DNA Looping and Unlooping by AraC Protein

ROBERT B. LOBELL* AND ROBERT F. SCHLEIF

Expression of the L-arabinose BAD operon in *Escherichia coli* is regulated by AraC protein which acts both positively in the presence of arabinose to induce transcription and negatively in the absence of arabinose to repress transcription. The repression of the *araBAD* promoter is mediated by DNA looping between AraC protein bound at two sites near the promoter separated by 210 base pairs, *araI* and $araO_2$. In vivo and in vitro experiments presented here show that an AraC dimer, with binding to half of *araI* and to $araO_2$, maintains the repressed state of the operon. The addition of arabinose, which induces the operon, breaks the loop, and shifts the interactions from the distal $araO_2$ site to the previously unoccupied half of the *araI* site. The conversion between the two states does not require additional binding of AraC protein and appears to be driven largely by properties of the protein rather than being specified by the slightly different DNA sequences of the binding sites. Slight reorientation of the subunits of AraC could specify looping or unlooping by the protein. Such a mechanism could account for regulation of DNA looping in other systems.

IN BOTH PROKARYOTES AND EUKARYOTES, DNA SEQUENCES located up to several kilobases or more away from the main site of action participate in regulating processes of transcription initiation, DNA replication, and recombination (*1, 2*). Some direct and much indirect data have accumulated indicating that these "action at a distance" events result from DNA looping in which proteins bound to two or more well-separated sites directly interact, thereby looping out the intervening DNA (*2–4*). However, little information has been obtained showing how the formation or dissolution of loops may be regulated.

DNA looping was first proposed and demonstrated in studies of gene regulation in the arabinose *araCBAD* operon of *Escherichia coli* (*2*). Products of the *araBAD* genes are proteins that permit the cells to catabolize arabinose as a source of carbon and energy. The protein AraC regulates the synthesis of *araBAD* gene products by regulating their transcription (Fig. 1), acting positively when arabinose is present and negatively when arabinose is absent (*5*). The positive action requires AraC binding at a site in the *araI* gene, while the negative action requires AraC binding not only at *araI* but also at $araO_2$, which is located 210 base pairs (bp) away. AraC when bound to these two sites directly interacts, forming a DNA loop between the sites (*2, 3*). Two facts suggest that the normal response to the addition of arabinose is the disappearance of the loop. First, normal induction does not require $araO_2$ (*6, 7*)—it can be deleted; and second, the initial response to the addition of arabinose is a rapid disappearance of AraC protein from $araO_2$ (*6*). The cyclic AMP (adenosine monophosphate) receptor protein, CRP, plays a role in induction by assisting opening of the loop (*8*) in addition to stimulating transcription of the BAD promoter by other as yet unknown mechanisms.

Using the *E. coli* system (*2*) for studying gene regulation, we have obtained data that encompasses the following four major points. (i) In the absence of arabinose, a DNA loop is maintained by an AraC dimer that makes contact with the $araO_2$ site and only half of the *araI* site, termed $araI_1$; (ii) arabinose breaks the $araO_2$-$araI_1$ loop, and leads to occupancy at the other half of *araI*, termed $araI_2$; (iii) loop breaking and loop re-formation do not require binding of additional AraC protein, implying that an arabinose-induced conformational change shifts part of the protein from contacting $araO_2$ to contacting *araI*; and (iv) the change in occupancy resulting from arabinose-induced loop breaking is not completely specified by the DNA sequences involved. We suggest that the loop breaking process is predominantly driven by an arabinose-induced conformational change in the relative orientation of the two subunits of AraC.

One component to the looping phenomena summarized above is that AraC protein contacts $araI_1$, but not $araI_2$, in the absence of arabinose when looping is possible; but it contacts both $araI_1$ and $araI_2$ in the presence of arabinose. Precisely such a binding response has been observed in the *araCBAD* system with deoxyribonuclease (DNase) footprinting on linear DNA under a condition where looping does not normally occur (*9*); on that basis, several of the results we demonstrate here had been suggested earlier (*10*).

