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Functional domains of the AraC protein

(arabinose operon/dimerization/DNA binding/transcriptional activation)

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ABSTRACT **The AraC protein, which regulates the L-arabinose operons in *Escherichia coli*, was dissected into two domains that function in chimeric proteins. One provides a dimerization capability and binds the ligand arabinose, and the other provides a site-specific DNA-binding capability and activates transcription. *In vivo* and *in vitro* experiments showed that a fusion protein consisting of the N-terminal half of the AraC protein and the DNA-binding domain of the LexA repressor dimerizes, binds well to a LexA operator, and represses expression of a LexA operator–β-galactosidase fusion gene in an arabinose-responsive manner. *In vivo* and *in vitro* experiments also showed that a fusion protein consisting of the C-terminal half of the AraC protein and the leucine zipper dimerization domain from the C/EBP transcriptional activator binds to *araI* and activates transcription from a p_{BAD} promoter–β-galactosidase fusion gene. Dimerization was necessary for occupancy and activation of the wild-type AraC binding site.**

Most of the proteins whose structures have been determined by x-ray crystallography are compact and globular (1). It has, therefore, come as a surprise to discover that many eukaryotic transcription factors contain nearly independent domains and in all likelihood are not compact (2). What about prokaryotic gene regulatory proteins? Some regulators, including TrpR repressor, Met repressor, and Cro repressor, are compact and globular (3–5), but others, such as the LexA, phage λ, and phage 434 repressors, consist of a DNA-binding domain connected to a dimerization domain (6–12). Since the dimeric regulatory protein of the L-arabinose operon in *Escherichia coli*, AraC, can bind to two half-sites in a direct repeat orientation or to two half-sites in inverted repeat orientation (13), each monomer of the protein is likely to possess significant flexibility and could well consist of a dimerization domain loosely connected to a DNA-binding domain.

The generation of protein chimeras involving AraC could facilitate the demonstration and identification of independent domains within the protein. Fusing the DNA-binding domain of AraC to a heterologous dimerization domain could generate chimeric proteins capable of binding to a normal AraC binding site. Such binding could be detected *in vivo* by the ability of such a protein to repress the p_{araC} promoter (p_C) and detected *in vitro* by a simple DNA mobility-shift assay. Analogously, a dimerization domain from AraC could potentiate the specific binding by a DNA-binding domain from another, normally dimeric, protein. Of course, in both cases, the functioning of the chimeric protein would require an appropriate linker connecting the domains.

Well-characterized domains are available for use in the construction of chimeric proteins. The leucine zipper regions from the eukaryotic transcription factors GCN4, Fos, and Jun have been used as dimerization modules (10, 14, 15). Also, the DNA-binding domains of the dimeric λ phage repressor and LexA repressor have been used as DNA-binding modules (6, 10, 14, 15). Several lines of evidence suggest that the N-terminal half of AraC contains the dimerization capability and that the C-terminal half of the protein contains at least part of the DNA-binding capability: direct contacts between bases in DNA and several of the amino acid residues in the C-terminal third of the protein (16), the existence of a dominant negative nonsense mutation in the same region (16), and a report that the final half of the protein encodes both DNA-binding specificity and transcription activation capabilities (17). We therefore constructed two types of chimeras utilizing parts of AraC: the AraC N-terminal half fused to the LexA DNA-binding domain and the AraC C-terminal half fused to the leucine zipper from the eukaryotic transcriptional activator C/EBP (18). These chimeras functioned well *in vivo* and *in vitro*, and here we show that AraC contains functional domains for dimerization, arabinose binding, DNA binding, and activation.

To perform these experiments it was necessary to utilize a number of the *in vivo* and *in vitro* properties of AraC and the arabinose operon. In the absence of arabinose, AraC contacts the $araI_1$ half-site and the $araO_2$ half-site of the *araBAD* regulatory region, forming a DNA loop between the two (Fig. 1) (19). When arabinose is added, the subunit that formerly contacted $araO_2$ releases and shifts to the $araI_2$ half-site, from which it activates transcription from the *araBAD* promoter (p_{BAD}) (19), results that extend a prior observation that the presence of arabinose can extend the region of $araI_2$ that AraC protects from DNase I digestion (20). AraC also represses its own synthesis by binding to the $araO_1$ site that partially overlaps the p_C promoter (21, 22).

