J. Mol. Biol. (1991) **218**, 45–54

AraC–DNA Looping: Orientation and Distance-dependent Loop Breaking by the Cyclic AMP Receptor Protein

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(Received 29 May 1990; accepted 22 October 1990)

The arabinose operon promoter, p_{BAD}, is negatively regulated in the absence of arabinose by AraC protein, which forms a DNA loop by binding to two sites separated by 210 base-pairs, $araO_2$ and $araI_1$. p_{BAD} is also positively regulated by AraC–arabinose and the cyclic AMP receptor protein, CRP. We provide evidence that CRP breaks the $araO_2$–$araI_1$ repression loop *in vitro*. The ability of CRP to break the loop *in vitro* and to activate p_{BAD} *in vivo* is dependent upon the orientation and distance of the CRP binding site relative to $araI_1$. An insertion of one DNA helical turn, 11 base-pairs, between CRP and *araI* only partially inhibits CRP loop breaking and activation of p_{BAD}, while an insertion of less than one DNA helical turn, 4 base-pairs, not only abolishes CRP activation and loop breaking, but actually causes CRP to stabilize the loop and increases the $araO_2$-mediated repression of p_{BAD}. Both integral and non-integral insertions of greater than one helical turn completely abolish CRP activation and loop breaking *in vitro*.

1. Introduction

Many transcription, recombination and replication systems are regulated by the interaction between proteins bound at distantly spaced sites along the DNA, a process known as DNA looping (for an example of each, see Dunn *et al.*, 1984; Moitoso De Vargas *et al.*, 1989; Mukherjee *et al.*, 1988). The formation of a DNA loop requires the two sites to be brought into close proximity by both bending and twisting the DNA, processes that can cost considerable energy. Factors that affect DNA topology, such as supercoiling and bending, should therefore affect DNA looping; and, indeed, this has been found to be so. Looping in the *ara* and *lac* operons is affected by supercoiling (Hahn *et al.*, 1986; Whitson *et al.*, 1987; Krämer *et al.*, 1987). Also, the integration of phage lambda into the *Escherichia coli* chromosome involves DNA loop formation by Int protein. IHF protein, a protein that bends DNA, facilitates this looping interaction (Moitoso De Vargas *et al.*, 1989), and can be replaced by either a naturally bent DNA sequence or another protein that bends DNA, the cyclic AMP receptor protein, CRP‡ (Goodman & Nash, 1989).

In this paper, we demonstrate that DNA looping can also be negatively affected by a protein that bends DNA. CRP prevents formation of the repression DNA loop in the *araBAD* operon, a phenomenon that could contribute to the transcriptional activation of the operon by CRP.

A complex regulatory system controls the expression of the *araBAD* operon promoter, p_{BAD}. The promoter is regulated both positively and negatively by AraC (Sheppard & Englesberg, 1967; Greenblatt & Schleif, 1971; Schleif & Lis, 1975; Dunn *et al.*, 1984), a protein that binds to three operator sites upstream from p_{BAD} (see Fig. 1) (Ogden *et al.*, 1980; Lee *et al.*, 1981; Dunn *et al.*, 1984). Positive regulation requires the interaction of AraC–arabinose at *araI* (Ogden *et al.*, 1980), a site immediately adjacent to the RNA polymerase site

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‡ Abbreviations used: CRP, cyclic AMP receptor protein; bp, base-pairs.

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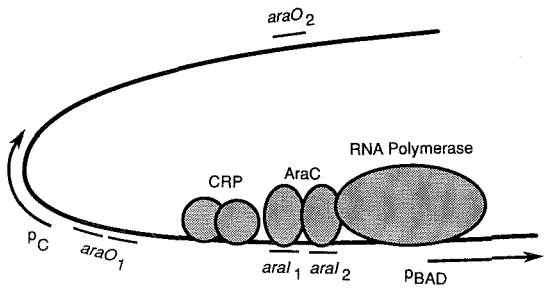


Figure 1. Binding sites and regulatory proteins in the *araCBAD* operon. The positions of the 3 AraC binding sites, as determined by DNase I footprinting, relative to the start of p_{BAD} transcription at +1 are $araO_2$, −252 to −288; $araO_1$, −110 to −148; *araI* is subdivided into 2 regions, $araI_2$, −35 to −54; and $araI_1$ −55 to −75. The start site of P_C is at position −148. The CRP binding site is from position −80 to −110.

of p_{BAD}. Negative regulation requires a DNA loop between AraC bound at $araO_2$ and *araI* (Dunn *et al.*, 1984; Martin *et al.*, 1986). The DNA loop also represses the promoter of the AraC-encoding gene, P_C (Huo *et al.*, 1988; Hamilton & Lee, 1988), which is transcribed divergently in relation to p_{BAD}.

The *araBAD* genes are also regulated by the cyclic AMP receptor protein, CRP, which mediates catabolite repression in operons that control the metabolism of a variety of sugars. CRP activates both the p_{BAD} and P_C promoters from a single site located between $araO_1/P_C$ and *araI* (Lee *et al.*, 1981; Dunn & Schleif, 1984; Stoltzfus & Wilcox, 1989). The mechanism by which CRP activates p_{BAD} is of interest for two reasons. First, the mechanism of the protein's action is not known for any system, and second in the *ara* system AraC protein lies between CRP and RNA polymerase on the DNA, whereas in most operons CRP binds at a site adjacent to RNA polymerase. The mechanism of activation of p_{BAD} by CRP apparently does not involve stimulation or stabilization of AraC binding to *araI*, since no cooperativity is observed between the binding of these proteins to their sites *in vitro* (Hendrickson & Schleif, 1984).

Two findings suggest that part of the CRP activation of p_{BAD} involves opening the $araO_2$-mediated repression loop. Hahn *et al.* (1984) found that p_{BAD} is uninducible in cells that are deficient in CRP binding due to a lack of cyclic AMP synthesis, but becomes inducible when $araO_2$ is deleted, and almost full induction of p_{BAD} is achieved when AraC is overexpressed. The idea that CRP activates p_{BAD} by opening the repression loop was further supported by an *in vitro* study that showed that the requirement for CRP in the transcription of p_{BAD} from supercoiled templates is greatly reduced by either deleting $araO_2$ or by misorienting the $araO_2$ and *araI* sites (Hahn *et al.*, 1986).