Formation of DNA loops in the absence of arabinose. We used a simple electrophoretic mobility assay to study AraC-mediated DNA looping on small supercoiled DNA molecules, or minicircles. We showed previously that AraC-mediated looping requires supercoiling of the DNA (*9*), and the "minicircle" assay now provides a convenient means of studying looping in the *araCBAD* regulatory system. The minicircle assay, first applied to a study of DNA looping in the *lac* system by Krämer *et al.* (*11*), revealed two properties characteristic of DNA loops. (i) Looped complexes formed on negatively supercoiled minicircles migrate during electrophoresis at a faster rate than unbound minicircles or minicircles containing protein bound at only one site. (ii) The additional protein-DNA contacts afforded by the interaction of the second DNA site with the protein greatly increase the binding energy, and as a result dramatically increase the lifetimes of the protein-DNA complexes in the looped state compared to the lifetimes of a state in

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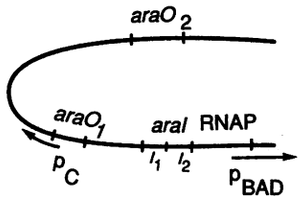


Fig. 1. Regulatory sites of the *araC-BAD* operon. Locations of the AraC binding sites, defined by DNase I footprinting, are shown relative to the transcriptional start site of the promoter p_{BAD} at +1. The *araI* site (*5*) divided into two "half" sites (*10, 22*); $araI_1$ extends from −56 to −78, and $araI_2$, from −35 to −51. $araO_1$ extends from −106 to −144 (*5*), and $araO_2$ from −265 to −294 (*2*). The p_C promoter controls expression of the araC gene, and is divergently transcribed relative to p_{BAD} from position −148.

which the protein binds to only one site.

The complex formed between AraC and negatively supercoiled minicircles containing the $araO_2$ and *araI* operators has the properties expected of a DNA loop (*12*). The migration of the complex is faster than that of free minicircles, and AraC dissociates from the DNA much more slowly than from similar DNA in which either the *araI* site or the $araO_2$ site has been changed from its normal sequence (Fig. 2). At 100 mM KCl and in the absence of arabinose, AraC dissociates from this DNA with a half-time of approximately 100 minutes, compared to a half-time for dissociation of less than 10 minutes from minicircles containing point mutations in $araO_2$ or *araI*. We conclude that a stable repression loop is maintained by cooperative interactions between the protein and both *araI* and $araO_2$.

The foregoing experiment measures the overall stability of the looped DNA. Dissociation cannot be distinguished from unlooping because bare minicircles and minicircles with bound AraC but which are unlooped have similar mobilities. We show below that the dissociation rate of AraC protein from *araI* is greatly reduced as a result of interactions with $araO_2$. This is an action at a distance, and the effect is best explained by DNA looping. After the formation of loops on minicircles, dissociation was allowed to occur. The amount of *araI* site still occupied by AraC protein was then monitored by linearizing the DNA with a restriction enzyme and performing a standard gel retardation assay in which free DNA migrates faster than DNA with bound protein (*13*). The dissociation rate of AraC from *araI* was reduced by at least a factor of 10 when an intact $araO_2$ site was present on the supercoiled minicircles (Fig. 3).

Loop breaking and reversibility. As described above, the addition of arabinose probably breaks the loop between $araO_2$ and *araI* in vivo. As we expected, arabinose breaks the loop in our in vitro system. When arabinose was added to a reaction containing looped complexes, no looped species was detected on electrophoresis (Fig. 4A). Alternatively, looped complexes were allowed to migrate into the gel, then electrophoresis was halted, and arabinose was added to the electrophoresis buffer. After a period of time during which arabinose could diffuse into the gel, electrophoretic migration was continued (Fig. 4B). This experiment shows that (i) arabinose opens the loops, even when the complex is within the gel, and (ii) free AraC protein, which does not migrate into the gel, is not required for loop opening.

The addition of arabinose opens loops formed on supercoiled minicircles. We now show that arabinose-induced loop breaking is reversible. AraC is first bound at *araI* in the presence of arabinose so that AraC binds as a dimer to *araI*, but does not bind to $araO_2$. Under these conditions a loop does not form. Loops subsequently form when the arabinose concentration is decreased by diluting the sample (Fig. 5). The loop was formed on $araO_2$-*araI* minicircles in the presence of excess competitor DNA, and then broken by the addition of arabinose. The reaction mixture was then diluted into buffer containing excess competitor DNA with or without arabinose. The data show that loops re-formed on the samples diluted into buffer lacking arabinose (*14*). The presence of the large excess of competitor DNA ensured that the loop re-formation process did not proceed by a pathway involving the binding of free AraC protein and that no AraC protein could remain at $araO_2$ after opening of the loop by the addition of arabinose (*15*). These results demonstrate that loop re-formation, in addition to loop breaking, can occur in the absence of free AraC.

