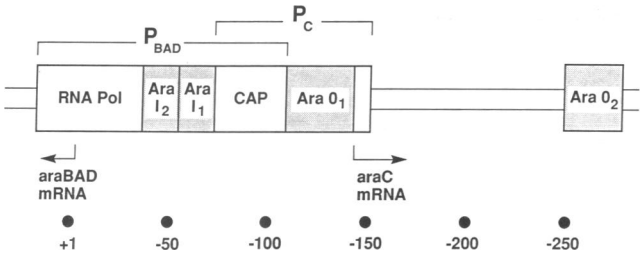

FIG. 1. Regulatory region of the *araBAD* and *araC* operons. The *araBAD* (P_{BAD}) and *araC* (P_C) promoters are shown along with the locations of the binding sites for RNA polymerase (RNA Pol), AraC protein ($araI_1$, $araI_2$, $araO_1$, and $araO_2$), and cAMP binding protein (CAP). Numbers mark positions of nucleotides relative to the start point of *araBAD* transcription.

araC766 [a deletion from *ara* nucleotides (nt) −626 to −1698] and in 30-387 by *araC719* (a deletion from *ara* nt −144 to −1114) (12). Strain 31-024, a derivative of NL20-000 (Ara^+Leu^-*lac514 rpsL*) has its *araC* gene replaced by an *araC:lacZ* fusion. The fusion was constructed by joining the first six codons of *araC* to the eighth codon in *lacZ* (10).

Methylation *in Vivo*. *In vivo* methylation data were obtained essentially as described (16, 17).

RESULTS

Mutant *araC* Genes Lacking Up to 60% of the Wild-Type Coding Sequence Retain the Ability to Activate P_{BAD}. To reduce the size of the AraC protein, the plasmid pKM19 (Fig. 2), which carries a copy of the wild-type *araC* gene, was treated with exonuclease to generate a series of overlapping deletions that removed various lengths of the proximal end of the *araC* coding sequence. The remaining distal ends were fused to the first 16 amino acids of *lacZ* and placed under the control of the *lac* promoter (P_{lac}). The plasmids that retained *araC* activation function were selected by their ability to give rise to arabinose-positive ampicillin-resistant transformants in 30-387, a strain isogenic with JM101 (12) except for the presence of the *araC719* deletion. Fig. 3 shows the distribution of the deletion end points. A substantial number of amino acid residues can be removed without abolishing the activation function of the protein. The most extended deletion, C170, removed the N-terminal 58% of the *araC* polypeptide. None of the truncated AraC proteins retained 100% activation of P_{BAD} (4.7–27.1%), and there is no apparent correlation between the degree of activation and the number of N-terminal amino acids removed (Fig. 3).

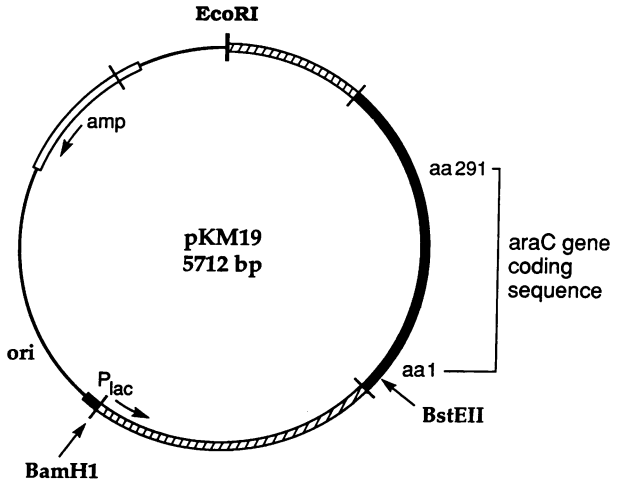

FIG. 2. Construction of AraC deletion mutants. pKM19 is a derivative of the plasmid pUC19. The *lacα* in the latter plasmid was replaced with the entire *lacZ* gene to provide nonessential DNA that could be removed by the BAL-31 exonuclease treatment. A *Mlu* I fragment that carries the entire *araC* gene (*ara* nt −146 to −1638) was inserted in the *Mlu* I site at the distal end of *lacZ*. pKM19 was linearized at a *Bst*EII restriction site at *ara* nt −203 and treated with BAL-31 for various periods of time. A second digestion within the multiple cloning site of pUC19 (with the enzyme *Bam*HI), followed by DNA repair and ligation, joined various lengths of *araC* distal sequences to the P_{lac} and its translational start. The ligation mixture was transformed into an $AraC^-$ strain (30-387) and plated on MacConkey arabinose ampicillin medium. Plasmids that carried *araC* genes capable of activating *araBAD* gave rise to red colonies on this medium and were screened for the sizes of deletions by digestion with *Hin*fI and subsequent analysis on 5% polyacrylamide gels (18).