MATERIALS AND METHODS

General procedures were as described (23, 24). β-Galactosidase activity (25) is presented as the average of at least three independent experiments.

E. coli strains, with only relevant markers listed, were JL1436 [F′ *lacI^q lacZΔM15*::Tn*9*/*lexA71*::Tn*5 recA*⁺ *sulA211* (λ*sulA*::*lacZ* cI *ind*⁻)] (26), TR321 (F′ *proAB lacI^q lacZΔM15*::Tn*10*/Δ*araC–leu1022 araB*⁺ *A*⁺*D*⁺ Δ*lac74 galK*⁺ Str^r (27), SH242 (F⁻ p_C–*lacZ araB180 leu*⁻ *thr*⁻ Δ*lac74* Str^r *thi*) (28), and JL797 (*lexA71*::Tn*5*/F′::Tn*3 lacI^q lacZΔM15*) (8).

DNA fragments were generated by PCR with conditions as described (13), using oligonucleotides with homology to the desired region plus additional nonhomologous sequence tails with unique restriction endonuclease sites for cloning. Plasmid pMSV-C/EBP and pAB1003 were templates for the C/EBP leucine zipper and the AraC domains, respectively (16, 18). As template for the *lexA* gene we used *E. coli* chromosomal DNA. Plasmid pSE380 (Invitrogen, San Diego) was used as the expression vector for all the constructs not otherwise noted. It is a derivative of pTrc99A (29), which carries a strong regulated hybrid *trp*/*lac* promoter, the *lacZ* ribosome binding site, a multiple cloning site, the *rrnB*

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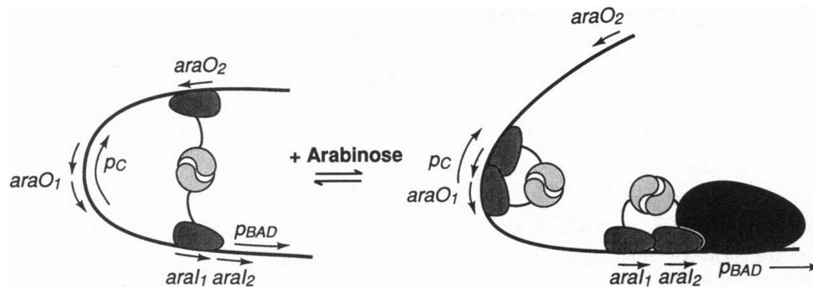

FIG. 1. Schematic representation of the *araCBAD* regulatory region. The presence of arabinose breaks the repression loop by reorienting the AraC subunits to contact adjacent DNA sites, resulting in activation of p_{BAD}.

transcription terminators, and the *lacI^q* gene. DNA fragments obtained by PCR were purified in agarose gels, recovered by electroelution, digested, and cloned into the respective restriction sites of pSE380. The sequences at the junction points were verified by dideoxy sequencing (30). Clones were obtained from color indicator plates on which >95% of the colonies showed the expected phenotype.

For DNA mobility-shift assays (31), radiolabeled DNA fragments were obtained by PCR using 100 ng of the 5′-end-labeled oligonucleotides with 150 ng of the unlabeled counterpart and the desired template. We used *E. coli* chromosomal DNA as template for the LexA operator sites of the *sulA* gene. The DNA fragments were separated by 6% PAGE and the DNA was recovered by electroelution (24). The binding reactions were done in the presence of 1.5 μg of sheared calf thymus DNA per 20-μl volume and, in some cases, 50 mM arabinose. Binding reactions were incubated at 37°C for 10 min.