Stoichiometry of the looped complex. AraC protein is predominantly a dimer in solution (*16*) and binds under most conditions as a dimer to *araI* on linear DNA (*17, 18*). Therefore, we originally

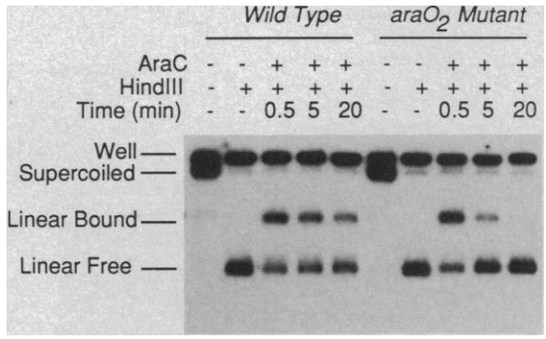
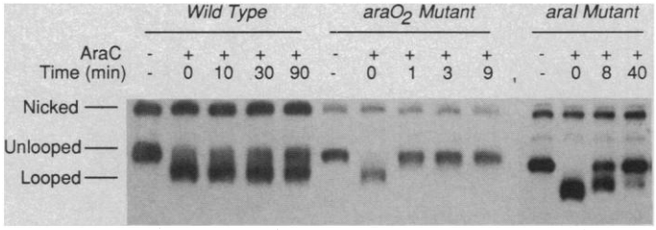


Fig. 3. Looping stabilizes AraC binding to *araI*. AraC was incubated with minicircles for 10 minutes in binding buffer plus 5 mM $MgCl_2$ and 50 mM KCl; excess unlabeled *araI* DNA was then added. Complexes were allowed to dissociate from minicircles for varying lengths of time, as indicated, and then arabinose was added to a concentration of 50 mM and incubated for 1 minute. Samples were then incubated with 40 units of Hind III for 4 minutes, and then with 750 ng of sonicated calf thymus DNA for 1 minute before electrophoresis. Calf thymus DNA was added to compete with the labeled DNA for restriction enzyme binding; thus, Hind III–DNA complexes are not observed. Protein was bound only to *araI* after cleavage of the minicircle because (i) in 50 mM KCl, 5 mM $MgCl_2$, and 50 mM arabinose, AraC completely dissociates from $araO_2$ in less than 1 minute on linear DNA; and (ii) the AraC monomer bound to $araO_2$ is transferred to $araI_2$ upon addition of arabinose. In this and other experiments, salt concentrations were chosen so that dissociation rates would be convenient.

Fig. 2. Dissociation of looped complexes formed on wild-type, $araO_2$ mutant, and *araI* mutant minicircles. A 404-bp Hind III fragment (*19*) containing the $araO_2$ and *araI* sites separated by 160 bp was ^{32}P end-labeled with T4 polynucleotide kinase, and ligated intramolecularly in the presence of 9 μM ethidium bromide to generate minicircles. The superhelical density of the minicircles was measured (*29*); the minicircles consisted of a mixture of supercoiled molecules, with an average of three negative superhelical turns, and nicked molecules. The $araO_2$ mutant contains a point mutation at position −271 (*3*). The *araI* mutant contains point mutations at positions −69 and −48 (*22*). Minicircles were incubated with purified AraC (*26*) at about 10^{-10} M at 30°C in binding buffer [10 mM tris-acetate, pH 7.4, 1 mM EDTA (potassium salt), 5 percent glycerol, 0.05 percent NP-40] plus 100 mM KCl. Similar AraC concentrations were used in other experiments. An excess of an unlabeled 40-bp DNA fragment containing *araI* was added and



incubated, for various lengths of time, before electrophoresis at 15 V/cm for 4 hours in a 5.5 percent acrylamide, bis-acrylamide (30:1) gel. The electrophoresis buffer (10 mM tris-acetate, pH 7.4, 1 mM EDTA) was maintained at 30°C.