There was a notable decrease in the accumulation of *araC* product *in vivo* when the deletions extended beyond the first 154 amino acid residues. The AraC polypeptide disappeared from whole cell extracts, as revealed by Na_2DodSO_4/polyacrylamide gel analysis (Figs. 3 and 4). Thus, C157, C161, C164, and C170, although showing degrees of P_{BAD} activation similar to those found in other mutants (Fig. 3), did not accumulate comparable amounts of soluble protein products. This finding indicates that very small amounts of some of the mutant proteins are sufficient for maximal activation. It suggests that the specific activities of these four mutant proteins are very close to that of the wild type, since wild-type *araC*, when present in single copy, also does not produce sufficient AraC to be detectable in gels.

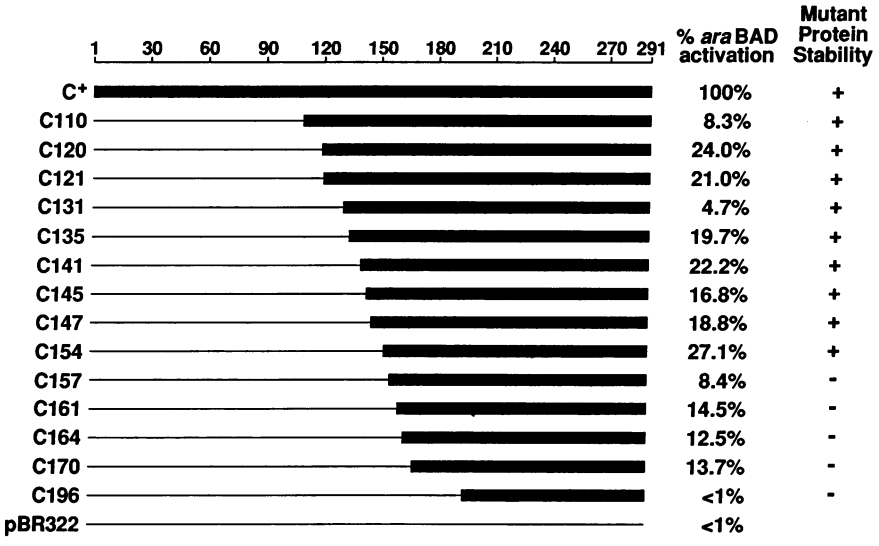

FIG. 3. Map of the *araC* deletions. The extent of each *araC* deletion as determined by DNA sequence analysis is shown. The bars represent the portion of the wild-type AraC protein that was retained. Numbers on the top line mark the positions of amino acids of the wild-type AraC protein. Activation *in vivo* was measured by L-arabinose isomerase (*araA* gene product) assays. (Procedure is described in Table 1.) The values shown are expressed as percent of the wild-type activation level. Stability was investigated by analyzing whole-cell extracts on a 12.5% Na_2DodSO_4/polyacrylamide gel. (Procedure is described in Fig. 4.) The presence (+) or the absence (−) of a polypeptide is indicated.

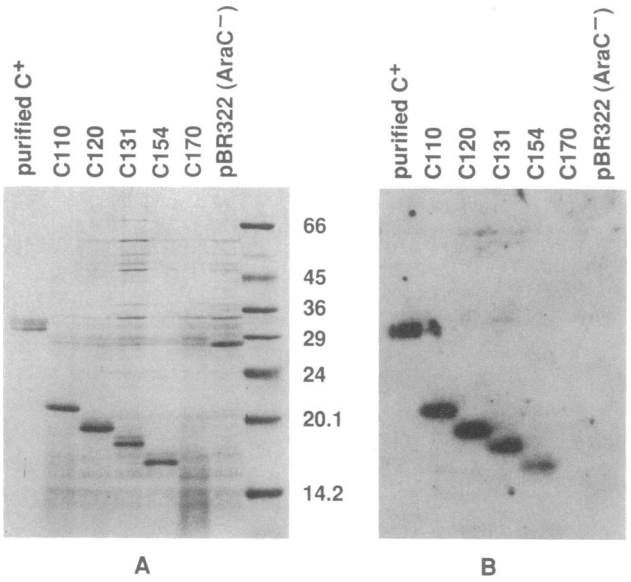

FIG. 4. Na_2DodSO_4/polyacrylamide gel analysis of deletion mutants C110, C120, C131, C154, and C170. Isopropyl β-D-thiogalactopyranoside-induced whole cell extracts were analyzed on a 12.5% $NaDodSO_4$/polyacrylamide gel. Gels were stained with Coomassie blue (*A*) or immunoblotted (*B*) as described (12). Molecular masses in kDa are indicated.