CRP can also activate p_{BAD} by an $araO_2$-independent mechanism. Both *in vivo* and *in vitro*, CRP can stimulate transcription when looping is impossible due to the absence of the $araO_2$ site (Hahn *et al.*, 1986; Lichenstein *et al.*, 1987; Stolzfus & Wilcox, 1989). As is the case in the $araO_2$-dependent activation mechanism, the amount of the CRP stimulation is affected by the concentration of AraC.

DNA looping by AraC *in vitro* requires supercoiled DNA templates (Hahn *et al.*, 1986). Recently, we utilized an electrophoretic technique to study AraC DNA looping on small supercoiled DNA molecules, or minicircles, and showed that the DNA repression loop in the *araCBAD* operon is maintained by a single AraC dimer bound between $araO_2$ and half of the *araI* site, $araI_1$ (Lobell & Schleif, 1990). Additionally, we showed that when arabinose is added to the looped complex, the loop breaks, and the AraC dimer remains bound to $araI_1$ while shifting its contacts from the distal $araO_2$ site to the previously unoccupied half of *araI*, $araI_2$. In this paper, we extend our study of the regulation of $araO_2$–$araI_1$ looping on minicircles, and explore the effect of CRP on this process.

We find that CRP increases the dissociation rate of AraC from $araI_1$ on minicircles from a slow rate characteristic of the looped state to a much faster rate characteristic of the unlooped state. This result shows that CRP breaks the $araO_2$–$araI_1$ repression loop *in vitro*. Insertions of DNA between the CRP binding site and *araI* have similar effects on the ability of CRP to break the loop *in vitro* and to activate p_{BAD} *in vivo*. CRP can activate p_{BAD} and break the loop only when its binding site is positioned on the same face of the DNA as in the wild-type, and when the distance between the CRP and *araI* sites is not increased by more than one DNA helical turn. When the CRP binding site is misoriented on the opposite face of the DNA helix relative to its wild-type position, CRP actually stabilizes the loop *in vitro* and enhances $araO_2$-mediated repression of p_{BAD} *in vivo*. These results support the hypothesis that part of the activation of p_{BAD} by CRP occurs through CRP-mediated loop breaking.

2. Materials and Methods

(a) *General methods and materials*

DNA manipulations, growth of cells and other general methods were performed as described by Schleif & Wensink (1981) and Maniatis *et al.* (1982). Sequencing was performed as described by U.S. Biochemical Co. for use with their modified phage T7 DNA polymerase, Sequenase. Restriction endonucleases were obtained from New England Biolabs. Phage T4 DNA ligase and T4 DNA kinase were obtained from U.S. Biochemical Co. [gamma-^{32}P]ATP used for DNA end-labeling was obtained from New England Nuclear. All other reagents were obtained from Sigma Chemical Co., Fisher Scientific or Biorad Laboratories. β-Galactosidase assays were performed as described (Miller, 1972).

(b) *Plasmid constructs of minicircles*

A 404 bp *Hin*dIII DNA fragment containing $araO_2$ and *araI* separated by 160 bp was isolated from pRL515 and