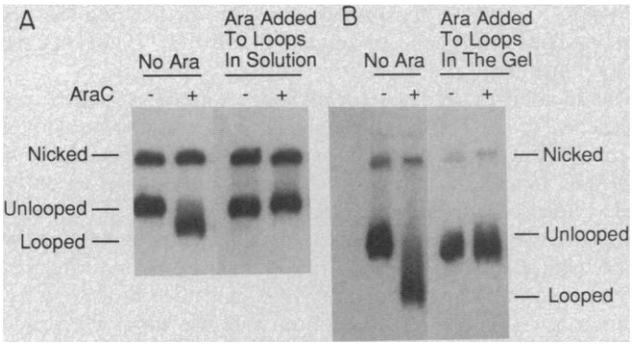


Fig. 4. Arabinose breaks the $araO_2$-*araI* loop. (**A**) Looped complexes were formed in binding buffer plus 100 mM KCl; excess unlabeled *araI* DNA was then added and the reaction was incubated for 10 minutes. The samples were either placed directly on a gel without arabinose, or arabinose was added to 50 mM just before the sample was added to a gel containing 50 mM arabinose in the electrophoresis buffer. (**B**) Looped complexes were formed as in (A) in the absence of arabinose and then subjected to electrophoresis for 1 hour at 15 V/cm in two different gels. The electrophoresis was stopped, and arabinose was added to 50 mM to one of the gels and allowed to equilibrate for 20 minutes; electrophoresis was then resumed for 4 hours.

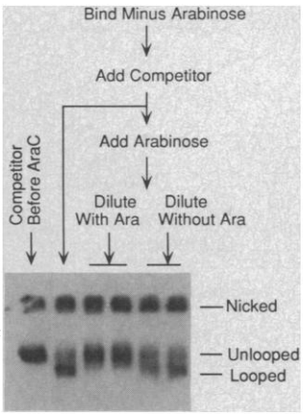


Fig. 5. Loop breaking and re-formation. AraC was incubated with minicircles in binding buffer plus 100 mM KCl, and then incubated for 8 minutes with a 500 molar excess of unlabeled *araI* DNA. Arabinose was added to 50 mM to part of the sample, incubated for 5 minutes, and then some of the sample was diluted (1:200) into either arabinose free buffer, or into buffer containing 50 mM arabinose. Dilution buffer also contained a 500 molar excess of the unlabeled *araI* DNA. Samples were incubated for an additional 15 minutes, and then subjected to electrophoresis. No binding of AraC to the minicircle was observed if the unlabeled *araI* competitor DNA was added before AraC (lane 1). A control experiment (not shown) showed that loop breaking by arabinose is complete within 1 minute.

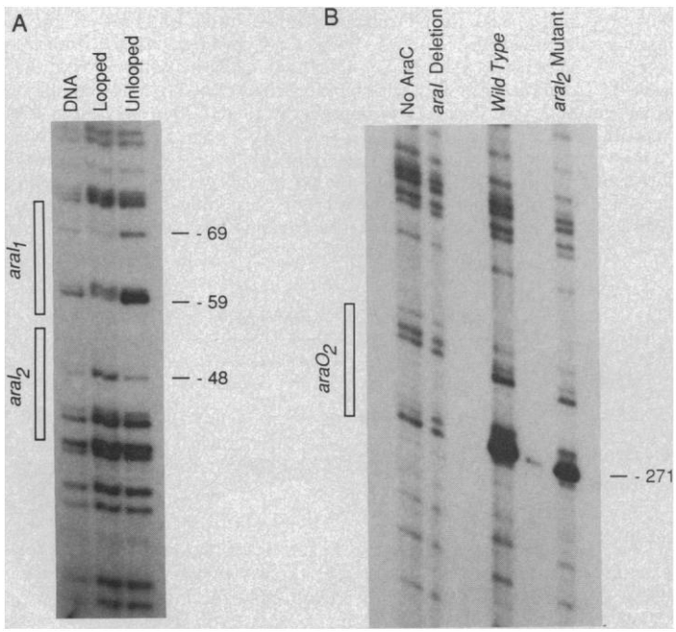


Fig. 6. Methylation interference and in vivo footprinting show that $araI_2$ is not contacted in the $araO_2$-*araI* loop. (**A**) The 404-bp Hind III fragment (*19*) was ^{32}P end-labeled, ligated into minicircles, and treated with dimethyl sulfate (DMS) (*17, 20*). Looped complexes were formed on methylated minicircles in binding buffer plus 50 mM KCl and allowed to dissociate for 20 minutes in the presence of excess unlabeled *araI* DNA. Reactions were subjected to electrophoresis; minicircle DNA was recovered from the looped and unlooped complexes and cleaved with Hind III restriction enzyme, regenerating linear ^{32}P end-labeled fragments. Bottom strand contacts at *araI* were visualized by cleaving off the top strand label as a small DNA fragment with PflM I (−37) restriction enzyme. The DNA was cleaved with piperdine at methylated bases, and subjected to electrophoresis in a 6 percent denaturing polyacrylamide gel. (**B**) In vivo DMS footprinting was performed (*3*), and DMS-modified plasmid was isolated from cells, end-labeled at the BstE II site (−203), cleaved at methylated base pairs, and subjected to electrophoresis in a 6 percent denaturing gel. Bottom strand guanine bases in and around $araO_2$ are shown. The $araI_2$ mutant contains four base pair changes, CG to AT, GC to AT, AT to TA, TA to GC, in positions −48, −49, −50, and −51, respectively. Binding of AraC to $araO_2$ is reflected by the intensity of the DMS enhancement at −271, and requires looping to AraC bound at *araI* (*3*). Lane 1 (no AraC) shows wild-type plasmid from a cell line containing a chromosomal deletion of the *araC* gene. No DMS enhancement at −271 was seen when the *araI* site was deleted (lane 2); in this plasmid, *araI* was mutated in the "upper case" positions: −74 TCGCA-TattttaGAACAGCTGCGTTA −47. As described (*19*), $araO_1$ was deleted in these constructs.