To define an upper limit to the size of the deletions permitted by the selection, an in-frame fusion of P_{lac} with the distal amino acid residues 196–291 (Fig. 3) was constructed. This mutant, C196, was found to be incapable of P_{BAD} activation and did not produce a detectable peptide. Thus residues 170–195, or a portion thereof, are essential either for the activation function or for maintaining a minimum product stability required for activation.

Production of Stable Immunologically Reactive Peptides in C110, C120, C131, and C154. Strain 30-387 transformed with pKM19-C110, −C120, −C131, or −C154 and induced with isopropyl β-D-thiogalactopyranoside contains large amounts of the truncated proteins (Fig. 4*A*). The molecular masses of these polypeptides, determined from their electrophoretic mobilities, were 22.2 kDa, 19.4 kDa, 18.2 kDa, and 16.5 kDa, in close agreement with values calculated from DNA sequence data: 22.5 kDa, 21.2 kDa, 19.9 kDa, and 17.5 kDa, respectively. The mutant peptides are immunologically active (Fig. 4*B*), suggesting that the major antigenic determinants are largely retained in the truncated proteins.

C110, C154, and C170 were further characterized with respect to their response to inducer and their ability to repress P_{BAD} and P_C. The results presented in the following sections are for a representative mutant, C154.

Constitutive Activation of P_{BAD} by C154 Protein. Unlike wild-type AraC protein, the mutant C154 protein does not require the inducer L-arabinose for P_{BAD} activation. In the absence of arabinose, a strain bearing a wild-type *araC* allele on the plasmid pBM1 showed P_{BAD} activity indistinguishable from that found in cells carrying an $AraC^-$ plasmid, pBR322 (Table 1). When arabinose was added to the culture, wild-type *AraC* activated P_{BAD} about 80-fold (Table 1). This induction ratio is normal for cells grown in rich medium. Strikingly, cells carrying the pKM19-C154 plasmid were not induced at all by arabinose; the activation of P_{BAD} by the mutant AraC protein was completely constitutive (Table 1). The lack of inducer response was also found in two other mutants tested, C110 and C170 (data not shown).

The constitutivity of P_{BAD} activation by C154 indicates that the C-terminal half of AraC protein forms the functional transcriptional activator, irrespective of the presence of the inducer arabinose. It further suggests that the N-terminal half of the protein prevents activation, either by masking the activator domain in the C-terminal half or by imparting to the latter an alternate tertiary structure that disallows activation. The function of the inducer would then be the removal of the constraint, analogous to a deletion of the N-terminal portion of the protein.

Table 1. Comparison of wild-type and mutant C154 protein in P_{BAD} activation and repression

Host strain	Plasmid	Isomerase specific activity, units/mg of protien	
		− arabinose	+ arabinose
Activation of the *araBAD* promoter by mutant C154			
30-391	pBM1 (C+)	0.79 ± 0.02	65.33 ± 3.62
	pKM19-C154	20.53 ± 1.38	21.18 ± 1.49
	pBR322	0.53 ± 0.06	0.79 ± 0.01
Repression of the *araBAD* promoter by mutant C154			
30-391 ($araO_2^+$)	pBM1 (C+)	0.79 ± 0.02	65.33 ± 3.62
30-387 ($araO_2^-$)	pBM1 (C+)	9.38 ± 0.49	90.71 ± 3.12
30-391 ($araO_2^+$)	pKM19-C154	20.53 ± 1.38	21.18 ± 1.49
30-387 ($araO_2^-$)	pKM19-C154	29.6 ± 3.12	18.29 ± 1.33
30-391 ($araO_2^+$)	pBR322	0.53 ± 0.06	0.79 ± 0.01
30-387 ($araO_2^-$)	pBR322	0.55 ± 0.06	0.8 ± 0.15