Assays were performed with pure AraC or with chimeric proteins obtained from crude extracts of *E. coli* cells carrying the appropriate overexpression plasmid. Crude extracts were obtained from 2 ml of cells (4×10^9 cells). After centrifugation, cells were resuspended in 0.5 ml of 0.1 M potassium phosphate, pH 7.4/50 mM KCl/1 mM EDTA/10% (vol/vol) glycerol/0.1 mM $ZnCl_2$/1 mM phenylmethylsulfonyl fluoride with 50 mM arabinose (where indicated) and sonicated for seven 5-sec pulses. The cell debris was removed by centrifugation for 10 min at 12,000 × *g* and the supernatant was stored in 30% glycerol at −70°C for up to several weeks.

RESULTS

DNA Binding and Dimerization by Chimeras Containing Parts of AraC. DNA was constructed encoding the N-terminal half of AraC fused to the LexA DNA-binding domain ($AraC_{Dimer}$–$LexA_{DNA}$), the C-terminal half of AraC fused to the leucine zipper region of C/EBP (Zip_{Dimer}–$AraC_{DNA}$), and the C/EBP leucine zipper fused to the LexA DNA-binding domain (Zip_{Dimer}–$LexA_{DNA}$) (Fig. 2). The ability of the $AraC_{Dimer}$–$LexA_{DNA}$ chimera to bind specifically to the LexA operator was assayed *in vivo* by the ability of the protein to repress β-galactosidase expression from p_{sulA}–*lacZ*. Table 1 shows that the LexA DNA-binding domain itself repressed only by a factor of 2 but that fusing this to the C/EBP leucine zipper dimerization domain or the N-terminal half of AraC led to repression by a factor of 14 or 12, respectively. The native LexA protein repressed by a factor of 26.

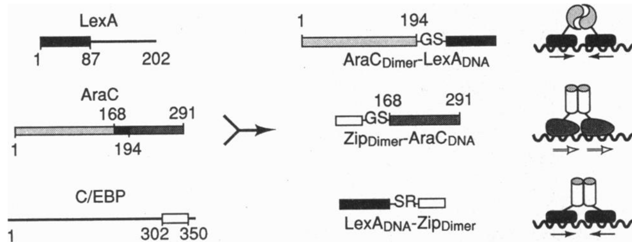

FIG. 2. (*Left*) Regions used to construct the chimeric proteins. Boundaries of the relevant domains are indicated by amino acid position numbers. (*Center*) Linear structures of the chimeric proteins; capital letters joining the different segments indicate the amino acids added during cloning. (*Right*) The proteins interacting with their respective DNA binding regions, depicted as wavy dark lines. Arrows below the DNA show the orientation of the native half-sites.

The ability of the converse chimera, Zip_{Dimer}–$AraC_{DNA}$, to recognize the AraC binding site was assayed *in vivo* by the ability of the protein to repress β-galactosidase expression from a p_C–*lacZ* fusion. As above, the DNA-binding domain itself did not repress (Table 2), but when it was fused to a dimerization domain from C/EBP, the chimeric protein repressed p_C–*lacZ* by a factor of 13. The results from both fusion experiments indicate that AraC contains separate dimerization and DNA-binding domains and locate these domains within the sequence of protein.

The Chimeras Can Dimerize *in Vivo*. The finding that the $AraC_{Dimer}$–$LexA_{DNA}$ fusion protein efficiently repressed the expression of p_{sulA}–*lacZ*, whereas the LexA DNA-binding domain alone did not, suggested that the addition of the AraC dimerization region did indeed dimerize the chimeric protein. Among the potential artifacts, however, was the possibility that the addition of the AraC sequence merely stabilized the LexA DNA-binding domain. We therefore showed that both of the chimeras we constructed using segments of AraC protein were capable of dimerizing.

If the $AraC_{Dimer}$–$LexA_{DNA}$ chimeric protein can dimerize, then its high-level expression in a strain possessing the wild-type *araC* gene should engage most of the wild-type AraC protein in nonfunctional dimers of the form AraC/$AraC_{Dimer}$–$LexA_{DNA}$. Because these heterodimers lack two DNA-binding domains with *ara* selectivity, they should be unable to bind and activate p_{BAD} transcription from the *araI* site, which consists of $araI_1$ and $araI_2$ half-sites. That is, the $AraC_{Dimer}$–$LexA_{DNA}$ chimera should act in a trans-dominant negative way to inactivate wild-type AraC. This effect was observed; AraC activated p_{BAD} 66-fold, but when $AraC_{Dimer}$–$LexA_{DNA}$ was also expressed, activation dropped to 8-fold (Fig. 3*A*).