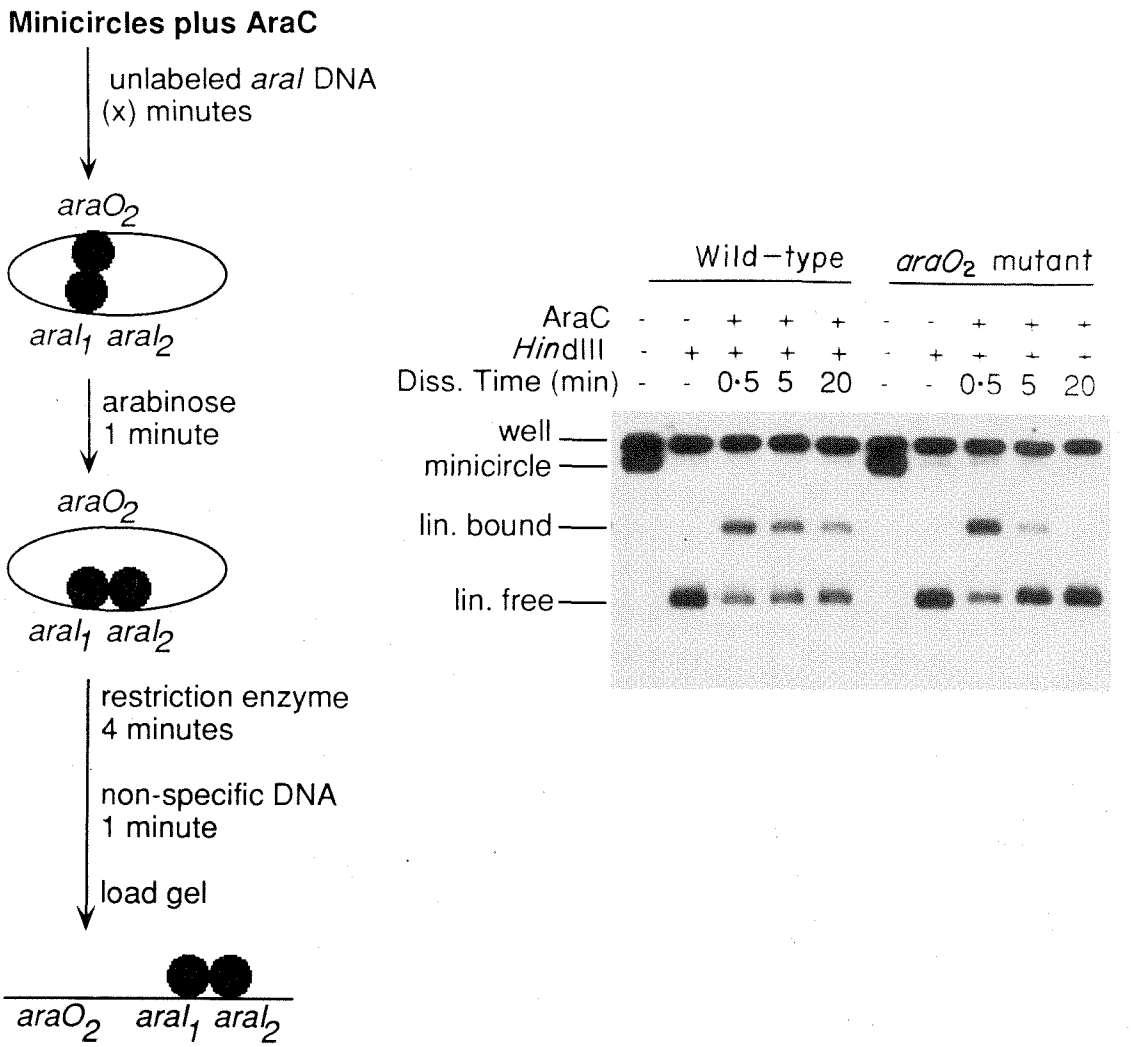


Figure 2. The looping–restriction cutting assay shows that $araO_2$ stabilizes binding of AraC to $araI_1$. A flow chart of the looping–restriction cutting assay is shown on the left. Three relevant states of AraC binding are depicted, a looped state with AraC binding to $araO_2$ and $araI_1$, an unlooped state with AraC–arabinose binding to $araI_1$ and $araI_2$, and AraC–arabinose binding to *araI* on the linearized DNA. The dissociation rate of AraC from $araI_1$ on minicircles containing either the wild-type $araO_2$ site, or a point mutation at position −271 within $araO_2$, was measured by the looping–restriction cutting assay; the data are shown in the gel on the right side of the Figure.

used to generate "wild-type" minicircles. pRL515 is a derivative of pES27, which is similar to pTD3 (Dunn & Schleif, 1984), except that it contains unique restriction sites created by the following mutations in positions outside 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. pRL515 was derived from ES27 by making the following modifications. A fragment containing $araO_1$ was deleted by filling in the ends with the Klenow fragment of DNA polymerase at *Sty*I(−166) and *Cla*I(−110) followed by ligation. pRL515 contains a *Hin*dIII site at position +50, and a second *Hin*dIII site at position −409, introduced by filling in the ends with Klenow at the *Eco*RI site (−399) and ligation of the sequence: −410·AAGCTTGAG-TC·−400.

Minicircles containing an $araO_2$ mutant site were generated from a 404 bp *Hin*dIII fragment, which was isolated from pRL526, a derivative of pRL515 containing a C to T change at position −271 (Martin *et al.*, 1986).

A 408 bp *Hin*dIII fragment containing *araI*, with both $araO_2$ and $araO_1$ deleted, was isolated from pRL518 and used in the experiment shown in Fig. 2(b). pRL518 was derived from pES27 as follows. The *Ssp*I site (−606) within the pBR322 sequences of pES27 was filled in with the Klenow fragment of DNA polymerase, and a *Hin*dIII linker (5′ AAGCTTGAGTC 3′) was ligated in. The region containing the $araO_2$ site was deleted by filling in with Klenow at *Eco*RI (−400) and *Bst*EII (−203) followed by ligation. The region containing $araO_1$ was deleted by filling in with Klenow at *Sty*I(−166) and *Cla*I(−110) followed by ligation.

(c) *Plasmid constructs for CRP–*araI *spacing mutations*

pRL41, a derivative of pDL3, which contains 440 bp of the p_{BAD} upstream region fused to the *galK* leader region

and the *lacZ* structural gene (Lee & Schleif, 1989), was the starting plasmid used in the creation of plasmids containing different spacings between the CRP site and *araI*. pRL41 contains the following point mutations outside regulatory sites, which create unique restriction sites: G to C at −76, T to G at −77, T to C at −107, C to G at −162 and A to C at −166. The spacing between the CRP site and *araI* was varied by inserting the following DNA sequences between positions −80 and −81 of *aracBAD*:

+44 bp CTATCTAGAGGCCTACTAGTCCCTAGGCGCTTGCTAGCTGCACG
+40 bp CTAGAGGCCTACTAGTCCCTAGGCGCTTGCTAGCTGCACG
+33 bp CTATCTAGAGGCCTACTAGTCCCTAGGCGCTTG
+26 bp CTATCTAGAGGCCTACTAGTCCCTAG
+22 bp CTATCTAGAGGCCTACTAGTCC
+15 bp CTATCTAGAGGCCTA
+11 bp CTATCTAGAGG
+4 bp CTAT

The constructs containing insertions of 4, 15 and 26 bp between CRP and *araI* also contain an insertion of 7 bp (5′ CGATCAT 3′) at the *Cla*I site (−110), such that a total of an integral number of DNA helical turns is inserted between $araO_2$ and *araI*. The CRP mutant binding site constructions contain a C to T mutation at −89 and a G to A mutation at −98.

Minicircles containing insertions of 4, 11 and 40 bp between CRP and *araI* were generated from *Hin*dIII fragments derived from pRL630, pRL620 and pRL600, respectively; these plasmids are derived from pRL515, and contain insertions of 4, 11 and 40 bp (the DNA sequence of the insertions is listed above) between positions −80 and −81.

(d) *Looping–restriction cutting assay*

Minicircles were generated by intramolecular ligation of ^{32}P end-labeled, *Hin*dIII DNA fragments in the presence of 9 μM-ethidium bromide. After ligation, the DNA was extracted with 1% SDS, 1 M-sodium acetate, and an equal volume of chloroform, and then precipitated with ethanol. The superhelical density of the minicircles in the ligation mixture was determined as described by Nordheim & Meese (1988). The ligations contain a mixture of nicked and supercoiled molecules; the distribution of topoisomers in a typical ligation mixture is 20% nicked molecules, 20% (−2) topoisomer, 40% (−3) topoisomer and 20% (−4) topoisomer.

Minicircles were incubated with purified AraC for 10 min (as described by Hendrickson & Schleif, 1984) at 30°C in binding buffer (10 mM-Tris–acetate (pH 7·4), 1 mM-KEDTA, 5% (w/v) glycerol, 0·05% (w/v) Nonidet P-40 (Shell Oil) plus 50 mM-KCl, 5 mM-$MgCl_2$ and, where indicated, 1 mM-cyclic AMP. Where indicated, purified CRP was then added and incubated 10 min. An excess of unlabeled DNA fragment containing *araI* was then added as a competitor for AraC, and complexes were allowed to dissociate for various lengths of time. The following were then added in order, and incubated for the indicated lengths of time, prior to loading the samples onto a gel: (1) arabinose to 50 mM, 1 min; (2) 40 units of *Hin*dIII, 4 min; (3) 750 ng sonicated calf thymus DNA, 1 min. Electrophoresis was for 3 to 5 h at 15 V/cm on 5·5% (30 : 1 (w/v), acrylamide : bisacrylamide) gels in 10 mM-Tris–acetate (pH 7·4), 1 mM-EDTA buffer. The calf thymus DNA acts as a competitor for the restriction enzyme, and therefore labeled DNA–*Hin*dIII complexes are not observed.