L-Arabinose isomerase was measured by the procedure described in ref. 19 and expressed as units of isomerase per mg of cell protein. A unit is the activity that produces 1 μmol of ketosugar per hr. Induced and uninduced cells were grown eight generations in Bacto-peptone medium with and without 0.4% L-arabinose, respectively. The cells were harvested at $A_{600} \approx 1.0$, cell-free extracts were prepared and assayed for L-arabinose isomerase activity (19) and protein concentration (20). The C154 values are the average obtained from 12 independent cultures. The $AraC^+$ (pBM1) and $AraC^-$ (pBR322) values are averages from 6 independent cultures each. Plasmid pBM1 has the fragment from a *Bam*HI site (at position −44) to a *Cla* I site (at position −2003) containing the *araC* gene cloned into the *Bam*HI and *Cla* I sites of pBR322. All strains were cultivated in Bacto-peptone broth because the mutant strain was severely inhibited by arabinose in minimal medium. All cultures of strains carrying pKM19 derivatives contained 100 μM isopropyl β-D-thiogalactopyranoside to derepress the P_{lac} promoters of the fused *araC* alleles.

C154 Protein Does Not Repress P_{BAD}. Repression of P_{BAD} results in a greatly decreased basal level of *araBAD* expression. For repression to occur, the cell must have two AraC-binding sites $araI_1$ and $araO_2$. Repression assays are carried out by comparing otherwise isogenic $araO_2^+$ and $araO_2^-$ strains. The results are shown in Table 1. In the absence of inducer, wild-type AraC repressed P_{BAD} about 12-fold. In contrast, mutant C154 did not repress P_{BAD}. The absence of repression was also found in two other mutants tested, C110 and C170 (data not shown). It thus appears that the N-terminal half of AraC is required for repression of P_{BAD}.

C154 Protein Does Not Autoregulate Its Own Synthesis. Transcription of *araC* gene is repressed by AraC protein in the absence and in the presence of inducer (21). This repression is sensitive to *araC* gene dosage (15). When wild-type *araC* was provided by a multicopy plasmid, P_C activity was

Table 2. Autoregulation of the P_C promoter by mutant C154 in host strain 31-024

Plasmid	β-Galactosidase, units	
	− arabinose	+ arabinose
pBR322	128.0 ± 0.4	129.1 ± 0.4
pBMI (C+)	2.85 ± 0.05	2.3 ± 0.35
pKM19-C154	111.1 ± 4.4	79.5 ± 6.9

β-Galactosidase assays were done according to the procedure of Miller (22); numbers represent the average values from four independent cultures. Culture conditions were as described in Table 1.

reduced approximately 50-fold in the absence and in the presence of arabinose (Table 2). The mutant pKM19-C154 plasmid effected less than 2-fold reduction in P_C activity, with or without the addition of arabinose. Despite the high copy number, the mutant AraC protein did not significantly repress P_C. The C154 deletion appears to have removed a portion of AraC protein essential for autoregulation.

C154 Protein Binds AraC Protein Cognate Sites on the DNA *in Vivo*. Specific protection against methylation of guanine residues within AraC protein binding sites was demonstrated in cells carrying pKM19-C154. Representative gels are shown in Fig. 5. Similar methylation protection pattern at all the AraC-binding sites was also observed with C170 (data not shown). The protection of the guanine residue at position −70 within $araI_1$ (Fig. 5*A*) is evident in both C^+ and C154 cells and in either glycerol or arabinose medium. $araI_1$ is a high-affinity binding site and is occupied by wild-type AraC in the presence and the absence of arabinose (7). The protection of guanine residues in $araI_2$, at positions −49 and −36, and the enhanced methylation of the guanine residue at position −47 in the induced C^+ lane showed that this weak binding site is occupied by wild-type AraC only in the presence of the