By the same reasoning, we showed that Zip_{Dimer}–$AraC_{DNA}$ also dimerizes *in vivo*. In this case we arranged for synthesis of the leucine zipper domain itself. Its expression led to the

Table 1. β-Galactosidase activity from p_{sulA}–*lacZ*

Protein expressed	β-Galactosidase units	Repression factor
None (strain only)	2300	—
None (+ pBR322)	2400	—
LexA	90	26
$LexA_{DNA}$	1300	2
$LexA_{DNA}$–Zip_{Dimer}	160	14
$AraC_{Dimer}$–$LexA_{DNA}$	190	12

Plasmids expressing the indicated proteins were transformed into the LexA operator strain JL1436. β-Galactosidase levels represent expression from p_{sulA}–*lacZ* fusion. Measurements were performed in the absence of arabinose.

Table 2. β-Galactosidase activity from p_C–*lacZ*

Protein expressed	β-Galactosidase units	Repression factor
None (strain only)	160	—
None (+ pBR322)	175	—
AraC	10	16
$AraC_{DNA}$	120	1
Zip_{Dimer}–$AraC_{DNA}$	12	13

Plasmids expressing the indicated proteins were transformed into the AraC operator strain SH242. β-Galactosidase levels represent expression from p_C–*lacZ* fusion. Measurements were performed in the absence of arabinose.

formation of Zip_{Dimer}/Zip_{Dimer}–$AraC_{DNA}$ dimers that had a trans-dominant negative effect on the ability of Zip_{Dimer}–$AraC_{DNA}$ chimera to repress p_C–*lacZ* (Fig. 3*B*). The first dominance experiment shows that $AraC_{Dimer}$–$LexA_{DNA}$ can dimerize, and the second shows not only that Zip_{Dimer}–$AraC_{DNA}$ can dimerize but also that dimerization is necessary for function. Neither experiment addresses the question of the actual dimerization affinities.

Both DNA-Binding Domains of Zip_{Dimer}–$AraC_{DNA}$ Protein Simultaneously Contact DNA. The dimerization capability provided by the leucine zipper region of C/EBP in Zip_{Dimer}–$AraC_{DNA}$ permits the AraC DNA-binding domain to bind to the *$araO_1$* site. There is a symmetry mismatch, however, between the dimerization domain and the DNA binding site (Fig. 4). The leucine zipper domain forms a parallel, two-stranded coiled-coil of α-helices most suitable for the attached DNA-binding domains to contact a DNA site with inverted repeat structure. The chimeric protein appeared, however, to bind to the *$araO_1$* site, a site with direct-repeat symmetry (13). Therefore, it was important to show directly that both DNA-binding domains of Zip_{Dimer}–$AraC_{DNA}$ actually made specific DNA contacts. The alternative possibility was that one of the DNA-binding regions made specific contacts to its DNA binding site and that the other DNA-binding domain made nonspecific contacts to DNA. These two alternatives were distinguished by examining the DNA-binding properties of the chimeric protein.

Fig. 4 shows a DNA mobility-shift assay of AraC and Zip_{Dimer}–$AraC_{DNA}$ proteins after incubation with radiolabeled DNAs containing three different embedded sites: *$araI_1$–$araI_1$* direct repeat of the half-sites, *$araI_1$* single repeat half-site, and nonspecific DNA. At equal protein concentration, the Zip_{Dimer}–$AraC_{DNA}$ chimera formed a distinct specific complex with the direct repeat *$araI_1$–$araI_1$* site but not with the other DNA samples. The same was true of wild-type AraC. These results indicate that both of the DNA domains of the Zip_{Dimer}–$AraC_{DNA}$ chimera can contact DNA specifically.