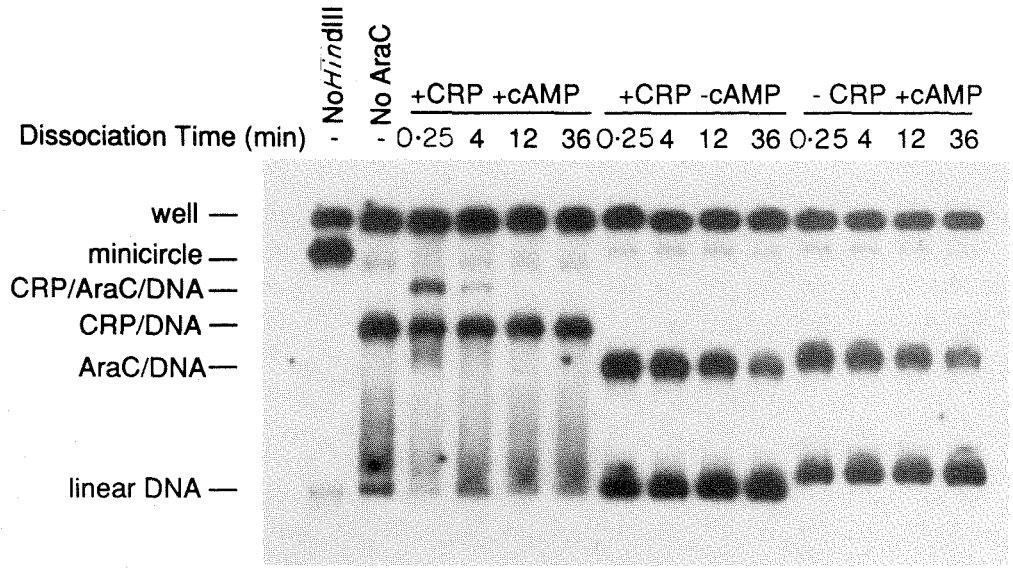
3. Results

(a) *CRP-induced loop breaking* in vitro

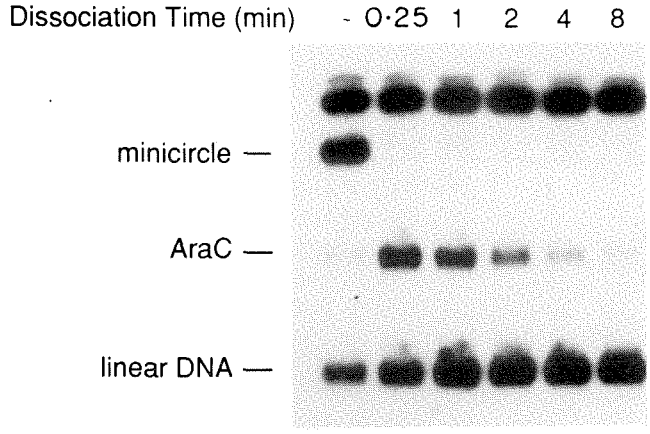
We studied the effect of CRP on the $araO_2$–$araI_1$ loop formed on supercoiled minicircles by examining the effect of CRP on the occupancy of $araI_1$ in the loop. The occupancy of $araI_1$ is a measure of the looping interaction, since the dissociation rate of AraC from this site is greatly reduced by looping to the $araO_2$ site (Lobell & Schleif, 1990). The dissociation rate of AraC from $araI_1$ on minicircles can be determined by an assay that is technically much less time-consuming than alternative methods, such as DNase I footprinting. This assay, referred to as the looping–restriction cutting assay, is shown in Figure 2 and described below. AraC–DNA loops are formed on supercoiled minicircles, an excess of unlabeled *araI* DNA fragment is added so that no additional binding of AraC to labeled DNA can occur. The complexes are allowed to dissociate for various lengths of time. Arabinose is then added, which breaks the loop and shifts the contacts of whatever AraC is still bound to the labeled minicircles from $araO_2$ to the previously unoccupied half of *araI*, $araO_2$. The minicircles, now containing AraC–arabinose bound only at *araI*, are briefly incubated with *Hin*dIII restriction enzyme, which linearizes the mini-circle. The reaction is then incubated with excess non-specific competitor DNA; this binds excess restriction enzyme and essentially removes the enzyme from the labeled DNA. The DNA is then subjected to electrophoresis to resolve free DNA from DNA containing AraC bound to *araI*. Since all samples are incubated for a fixed length of time after arabinose addition, the amount of linear DNA–AraC complex at each time point reflects the occupancy of *araI* in the loop.

We compared the dissociation rate from $araI_1$ in minicircles containing either wild-type $araO_2$ or a point mutation in this site by the looping–restriction cutting assay (Fig. 2). The data show that the dissociation rate from $araI_1$ is eight-fold slower on minicircles containing wild-type $araO_2$ compared to that from the mutant $araO_2$ site, demonstrating that $araO_2$ stabilizes AraC binding to $araI_1$.

The following demonstrates that cyclic AMP-CRP eliminates the $araO_2$-mediated stabilization of AraC binding to $araI_1$. Cyclic AMP-CRP was added to a reaction containing looped AraC–minicircle complexes, and then the dissociation of AraC was examined by the looping–restriction cutting assay. The data (Fig. 3(a)) show that AraC dissociates 16-fold faster in the presence of cyclic AMP-CRP. In the absence of CRP, AraC dissociates from the minicircles with a half-time of 40 minutes. In the presence of cyclic AMP-CRP, a complex is



(a)



(b)

Figure 3. CRP breaks the $araO_2$–$araI_1$ loop. (a) CRP increases the dissociation rate of AraC from $araI_1$ on minicircles in a cyclic AMP-dependent manner. Looped complexes were formed on $araO_2$–*araI* minicircles in the presence or absence of cyclic AMP, and then CRP was added. $araI_1$ occupancy was then probed as a function of dissociation time by the looping–restriction cutting assay. (b) Dissociation rate of AraC from *araI* in the unlooped state in the absence of arabinose. The dissociation rate in the absence of arabinose of AraC from *araI* on supercoiled minicircles deleted of both $araO_2$ and $araO_1$ was measured by the looping–restriction cutting assay.

formed that contains CRP and AraC bound to the DNA; AraC dissociates from this complex with a half-time of 2·5 minutes, resulting in a complex that contains only CRP bound to the DNA. In the absence of arabinose and CRP, AraC binds to *araI* in an unlooped state on minicircles containing a deletion of $araO_2$, and dissociates from the DNA with a half-time of two minutes (Fig. 3(b)). Thus, the presence of CRP shifts the dissociation rate of AraC from *araI* on minicircles to a value characteristic of unlooped DNA. It is most likely, therefore, that CRP breaks the loop, and forces AraC to bind to *araI* in the unlooped state.