expected that the loop would contain an AraC dimer bound at *araI* and a dimer bound at $araO_2$. On the other hand, the fact that a complex containing only a dimer of AraC bound at *araI* can re-form a loop in the absence of free AraC (Fig. 5) suggests that the loop consists of a single dimer of AraC.

We then measured the protein stoichiometry in the loop by comparing the ratio of protein to DNA in looped minicircles to the ratio of protein to DNA in a complex formed on linear DNA, which we know contains one AraC dimer. A complex of purified ^{35}S-labeled AraC with linear ^{32}P-labeled DNA containing both $araO_2$ and *araI* was made at a concentration of protein where only *araI* was bound. The AraC-*araI* complex was isolated from a gel, and the ^{35}S and ^{32}P radioactivities were determined. The ratio of the two isotopes in this sample standardizes the assay for one dimer per DNA molecule. Part of the same DNA sample used to derive the AraC-*araI* standard was ligated to make minicircles, and the looped complex formed with the same ^{35}S-labeled AraC was isolated and quantitated. These complexes contained the same ratio of ^{35}S to ^{32}P (*19*), indicating that they too contained single dimers of AraC protein. Similar data were obtained with three different [^{35}S]AraC preparations.

In a control experiment showing that our assay had the requisite sensitivity, we measured complexes formed on linear DNA containing the *araI* and $araO_1$ sites, each of which binds a dimer of AraC (*17*) on linear templates. This DNA was incubated with ^{35}S-labeled AraC such that two different bound complexes were formed; one containing AraC bound at either *araI* or $araO_1$, the other containing AraC bound at both sites. As expected, the ratio of protein to DNA in the band with both sites occupied was twice that of the band containing one site occupied (*19*).

Sequence contacts made by AraC in the loop. In that the *araI*-$araO_2$ loop is maintained by a dimer of AraC protein, it seems likely that only part of *araI* is utilized for looping. We tested this by two methods and found that $araI_2$ is not contacted in the looped state. We also found that, after arabinose is added and the loop opens, $araI_2$ is contacted.

The methylation interference technique (*20*) can be used to identify those guanine bases that, when methylated, weaken the DNA loop. In this type of experiment the DNA is lightly methylated before the binding reactions are performed. After addition of AraC protein, free protein is eliminated, and loops are allowed to

dissociate before electrophoretic separation of looped and unlooped species. The two DNA populations are cleaved at the positions of methylated guanines and separated on a DNA sequencing gel. The guanine residues whose methylation does not interfere with loop formation are enriched in the DNA from the looped molecules, and conversely, the guanine residues whose methylation does interfere with loop formation are enriched in the unlooped population. Of the three guanines on the bottom strand of *araI* whose methylation affects binding of AraC protein to linear DNA (*17*), methylation at −69 and −59 within $araI_1$ both interfere with loop formation, whereas methylation at position −48 which is within $araI_2$ does not (Fig. 6A) (*21*). These data indicate that, in the looped state, AraC contacts $araI_1$ but does not contact $araI_2$.

As a second demonstration that $araI_2$ is not contacted by AraC in the looped state, we altered four highly conserved (*22*) nucleotides in $araI_2$ and then examined looping in vivo and in vitro. If the protein contacts $araI_2$ only when unlooped, then alterations in $araI_2$ should have no effect on binding in the looped state, but should have a large effect on binding in the unlooped state. This is precisely what we found, both in vivo and in vitro. The in vivo experiment was based on the fact that AraC occupancy of $araO_2$, and consequently dimethyl sulfate reactivity there, is dramatically increased by DNA looping. These mutations do not affect the occupancy of AraC at $araO_2$ in vivo, and thus, do not affect looping in vivo (Fig. 6B). In vitro, the looped complexes formed on the wild-type and $araI_2$ mutant templates dissociated at the same rate (Fig. 7A), also showing that looping is independent of the sequence of $araI_2$. These data directly demonstrate that $araI_2$ is not involved in looping, as was previously suggested on the basis of genetic evidence (*23*).