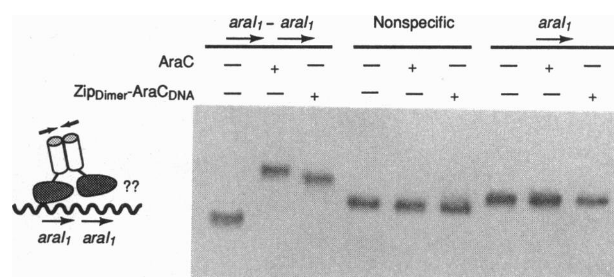

FIG. 4. Both DNA-binding domains of Zip_{Dimer}–$AraC_{DNA}$ specifically and simultaneously contact DNA. DNA mobility-shift assays used a 130-bp *$araI_1$–$araI_1$* fragment, a 320-bp *$araI_1$* fragment, and a 260-bp nonspecific fragment with pure AraC and with Zip_{Dimer}–$AraC_{DNA}$ prepared from a lysate carrying the overexpression plasmid. Lanes 1–3 were loaded 20 min prior to lanes 4–9. Electrophoresis continued for a total of 180 min.

Activation by the AraC DNA-Binding Domain. The preceding sections showed that the C-terminal half of the AraC protein comprises a domain capable of specific binding to DNA when it is connected to a dimerizing domain. Does the DNA-binding domain also possess the determinants necessary for activating transcription? We tested this possibility by cotransforming a plasmid carrying a p_{BAD}–*lacZ* fusion and the plasmid encoding the Zip_{Dimer}–$AraC_{DNA}$ hybrid protein into cells deleted of the *araC* and *lacZ* genes. Introduction of the chimeric Zip_{Dimer}–$AraC_{DNA}$ increased the β-galactosidase levels 30-fold, from 200 units to 6100 units (Table 3), indicating that the chimeric protein binds to the *araI* site and activates p_{BAD} *in vivo*.

The DNA-binding domain of the AraC protein, $AraC_{DNA}$, when not connected to a dimerization domain was unable to