When CRP is added to looped complexes in the absence of cyclic AMP, only AraC–DNA complexes are observed, and these dissociate with a half-time, 40 minutes, characteristic of the looped state (Fig. 3). This shows that the loop-breaking effect of CRP requires binding to the DNA, since CRP binding requires cyclic AMP. Additionally, we found that CRP has only a small, about a twofold, negative effect on the binding of AraC–arabinose to *araI* in the unlooped state (Fig. 4). This suggests that the mechanism of loop breaking is only partially due to a direct, negative interaction of CRP with AraC bound at *araI*; the majority of the

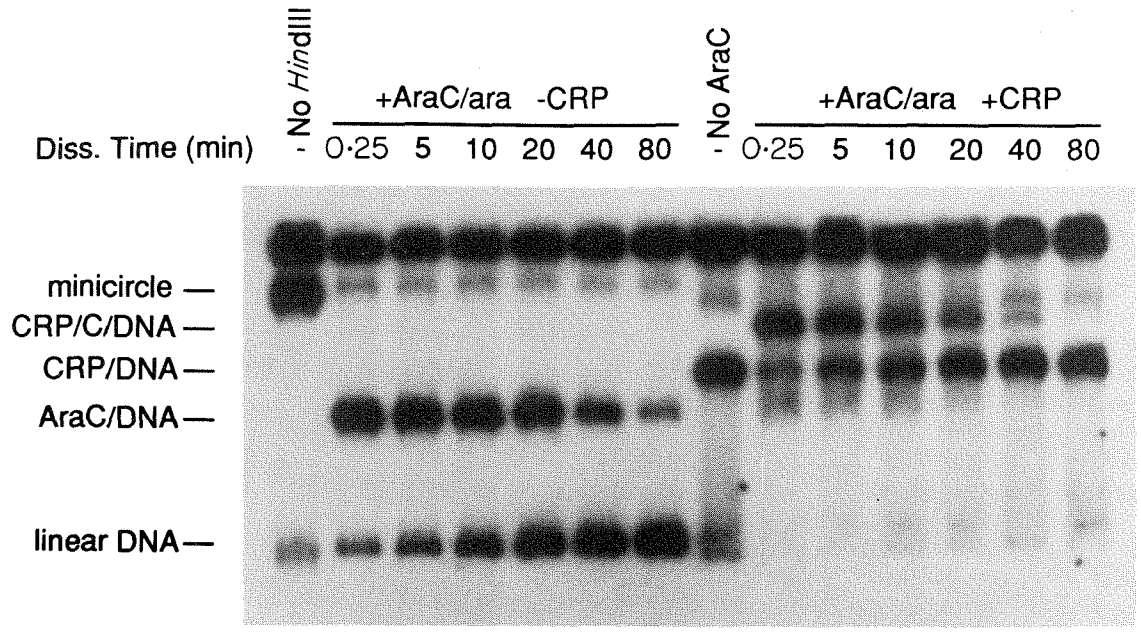


Figure 4. CRP has only a small effect on AraC binding to *araI* in the unlooped state. A limiting concentration of AraC was incubated with $araO_2$–*araI* minicircles in the presence of arabinose and cyclic AMP such that $araO_2$ is not occupied. CRP was added where indicated, and the occupancy of AraC at *araI* was examined as a function of the dissociation time by the restriction–cutting assay.

loop-breaking effect is directed at the looping interaction between AraC bound to $araO_2$ and $araI_1$.

(b) *CRP-induced loop breaking* in vitro *is dependent upon the orientation and distances of the CRP binding site relative to* araI

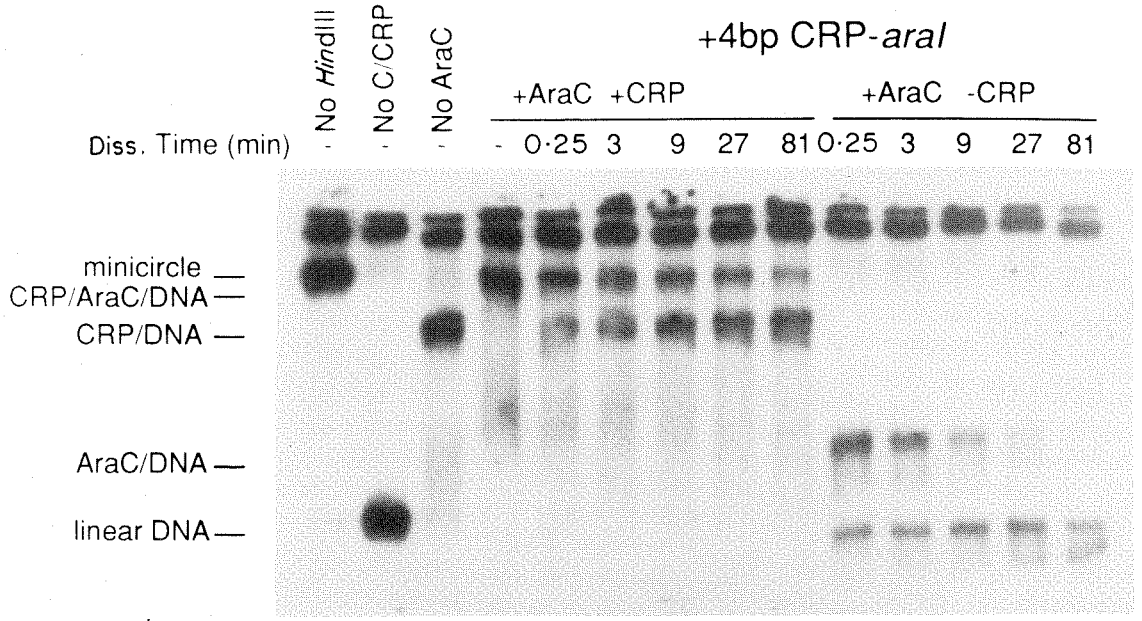
CRP bends the DNA when it binds to *lac* operon DNA (Wu & Crothers, 1984), and to other operons that it regulates, including *araCBAD* (Lichenstein *et al.*, 1987). $araO_2$–$araI_1$ loop breaking by CRP could be due to bending of the DNA by CRP in a manner that inhibits the formation of the repression loop. We reasoned that if a CRP-induced bend is responsible for loop breaking, then CRP might break the loop from other positions between $araO_2$ and *araI*. The following data, however, show that CRP can break the loop only when its binding site is located close to and oriented properly to *araI*.