The $araI_2$ mutations have a large effect on binding to *araI* in the unlooped state, as is expected from previous studies of AraC binding to *araI* on linear DNA (*17*). When looped complexes are broken with arabinose, cleaved with restriction enzyme, and subjected to electrophoresis on a gel, AraC remains bound to DNA containing wild-type *araI* but not on the $araI_2$ mutant (Fig. 7B). This shows in a different way that $araI_2$ sequences are not contacted by AraC in the looped state, but are contacted in the unlooped state.

Sequence independence of arabinose-induced loop breaking. What directs AraC protein to bind to $araI_1$ and $araO_2$ and thus form a loop, in the absence of arabinose, and to bind to $araI_1$ and $araI_2$, thereby opening the loop, in the presence of arabinose? The binding sites themselves could specify this behavior as each is slightly different from the others (*10*). Alternatively, in the absence of arabinose the protein may preferentially bind to nonadjacent sites and, as a result, form a loop, and, in the presence of arabinose, bind to adjacent sites and therefore not form a loop. We now show that loop breaking is not determined by the sequences contacted in the looped and unlooped states, but rather, suggest that loop breaking is due to an effect of arabinose on the protein.

Three lines of evidence indicate that arabinose-induced loop breaking is not determined by the *araI* or $araO_2$ sequence. First, a stable, faster mobility complex, characteristic of a loop, is formed between two $araI_1$ sites in the absence of arabinose, and arabinose breaks this loop. Second, on minicircles with $araO_1$ replacing *araI*, $araO_2$ stabilizes binding of AraC to $araO_1$ in the absence but not the presence of arabinose (*24*). This suggests that arabinose breaks a loop between $araO_2$ and $araO_1$. Third, loop breaking by arabinose is not dependent on the sequence of $araI_2$, since arabinose breaks the loop formed on minicircles containing the $araI_2$ mutations (*25*). Since arabinose breaks loops between $araI_1$ and $araI_1$, $araO_2$ and $araO_1$, and $araO_2$ and $araI_1$, we conclude that arabinose-induced loop breaking is not determined by a particular DNA sequence.

Model for AraC-mediated repression and induction. Our results show that the DNA loop between $araO_2$ and *araI*, which is required for repression of *araBAD*, consists of a dimer of AraC protein making contacts with half of *araI*, $araI_1$, and with $araO_2$. Arabinose breaks this loop, with the protein retaining its contacts to $araI_1$ while its contacts to $araO_2$ are broken and new contacts are formed to $araI_2$. This process is reversible; if arabinose is removed, the contacts at $araI_2$ are broken and new contacts are formed to $araO_2$. Arabinose-induced loop breaking is not determined by the DNA sequence to which AraC binds, and does not require contacts with $araI_2$.

One simple mechanism that explains the data is an arabinose-induced subunit reorientation. One subunit of AraC could contact $araI_1$, and the second subunit could contact either $araI_2$ or $araO_2$. In the absence of arabinose, the subunits of AraC protein would tend to be in an orientation requiring interaction with two nonadjacent DNA sites, and as a result, would favor looping. In the presence of arabinose, the monomers could reorient so that interactions with adjacent binding sites are more favorable although, we suspect, considerably less than shown in Fig. 8. Thus, looping could occur between any pair of nonadjacent and suitably oriented DNA binding sites. The addition of arabinose to such a complex would tend to orient the subunits for binding to adjacent sites. This would weaken looped complexes and strengthen nonlooped complexes, regardless of the sequences of the DNA binding sites, just as is seen experimentally (Figs. 4 and 7) (*26*).