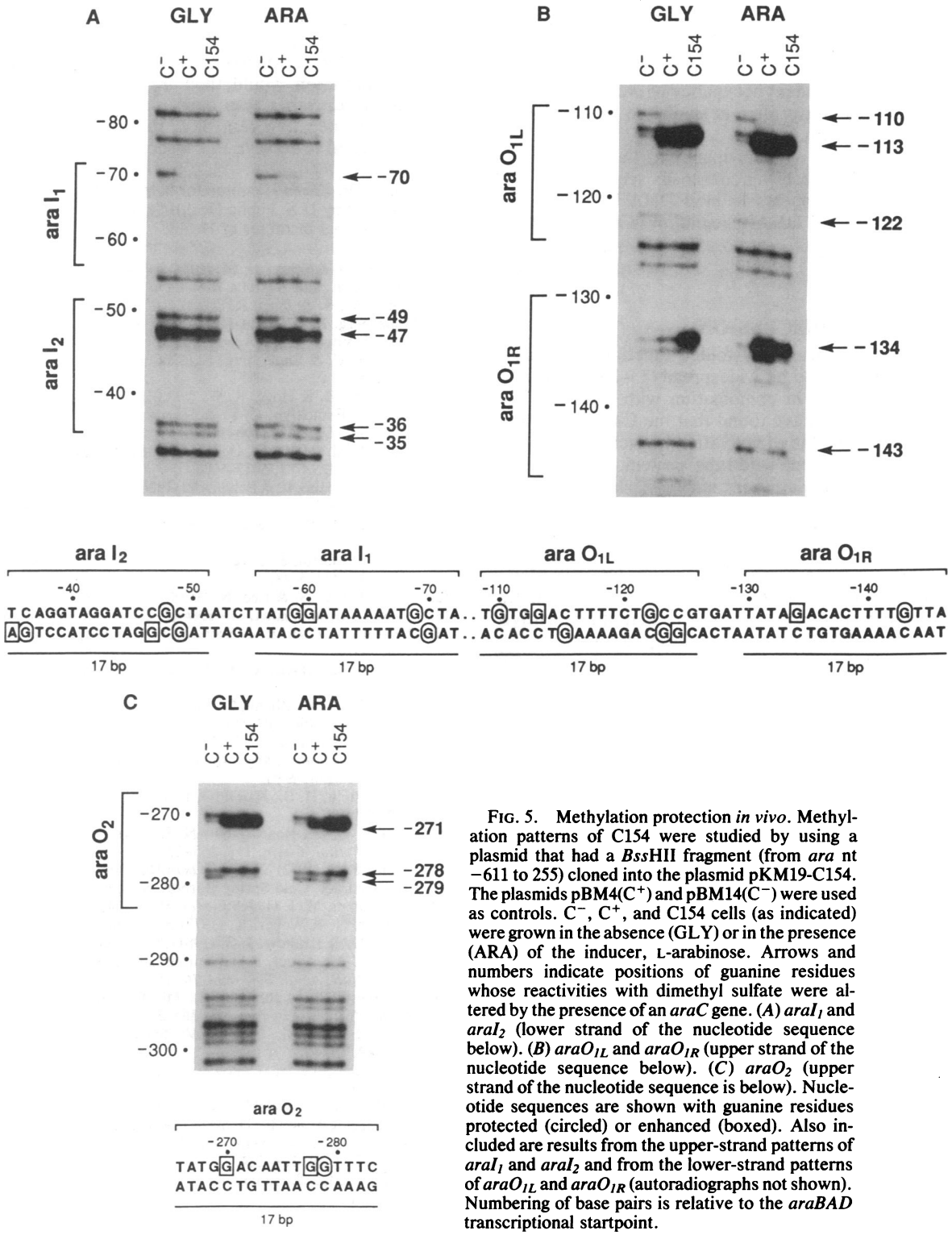

FIG. 5. Methylation protection *in vivo*. Methylation patterns of C154 were studied by using a plasmid that had a *Bss*HII fragment (from *ara* nt −611 to 255) cloned into the plasmid pKM19-C154. The plasmids pBM4(C^+) and pBM14(C^-) were used as controls. C^-, C^+, and C154 cells (as indicated) were grown in the absence (GLY) or in the presence (ARA) of the inducer, L-arabinose. Arrows and numbers indicate positions of guanine residues whose reactivities with dimethyl sulfate were altered by the presence of an *araC* gene. (*A*) $araI_1$ and $araI_2$ (lower strand of the nucleotide sequence below). (*B*) $araO_{1L}$ and $araO_{1R}$ (upper strand of the nucleotide sequence below). (*C*) $araO_2$ (upper strand of the nucleotide sequence is below). Nucleotide sequences are shown with guanine residues protected (circled) or enhanced (boxed). Also included are results from the upper-strand patterns of $araI_1$ and $araI_2$ and from the lower-strand patterns of $araO_{1L}$ and $araO_{1R}$ (autoradiographs not shown). Numbering of base pairs is relative to the *araBAD* transcriptional startpoint.

inducer. These observations with the wild-type cells are in agreement with our *in vitro* DNase I protection results (7).