We varied the location of the CRP binding site by inserting either 4, 11 or 40 base-pairs between the CRP and *araI* sites. The dissociation rate of AraC from minicircles containing these different spacings was measured in the presence or absence of CRP by the looping–restriction cutting assay. The data from these spacing mutations are shown in Figures 5 and 6, and summarized in Table 1, along with *in vivo* p_{BAD}–*lacZ* expression measurements (see section (c), below). CRP-induced loop breaking is dependent upon the orientation between the CRP and *araI* sites. When four base-pairs, nearly half a DNA helical turn, are inserted between CRP and *araI*, CRP not only fails to break the loop but appears to stabilize it; AraC dissociates three times more slowly in the presence of CRP in this spacing variant (Fig. 5(a) and Table 1). However, when the distance between CRP and *araI* is increased by 11 base-pairs, i.e. one complete DNA helical turn, CRP breaks the loop, although less effectively than when it is at the wild-type spacing. With the 11 base-pair insert, the half-time for dissociation of AraC from $araI_1$ decreases from 40 minutes to 4·5 minutes in the presence of CRP (Fig. 5(b)). The CRP-induced loop-breaking effect, that is, the amount of the increase in the AraC dissociation rate, is reduced from a 16-fold to a ninefold effect by inserting one DNA helical turn between the CRP and *araI* sites.

CRP-induced loop breaking is not only dependent upon the relative orientation between the CRP and *araI* sites, but also on the absolute distance between the sites. Thus, when the CRP–*araI* spacing is increased by 40 base-pairs, there is no observable effect of CRP on the dissociation rate of AraC (Fig. 6). The relatively slow dissociation of AraC in this construct shows that looping is still occurring, as expected. We conclude that CRP-induced loop breaking is dependent upon the distance and orientation of CRP relative to *araI*. When the CRP site is misoriented relative to its wild-type position within *araBAD*, CRP can actually enhance the looping interaction.

(c) *Activation of* p_{BAD} *by CRP* in vivo *is dependent upon the orientation and distance of the CRP binding site relative to* araI

Loop breaking by CRP could function in the activation of p_{BAD}. We would therefore expect that



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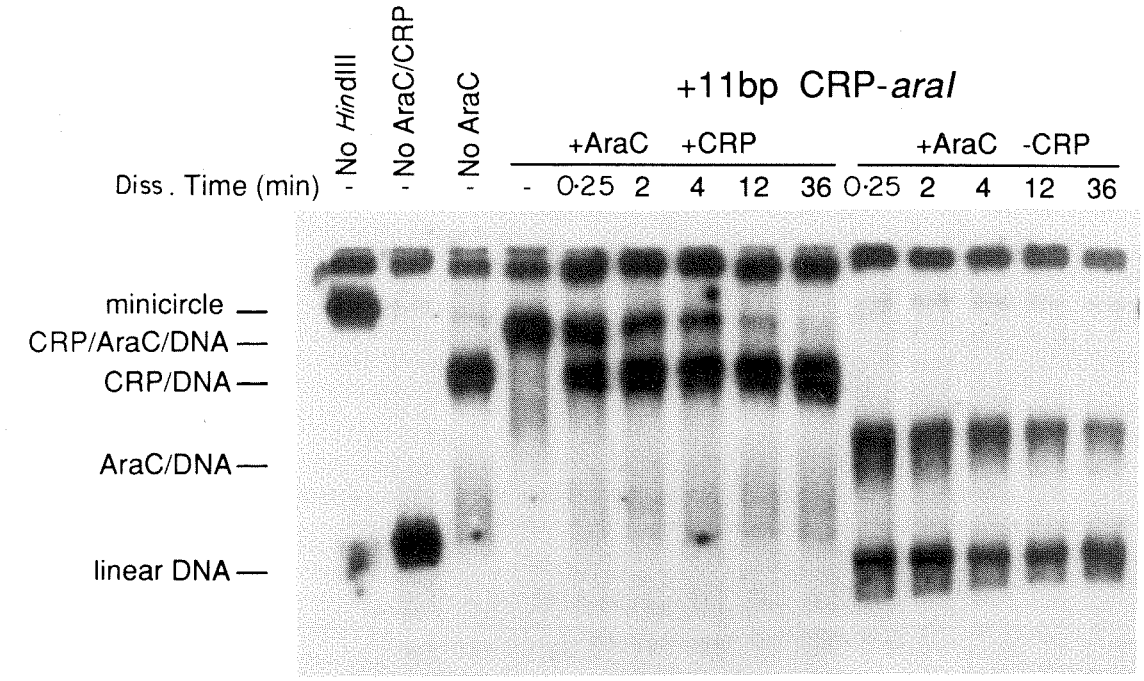

(b)

Figure 5. Loop breaking and loop stabilization by CRP in the +4 bp and +11 bp CRP–*araI* spacing mutants. (a) The dissociation rates from $araI_1$ in a spacing mutant containing 4 bp inserted between CRP and *araI* were compared in the presence and absence of cyclic AMP–CRP by the looping–restriction cutting assay. (b) Same as in (a) except a construct containing an 11 bp insertion between CRP and *araI* was assayed.

activation of p_{BAD} by CRP would depend on the distance and orientation of the CRP binding site relative to *araI*, as is seen with the CRP-induced loop breaking *in vitro*. The following shows that CRP activation *in vivo* is also dependent upon the distance and orientation between the CRP and *araI* sites.