The transition from the looped, repressed state to the unlooped, induced state of the *araCBAD* operon is regulated by an arabinose-induced increase in the occupancy of the $araI_2$ site. This places a subunit of AraC near the RNA polymerase binding site and could be a major part of the induction process. Breaking of the repression

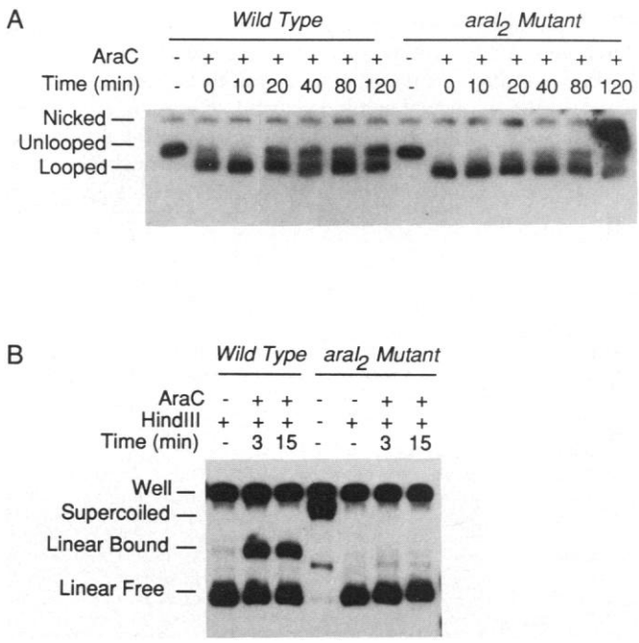


Fig. 7. $araI_2$ is contacted in the unlooped but not in the looped state. (**A**) Comparison of the dissociation rate of complexes formed on wild-type or $araI_2$ mutant templates. Complexes were formed in binding buffer plus 100 mM KCl, excess unlabeled *araI* DNA was added for varying lengths of time, and then subjected to electrophoresis. The $araI_2$ mutations are described in Fig. 6. (**B**) $araI_2$ was contacted when the loop was broken by arabinose. Looped complexes were formed in binding buffer plus 50 mM KCl, 5 mM $MgCl_2$, and then incubated for 5 minutes with excess unlabeled *araI* DNA. Arabinose was added to 50 mM and incubated for 3 or 15 minutes, and then Hind III (40 units) was added and the incubation continued for 4 minutes. Samples were incubated with 750 ng of calf thymus DNA for 1 minute and then subjected to electrophoresis.

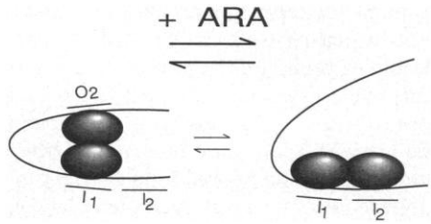


Fig. 8. In the absence of arabinose, the subunits of AraC protein could be in an orientation disfavoring binding to two adjacent sites, and therefore the protein would preferentially participate in looping. An arabinose-induced conformational shift could reorient the subunits so that contacting two adjacent DNA sites would be favored, and would therefore drive the transition from the looped to the unlooped state.

loop and the presumed resultant occupancy of $araI_2$, however, is insufficient to generate full induction because elimination of the repression loop by deletion of $araO_2$ results only in a small increase in promoter activity in the absence of arabinose (*2*). Arabinose must also be added to achieve full induction.

Although the structure of AraC is unknown, existing data are consistent with the idea that arabinose induces a conformational change in the protein. Fucose, an analog of arabinose that does not induce *araBAD* (*27*), binds to AraC but does not alter its intrinsic fluorescence; whereas binding of arabinose does change its intrinsic fluorescence (*28*). As expected, fucose does not break DNA loops in our in vitro system (*24*), which implies that fucose stabilizes the repressing conformation of AraC and thereby fails to induce *araBAD*.

In summary, we have demonstrated DNA loop formation and loop breaking in an in vitro system. A dimer of the protein generates loops in the absence of arabinose by contacting two well-separated AraC protein binding sites. On the addition of arabinose, which breaks the loops, the protein contacts two adjacent AraC protein binding sites. An efficient mechanism for regulation of such looping behavior is for the protein's subunits to shift their orientation, being oriented in the absence of arabinose such that looping interactions with nonadjacent sites are most favorable, and in the presence of arabinose such that binding interactions with adjacent sites are most favorable. Such a mechanism could regulate looping in a wide variety of systems.