Fig. 5*B* shows the *in vivo* occupancy at the $araO_I$ site. This site is composed of two 17-bp consensus binding sites, designated $araO_{IL}$ and $araO_{IR}$ (7). The $araO_{IL}$ binds AraC better than does $araO_{IR}$. In the absence of inducer, both $AraC^+$ and C154 cells showed protection of guanine residues within the high-affinity $araO_{IL}$ site (at positions −110 and −122 with enhancement of methylation at position −113). Occupancy at $araO_{IR}$ by wild-type $AraC^+$ protein occurs only in induced cells. This is evident in the methylation enhancement at position −134 and the protection at position −143 in induced C^+ cells but not in glycerol-grown C^+ cells. It is of interest to note that the occupancy at $araO_{IR}$ in C154 occurs without inducer. The methylation enhancement at position −134 is seen in both induced and uninduced C154 cells. This ligand-independent occupancy of $araO_{IR}$ by C154 indicates that the mutant protein is similar to the activator form of wild-type protein.

Fig. 5*C* shows the *in vivo* occupancy at the $araO_2$ site, which consists of a single 17-bp consensus sequence.

The mutant protein appears to bind DNA less well, although the specificity of DNA recognition is retained. The weaker contact with $araO_{IR}$ (at position −143) and the lack of protection in $araI_2$ are probably due to reduced overall binding affinity.

DISCUSSION

The existence of independently folded functional domains within proteins appears to be widespread (23–25). By using *in vitro* DNA resection in combination with selection for activation function, we have found that the C-terminal 40% of AraC protein is capable of activating transcription of *ara* genes at a level 5–27% of wild-type protein. Most of the truncated AraC proteins are very stable *in vivo* and retain near wild-type antigenic reactivity.

The dispensation of the requirement for inducer in transcriptional activation by truncated proteins was observed with several mammalian hormone receptors (26, 27). Considering the diversity of the sources of these activators (i.e., *E. coli*, rat, and human cells), we believe that other positive regulators may be found to share this property also.

We have found that removal of the N-terminal portion of AraC resulted in total loss of inducer response. Several lines of indirect evidence also suggest that the N-terminal half of the protein may contribute toward inducer-binding. Missense mutations showing constitutivity and D-fucose resistance map in the N-terminal half of the protein (refs. 28–30; and E. Hamilton and N.L.L., unpublished data). A single mutant with altered inducer specificity is located in the N-terminal half as well (E. Hamilton and N.L.L., unpublished data). On the other hand, none of several dozens of missense mutations in the C-terminal half of the protein appears to have lost the requirement for arabinose for induction (refs. 12 and 13; N.L.L., unpublished data). Thus, the responses to inducer in more than a hundred mutants are all consistent with the proposal that the binding of arabinose occurs in the N-terminal half of the protein.

Our finding that the C-terminal 40% of AraC protein binds DNA, making specific contacts as does full-length protein, albeit with lower affinities, provides direct evidence for the C-terminal location of the DNA-recognition domain(s). Two regions, one spanning amino acid residues 196–215 and one spanning amino acid residues 245–264, are homologous to sequences that form a "helix-turn-helix" motif in many DNA-binding proteins (12, 31, 32). Both regions fall within the C-terminal 40% of the protein and have been suggested to make specific contacts with DNA (12).

The loss of negative control functions in the deletion mutants poses an intriguing problem. If occupancy of operator sites alone suffices to reduce transcription, then one would expect C154 to retain negative control of P_{BAD} and P_C. Comparison of mutant and wild-type *in vivo* methylation patterns at DNA sites required for repression ($araI_I$ and $araO_2$) and for autoregulation ($araI_I$ and $araO_2$ in uninduced cultures and $araO_I$ and $araO_2$ in induced cultures) showed little difference between wild-type and mutant cells. The loss of negative control in the mutant, therefore, suggests that some features of the protein other than its DNA-binding property are indispensible for repression and autoregulation and that this is provided by the N-terminal portion of the protein. For example, if the N-terminal region of AraC were to participate in AraC–AraC associations while bound to DNA, loss of this region would prevent DNA loop formation. This interpretation would then argue that it is the DNA suprastructure, rather than operator occupancy, that is responsible for negative control of these promoters. Further work is needed to resolve this issue.

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