We varied the orientation and distance between the *araI* and CRP sites in a p_{BAD}–*lacZ* fusion construct by inserting either integral or non-integral numbers of DNA helical turns between the sites. Where non-integral numbers of turns were inserted, a compensatory insert between the CRP binding site and $araO_2$ was introduced so that the total insertion between $araO_2$ and *araI* was an integral number of DNA helical turns; this maintains the

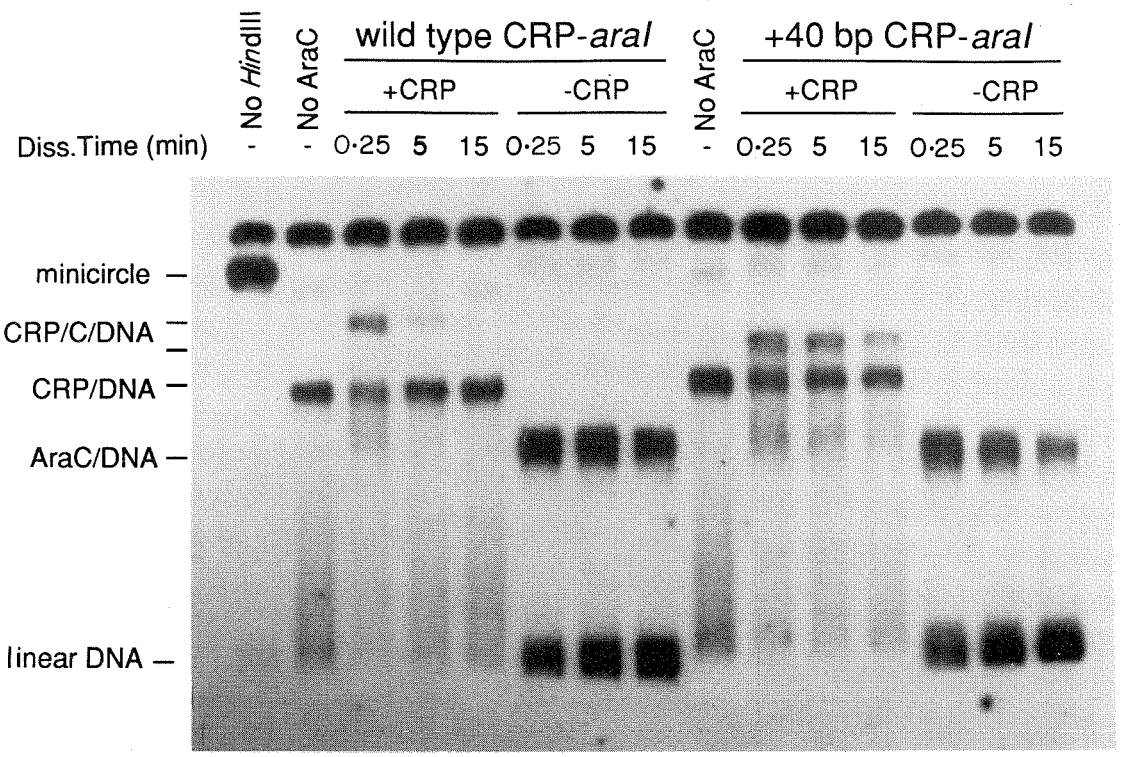

Figure 6. CRP does not affect looping when the CRP–*araI* spacing is increased by 40 bp. The dissociation rates of AraC from $araI_1$ in the presence and absence of CRP were compared in constructs containing either the wild-type CRP–*araI* spacing or a 40 bp insert between CRP and *araI* by the looping–restriction cutting assay.

proper orientation between $araO_2$ and *araI* and allows for the repression of p_{BAD} by $araO_2$ *in vivo* (Dunn *et al.*, 1984).

The insertions might alter the expression of the p_{BAD}–*lacZ* fusion not only by affecting the ability of CRP to activate, but also by affecting formation of the repression loop. To detect any effects of this nature we created identical spacing inserts that also contained point mutations in the CRP binding site and compared the expression level in the various

Table 1

*Effects of CRP–*araI *spacing on induction and AraC dissociation rate*

CRP–*araI* spacing	β-Galactosidase units		Factor stimulation of p_{BAD} by CRP	Half-time of AraC on *araI*		Factor of CRP stimulation
	CRP^+	CRP^-		+CRP	−CRP	
+0 (WT)	36,000	3500	10·3	2·5	40	16
+4	3200	9100	0·4	50	20	0·4
+11	20,000	3300	6·1	4·5	40	8·8
+15	6000	6800	0·9			
+22	7700	6100	1·3			
+26	7400	9500	0·8			
+33	8200	8800	0·9			
+40				17	22	1·3
+44	9400	8400	1·1			

Expression of the p_{BAD}–LacZ fusion from a multicopy plasmid containing the CRP^- and CRP^+ constructs and the various CRP–*araI* spacing mutations. The CRP mutations are C to T and G to A conversions at positions −89 and −98, respectively. Promoter activity is expressed as β-galactosidase units, and is an average of at least 3 independent measurements. The standard deviations of the measurements ranged from 10 to 20% of the total units. Shown also is the effect of CRP on the dissociation rate of AraC from $araI_1$ in several of the spacing mutants. Dissociation rate data are averages of 3 independent measurements. Dissociation half-times are reported as averages of 2 independent experiments, derived from autoradiograms that were scanned and quantified using a Biorad 620 densitometer. The dissociation half-times of complexes were determined relative to the 15-s time point. Typically between 45 and 65%, and not 100% of the DNA, is bound as AraC–DNA or AraC–CRP–DNA complexes at the 15-s time point. The lack of complete binding is due to dissociation of complexes after arabinose addition. Since the dissociation of complexes after arabinose addition is fixed for all of the time points, the dissociation half-time relative to the 15-s time point reflects the dissociation rate prior to arabinose addition. WT, wild-type.

spacing inserts containing the wild-type CRP binding site with the same spacing variants containing the mutant CRP binding site.

The data in Table 1 show that, like CRP-induced loop breaking, activation of p_{BAD} by CRP is dependent upon its orientation and distance from *araI*. p_{BAD} expression decreases tenfold when the CRP binding site contains point mutations at positions −89 and −98 (Table 1); the same order of decrease is seen in a deletion that removes the entire CRP binding site while maintaining the $araO_2$–*araI* looping interaction (data not shown). Thus, CRP activates p_{BAD} tenfold when its binding site is directly adjacent to *araI*.