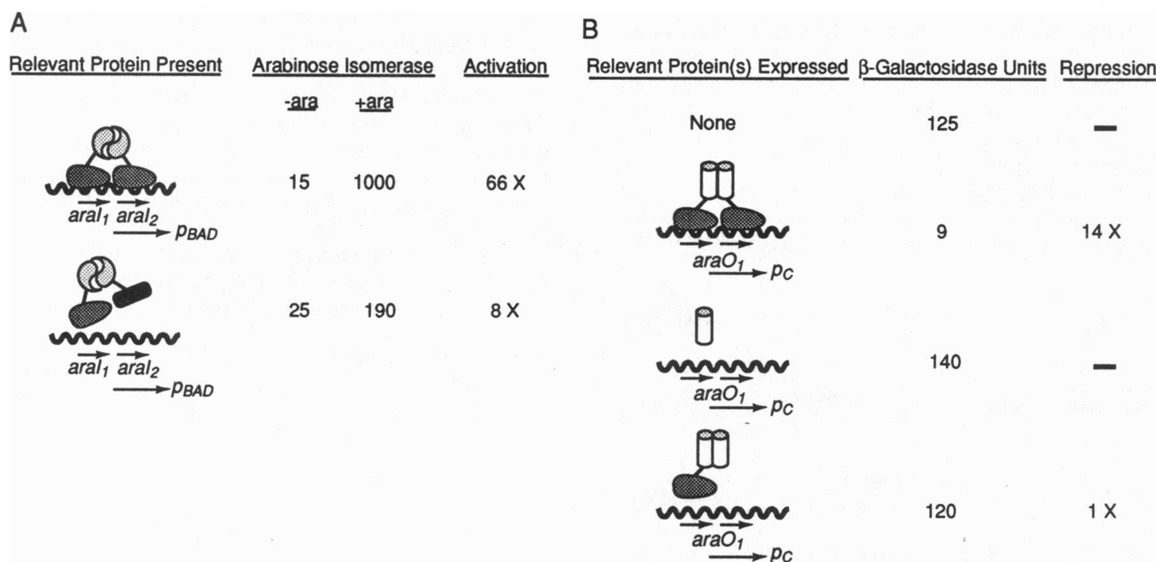

FIG. 3. Dimerization of the chimeric proteins *in vivo*. (*A*) An AraC dimer binds to the *$araI_1$* and *$araI_2$* half-sites and induces expression of p_{BAD}. Additional expression of $AraC_{Dimer}$–$LexA_{DNA}$ interferes with activation. Levels of arabinose isomerase enzyme in the presence or absence of arabinose (+ara or −ara) are shown in units per cell. (*B*) Expression of Zip_{Dimer}–$AraC_{DNA}$ from a modified pSE380 vector containing the p15A origin of replication (32) results in binding to *$araO_1$* and repression of p_C. Additional expression of the leucine zipper alone (white cylinder) interferes with repression.

Table 3. β-Galactosidase activity from p_{BAD}–*lacZ* promoters in the presence and absence of arabinose (ara)

	None	AraC		Zip_{Dimer}–$AraC_{DNA}$		$AraC_{DNA}$	
Fusion	+ ara	− ara	+ ara	− ara	+ ara	− ara	+ ara
$araI_1$–$araI_2$$p_{BAD}$–lacZ	200	90	25,000	6,100	9,000	100	120
$araI_1$–$araI_1$$p_{BAD}$–lacZ	200	22,500	24,300	21,600	19,800	2,300	3,700

Plasmids carrying *$araI_1$–$araI_2$$p_{BAD}$–lacZ* or *$araI_1$–$araI_1$$p_{BAD}$–lacZ* fusions were cotransformed with plasmid overexpressing AraC, Zip_{Dimer}–$AraC_{DNA}$, or AraC DNA-binding domain alone in TR321, a strain lacking *araC* and *lacZ* genes; the overexpression plasmids were derived from a modified pSE380 vector containing the p15A origin of replication (33). β-Galactosidase levels for different combinations were measured in the presence or absence of arabinose.

activate detectable transcription from the wild-type p_{BAD} promoter. This is as expected because AraC occupancy of the half-site closer to the RNA polymerase binding site is necessary for induction, but this half-site binds AraC only weakly (13, 27). To test whether the DNA-binding domain by itself could activate, we used a promoter generated in a previous study in which the half-site closest to the RNA polymerase has been changed to the more tightly binding sequence *$araI_1$* (27). The overall *$araI_1$–$araI_1$* site has been repositioned by two bases so that the −35 region of the promoter is still functional (27). This *$araI_1$–$araI_1$* site was activated 100-fold by wild-type AraC and 90-fold by Zip_{Dimer}–$AraC_{DNA}$. The AraC DNA-binding domain without a dimerization domain was able to stimulate this promoter 15-fold (Table 3). These results indicate that the C-terminal domain of AraC contains the information required to activate transcription; however, it fails to activate the *$araI_1$–$araI_2$* wild-type promoter because it is unable to interact with an *$araI_2$* half-site. Our results are in general agreement with previous work by Menon and Lee (17) which demonstrated that the C-terminal domain of AraC contained the information required to bind and activate transcription of the *araBAD* genes (17).

A Trans-Domain Interaction Between the AraC Dimerization Domain and the LexA DNA-Binding Domain. Results from earlier experiments (see *Discussion*) imply that the dimerization domain of AraC binds arabinose. These results also suggest that arabinose generates a substantial conformational change in AraC. Therefore, it seemed possible that the presence of arabinose could have a detectable effect on the $AraC_{Dimer}$–$LexA_{DNA}$ chimera. Remarkably, an effect, although small, was observed, both *in vivo* and *in vitro*. The addition of arabinose to cells containing the p_{sulA}–*lacZ* fusion and $AraC_{Dimer}$–$LexA_{DNA}$ increased repression of β-galactosidase synthesis (Fig. 5*A*) about 2-fold. Neither the indicator strain itself nor the strain containing LexA showed a similar response to arabinose.