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12. In the absence of arabinose, two complexes are formed on minicircles containing $araO_2$ and *araI*. A stable complex, which dissociates with a half-time of 100 minutes at 100 mM KCl (Fig. 2, wild type), is formed at low AraC concentrations in the absence of competitor DNA. The second complex is unstable, migrates slightly faster than the stable complex (Fig. 2, *araI* mutant, at 0 and 8 minutes) and is formed only at high AraC concentrations. Weakly associated AraC dissociates from the unstable complex with a half-time of less than 1 minute at 100 mM KCl, and in the process, the stable complex is formed. In our other experiments binding reactions are incubated with an excess of unlabeled competitor DNA containing AraC binding sites so that only the stable complex is present.
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14. The sample diluted in the presence of arabinose does not re-form the loop during the electrophoresis. This could be due to the low ionic strength of the electrophoresis buffer, 10 mM tris-acetate, pH 7.4, 1 mM EDTA, which may prevent AraC from dissociating from $araI_2$ and establishing contacts with $araO_2$.
15. Under the conditions of this experiment, $araO_2$ should have been completely unoccupied before the sample was diluted. AraC dissociates from $araO_2$ with a half-time of less than 1 minute at 50 mM KCl on linear DNA. Because the arabinose dilution experiment was performed at 100 mM KCl and the samples were incubated for 5 minutes before dilution, there could not be any AraC remaining at $araO_2$.
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18. On the basis of their DNase I protection experiments, Lee *et al.* (*10*) suggested that twice as much AraC might bind to *araI* in the presence of arabinose than in its absence. Three lines of evidence argue against this idea. (i) The retardation on electrophoresis of an *araI*-containing DNA fragment on the binding of AraC protein in the presence or absence of arabinose is the same, suggesting that the same amount of AraC binds in either case. In view of previous results (*17*), this is likely a dimer. (ii) Using the methods described above, we find that the same amount of AraC binds to *araI* on linear DNA in the presence and absence of arabinose (*24*). (iii) When linear DNA containing an AraC-arabinose-*araI* complex is incubated with less than stoichiometric amounts of an AraC monoclonal antibody, a single antibody-AraC-*araI* complex is observed on electrophoresis (*24*). If a dimer of AraC were to bind to each half of *araI*, two different antibody-AraC-*araI* complexes should have been observed; one complex with one antibody molecule, and one with two.
19. The ratio of ^{35}S to ^{32}P in the *araI*-$araO_2$ minicircle was 0.9 ± 0.1 ($n = 10$) times the value observed for binding of dimeric AraC to *araI* on linear DNA. The ratio in complexes on linear DNA with AraC binding at both $araO_1$ and *araI* was 2.1 ± 0.15 ($n = 4$) times the value observed for binding to *araI* alone. AraC was ^{35}S-labeled with a T7 RNA polymerase expression system [S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1074 (1985)]. The araC gene was fused to the T7 promoter by cloning a Bam HI–Hind III fragment containing AraC from pAB1003 (*22*) into the polylinker of pT713 (BRL). Cells were cultured and heat-shocked to induce T7 RNA polymerase and AraC and then labeled for 1.5 hours with 1 mCi of [^{35}S]methionine (700 Ci/mmol) per milliliter of cells. [^{35}S]AraC was purified as described (*17*). The DNA fragment containing $araO_2$ and *araI*, a 404-bp Hind III, was purified from pRL515, a derivative of pTD3 containing restriction sites created by the following mutations in positions outside of regulatory sites: G to C at −76, T to G at −77, T to C at −107, C to G at −162, A to C at −166. $araO_1$ was deleted by filling in the ends with the Klenow fragment of DNA polymerase at restriction sites Sty I (−166) and Cla I (−110) and ligation. The plasmid pRL515 contains Hind III sites at +50 and −409, introduced by filling in the ends of a Klenow fragment at the Eco RI site (−399) and ligation of the sequence: −410 -AAGCTTGAGTC- −400. This DNA was labeled and a portion was ligated to make minicircles (Fig. 2). The linear AraC-*araI* complex, which has a 100 times higher affinity than the linear AraC-$araO_2$ complex, was formed by the addition of a limiting amount of AraC. To ensure specific binding of AraC, excess unlabeled pRL515 was added to reactions containing linear DNA complexes, and an excess of unlabeled 40-bp *araI* DNA fragment was added to minicircle complexes before electrophoresis; the migration of the unlabeled DNA-AraC complexes in the gel was much different from that of the labeled linear *araI* complex or looped minicircles. The [^{35}S]protein-[^{32}P]DNA complexes were excised from the gel and counted (*17*). To ensure that the specific activity of the minicircle DNA and the linear DNA fragment were the same, supercoiled minicircles were isolated and cleaved with BstE II (−203) restriction enzyme, generating linear DNA with the ^{32}P label "internal" to the ends of the fragment. The ratios of ^{35}S to ^{32}P of the looped complex formed on the purified minicircle and the *araI* complex formed on the "internal" ^{32}P-labeled linear DNA were the same in three independent measurements.
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