Activation of p_{BAD} by CRP is abolished in all of the spacing variants except for the 11 base-pair insertion, which still allows a sixfold activation by CRP. The activation by CRP in the 11 base-pair spacing mutant is not dependent upon the particular sequence of the insert, as an insertion of a different 11 base-pair sequence also allows a similar factor of activation by CRP (data not shown). The failure of CRP to activate p_{BAD} in the other spacing mutations is not due to lack of $araO_2$-mediated repression, since p_{BAD} expression in all of the spacing mutants increases at least threefold when $araO_2$ is deleted (data not shown). Significantly, p_{BAD} is repressed by CRP in the four base-pair spacing mutant; the level of expression is 2·5-fold in the construct containing the wild-type CRP site compared to that in the mutant CRP site. The repression of p_{BAD} by CRP in the four base-pair spacing mutant is dependent upon $araO_2$ (data not shown). Additionally, the CRP-induced repression of p_{BAD} *in vivo* is consistent with the observed CRP-induced loop stabilization *in vitro* (Fig. 5(a)).

We conclude that activation of p_{BAD} by CRP requires the CRP binding site be located close to, and oriented properly to the *araI* site. There is a correlation between *in vivo* activation of p_{BAD} and the *in vitro* stimulation of loop breaking.

4. Discussion

We have shown that CRP breaks the $araO_2$–$araI_1$ loop *in vitro*. The ability of CRP to break the loop *in vitro* is dependent upon the distance and orientation of the CRP binding site relative to *araI*. Similarly, CRP activation of p_{BAD} *in vivo* shows the same dependence on the distance and orientation of the CRP site relative to *araI*. These results support the idea that part of the CRP stimulation of p_{BAD} is derived from hindering the AraC–DNA looping that represses p_{BAD}.

How does CRP break the loop *in vitro*? In short, we do not know. It merely summarizes the data to state that the loop is destabilized when the CRP binding site is near the AraC protein, but not otherwise. Whether the destabilization is due to the bend introduced by the binding of CRP or the mere presence of the protein was not determined by the experiments reported here.

Could CRP-induced loop breaking exist in other systems? As in *araCBAD*, the promoters of the *lac* and *deo* operons are repressed *via* DNA loop formation (Eismann *et al.*, 1987; Krämer *et al.*, 1987; Dandanell & Hammer, 1985; Amouyal *et al.*, 1989), and positively activated by CRP. Additionally, as in *araCBAD*, CRP binds adjacent to the promoter-proximal operator site within the DNA loop both in *lac* and *deo*. Therefore, as in *araCBAD*, CRP might also break the loop in *lac* and *deo*, and thereby indirectly activate their respective promoters.

The mechanism by which CRP activates independently of repression looping in *araCBAD* (Hahn *et al.*, 1986; Lichenstein *et al.*, 1987; Stolzfus & Wilcox, 1989), is likely to be different from that in other systems. In the *lac* operon, CRP binds cooperatively with RNA polymerase and enhances the isomerization rate of RNA polymerase from the closed to the open complex, from a site approximately 15 base-pairs from the *lac* P1 promoter (Straney *et al.*, 1989). This mechanism may not be possible in the $araO_2$-independent activation of p_{BAD} by CRP, considering that AraC is bound between CRP and RNA polymerase when the operon is both repressed and induced (Martin *et al.*, 1986; Lobell & Schleif, 1990).

The finding that CRP has a small, twofold, negative effect on AraC–arabinose binding to *araI* in the unlooped state (Fig. 4) suggests a mechanism that could account for the $araO_2$-independent activation of p_{BAD} by CRP. AraC binds to two half-sites within *araI* (Lee *et al.*, 1987), making strong contacts to $araI_1$ and weak contacts to $araI_2$ (Brunelle & Schleif, 1989). AraC binding to *araI* involves significant DNA bending (Lobell, 1990). Since both AraC and CRP bend the DNA and bind close to each other along the DNA, the proteins could interfere with each other's binding to the DNA. Thus, CRP might force AraC to lose some of its contacts with $araI_1$ and to "roll over" more onto $araI_2$, where AraC would be in closer proximity to contact and activate RNA polymerase. Since $araI_2$ is apparently a weaker binding site than $araI_1$, due to several positions of non-homology with the AraC direct repeat consensus binding sequence (Brunelle & Schleif, 1989), by forcing AraC onto $araI_2$, CRP would lower the affinity of AraC binding to *araI*, as we observed (Fig. 4). This model makes a testable prediction: the contacts made by AraC at $araI_2$ should be affected by CRP.

As suggested above, CRP-induced loop breaking could occur in *lac* and other operons. Additionally, the activation of the *lac* promoter and *araCBAD* by CRP is similar in that CRP shows the same orientation dependence in both operons; as in *araCBAD*, CRP can partially activate the *lac* promoter when its distance from the promoter is increased by 11 base-pairs, but not by five base-pairs, both *in vivo* (Mandecki & Caruthers, 1984) and *in vitro* (Straney *et al.*, 1989). CRP-induced DNA bending could be involved in the mechanism of CRP activation; the orientation dependence could be due to the requirement that the CRP-induced DNA bend be positioned correctly relative to the promoter.

Our finding that CRP breaks the $araO_2$–$araI_1$ DNA loop demonstrates a second mechanism, in addition to that of arabinose-mediated loop breaking (Lobell & Schleif, 1990), by which this looping interaction can be abolished. Conversely, Int-mediated DNA looping is enhanced by two proteins that bend DNA, IHF and CRP, as well as by naturally bent DNA (Moitoso de Vargas *et al.*, 1989; Goodman & Nash, 1989). We expect that DNA looping in other systems will be either positively or negatively effected by other proteins that bend the DNA, or otherwise effect the topology of a DNA loop.

This work was supported by grant GM18277 from the National Institutes of Health.

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Edited by P. von Hippel

Note added in proof. We wish also to bring to the reader's attention the following two recent papers, which investigate stimulation by CRP in the *lac* and *melR* promoters in which the location of the CRP binding site is systematically varied: Stringent spacing requirements for transcription activation by CRP, Gaston, K., Bell, A., Kolb, A., Buc, H. & Busby, S. (1990). *Cell,* **62**, 733–743; Helical phase dependent action of CRP: effect of the distance between the CRP site and the −35 region on promoter activity, Ushida, C. & Aiba, H. (1990). *Nucl. Acids Res.,* **18**, 6325–6330. In contrast to the present study, CRP stimulation in these systems appears not to be predominantly assisting the breaking of a DNA loop. Nonetheless, CRP stimulation in these systems as well is confined to spacings in which CRP occupies one face of the DNA helix.