In vitro experiments also showed that arabinose increased the binding of $AraC_{Dimer}$–$LexA_{DNA}$ to the LexA operator. $AraC_{Dimer}$–$LexA_{DNA}$/DNA complex did not form when a cell extract from the untransformed strain was present nor when a 100-fold molar excess of unlabeled specific competitor DNA was added to the binding reaction mixture before the labeled DNA. Detectable amounts of the $AraC_{Dimer}$–$LexA_{DNA}$/DNA complex formed only when arabinose was included in the binding assay buffer. This was added immediately or after 10 min to demonstrate that arabinose was not

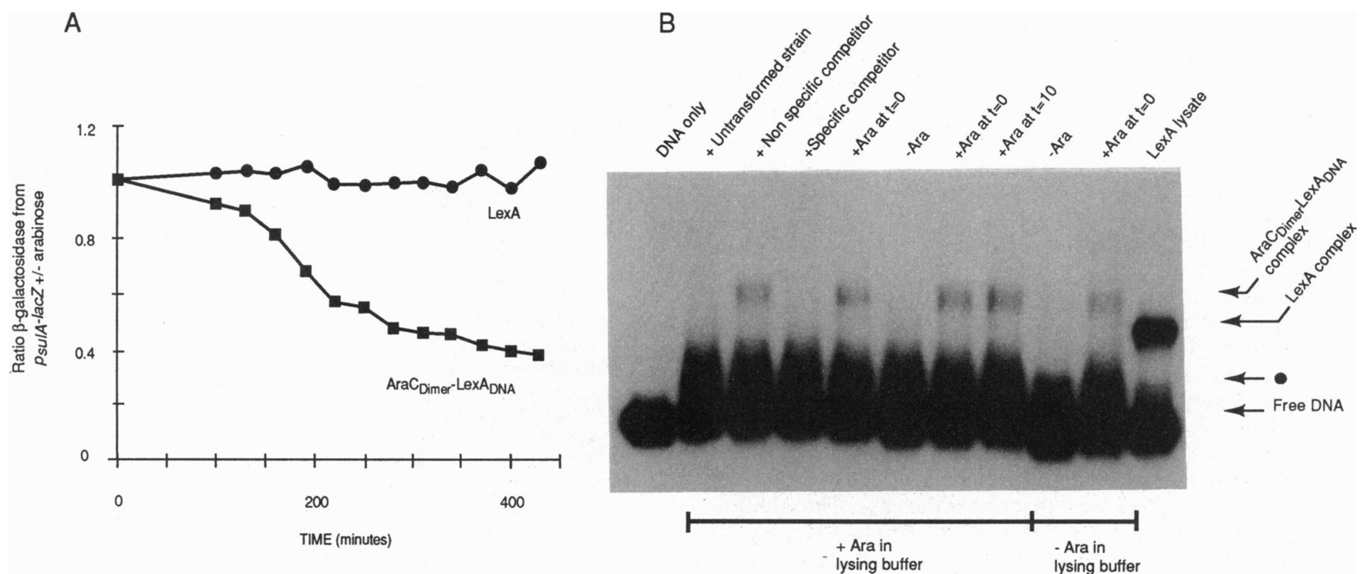

FIG. 5. Arabinose increases the binding affinity of the $AraC_{Dimer}$–$LexA_{DNA}$ for the LexA operator. (*A*) Ratio of β-galactosidase levels obtained from cells cultured in the presence or absence of arabinose. The indicator strain contained a p_{SulA}–*lacZ* fusion and was transformed with overexpression plasmids encoding LexA (●) or $AraC_{Dimer}$–$LexA_{DNA}$ (■). Cells were grown overnight in the absence of arabinose and at time zero were diluted into medium with and without arabinose. (*B*) DNA mobility-shift assays of radiolabeled DNA fragment containing the *sulA* operator, incubated with lysates from *lexA*⁻ cells alone (strain JL797) or transformed with overexpression plasmids expressing LexA or $AraC_{Dimer}$–$LexA_{DNA}$. The lysate used for each reaction, unless indicated above the lane, was from $AraC_{Dimer}$–$LexA_{DNA}$-expressing cells. The presence of arabinose in lysates is shown by the bars at the bottom. Unlabeled *$araI_1$–$araI_1$* fragment was used as nonspecific competitor in 100× molar excess. Arabinose was added at different times during the binding reaction, and the time (t) of addition is indicated in minutes. DNA–protein complexes are shown along with the unbound DNA. The black dot shows an undefined complex that was formed with most of the lysates, including that from the untransformed strain.

altering the stability of the protein (Fig. 5*B*). These results indicate that an arabinose-binding site is located in the dimerization domain in the N-terminal half of AraC and that this domain undergoes a change upon the addition of arabinose that affects the DNA-binding properties of a heterologous DNA-binding domain attached by a linker.

DISCUSSION

The experiments described here show that functional chimeric proteins can be constructed in which either their dimerization and arabinose-responsive abilities derive from the N-terminal half of AraC protein or their DNA-binding and transcription-activation abilities originate from the C-terminal half of AraC. Thus, functional domains of AraC specifying these four activities have been located. The experiments were designed to locate dimerization and DNA-binding domains, and it was not expected that these same chimeric proteins would also allow localization of the transcription-activation and arabinose-binding portions of AraC. The approach and constructs we used should prove useful in defining the minimum sequences for dimerization and DNA binding and may also be useful in the additional localization of the portions of the protein involved with arabinose binding and transcriptional activation.

As mentioned in the introduction, direct biochemical probing has located amino acid–DNA-base contacts between residues 208 and 212 of AraC and one of the two major groove regions of the DNA half-site that is contacted by each monomer of the AraC dimer (16). The location of the amino acids that contact the second major groove of each half-site is unknown. Our localization of all the amino acids necessary for specific DNA binding restricts the structure that contacts DNA to the second half of AraC but leaves unanswered the role of a second potential helix–turn–helix region that begins at amino acid 256.

Notwithstanding the results in this paper showing that the N-terminal half of AraC can participate in specific dimer formation, our results do not eliminate the possibility that *in vivo* the DNA-binding domains themselves of both AraC and LexA are unstable and are stabilized, but not dimerized, by additional protein. Then, while it is true that AraC is dimeric *in vitro* (32, 34) and normally requires both half-sites for binding to DNA *in vivo* (20, 27), our experiments could have misidentified the relevant dimerization domain of AraC. This possibility appears unlikely because other studies utilizing the DNA-binding domains of λ repressor or LexA found that dimerization by domains from GNC4, Fos, and Jun proteins was required for binding to their respective operators (10, 14, 15), the same result on which we base our conclusion of dimerization. Thus, the ability of $AraC_{Dimer}$–$LexA_{DNA}$, but not the LexA DNA-binding domain, to repress p_{sulA}–*lacZ* was more likely the result of the protein's dimerization.

Previous work suggested that arabinose does not directly affect the DNA binding of AraC by altering the structure of the DNA-contacting part of the protein (13). The experiments described here demonstrate that this is the case. The addition of arabinose had a measurable effect on the binding of $AraC_{Dimer}$–$LexA_{DNA}$ to the DNA. Since it has been inferred (13) and directly shown (J. Withey and R.F.S., unpublished work) that arabinose does not affect dimerization of AraC monomers, the effect of arabinose on the binding of $AraC_{Dimer}$–$LexA_{DNA}$ to the LexA operator most likely is the result of a conformational change in the N-terminal part of the protein. A similar trans-domain ligand-induced response has been observed in fusion proteins carrying the LexA DNA-binding domain and the hormone-binding site of the glucocorticoid receptor (35).

In summary, functional chimeras have been constructed in which the N-terminal half of AraC provided dimerization and arabinose responsiveness and the C-terminal half provided binding and transcription-activation capabilities. The construction of such functional chimeras demonstrates that AraC possesses a modular structure, with independent domains capable of dimerizing and of binding to DNA.

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