# Helical phase dependent action of CRP: effect of the distance between the CRP site and the −35 region on promoter activity

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## ABSTRACT

**A plasmid carrying a CRP-dependent promoter fused to the *lac* structural genes was manipulated to construct a set of spacing mutants that have varying lengths between the CRP binding site and the −35 region. The lengths of the spacer were changed over 45 bp by inserting or deleting nucleotides. DNase I footprinting analysis revealed that the spacer length did not affect the binding of cAMP-CRP to the CRP site. The effect of the spacer length on transcription activation by cAMP-CRP was tested *in vivo* by β-galactosidase and quantitative S1 assays with *crp*⁺ and Δ*crp* cells harboring plasmids. Insertions or deletions of non-integral helical turns, which displace the CRP site onto the opposite face of DNA helix compared to the original promoter, eliminated completely the activation of transcription. In contrast, changing the spacer length by integral helical turns allowed the promoter to respond to CRP, although the degree of activation varied with the length of the spacer. We conclude that stereospecific positioning of CRP and RNA polymerase on the DNA helix is strictly required for CRP action. The data support a model that CRP stimulates transcription by directly contacting RNA polymerase.**

## INTRODUCTION

The cAMP receptor protein (CRP or CAP) of *Escherichia coli* is a well-known DNA binding protein which regulates the transcription of a large number of genes (1−3). The protein is a dimer of two identical subunits composed of 209 amino acids (4−6). The CRP subunit has a two-domain structure. The larger N-terminal domain is responsible for cAMP binding and subunit-subunit interaction while the smaller C-terminal domain contains a helix-turn-helix motif involved in DNA binding (7−10). When complexed with its allosteric effector cAMP, CRP elicits a conformational transition (7, 8) and binds to specific sites within or near promoters to activate or repress transcription.

The CRP binding sites in various genes include variations of the consensus sequence which is 22-bp and exhibits a perfect two-fold symmetry (11−14). Importantly the CRP sites lie at different locations relative to the transcription start site in different promoters (2, 3). Although direct contact between CRP and RNA polymerase bound to DNA has been believed to play an important role in transcription activation, the variety of locations of the CRP site seemed to be incompatible with a simple protein-protein interaction model. To account for this problem, *lac* promoter mutants with altered spacing between the CRP binding site and the −35 region have been characterized (15, 16). The major conclusion of these previous studies was that the promoter with 11-bp insertion retained partially the ability to respond to CRP while the insertion of 5 bp eliminated the effect of CRP on transcription. Thus the activation by CRP seems to be dependent on the helical phase between two sites.

We are concerned here with how strictly the stereospecific positioning of the CRP and RNA polymerase binding sites is needed for CRP action. It also remains to be seen how far the CRP site can be separated from the transcription start site to exert the function. To answer these questions, we constructed and characterized a series of artificial promoters that have varying lengths of the spacer between the CRP site and the −35 region. Our data clearly indicated that activation by CRP is strictly dependent on the helical phase between two sites. A significant activation of transcription was observed even when the CRP binding site was as far as 92.5 bp from the transcription start site. In addition we showed that the CRP activation can occur most efficiently when the CRP site is located 61.5 bp upstream from the transcription start site.

## MATERIALS AND METHODS

### Plasmid constructions

The plasmids used in this work were all derived from pRCI3 that contains an artificial promoter activated by cAMP-CRP (17). Plasmid pUT0 was constructed by removing one of the two CRP sites from pRCI3. The nucleotide sequence and structural features around the promoter region of pUT0 are shown in Figure 1. To construct a series of plasmids with various distances between the CRP binding site and the −35 region, one or two unique restriction sites in the spacer region of pUT0 were cleaved. The linearized plasmids with staggered ends were treated with the Klenow fragment of DNA polymerase I or S1 nuclease and recircularized with T4 DNA ligase. In some cases synthetic

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oligonucleotides were inserted into unique restriction sites. The oligonucleotides used are *Bam*HI linker (dCGGATCCG) and *Sma*I linker (dCCCGGG). Construction of the spacing mutants is described in further detail in Table 1. The nucleotide sequences of the spacing mutants were determined according to the improved dideoxy method (18).

### β-Galactosidase assay

The isogenic *E. coli* strains TB100 (Δ*lac crp*$^+$ *cya*$^+$) and TB102 (Δ*lac* Δ*crp cya*$^+$) (17) were transformed with plasmids and used for β-galactosidase assay. Cells were grown to $OD_{600}$=0.5−0.7 in LB medium containing 50 μg/ml ampicillin. The β-galactosidase activity was determined according to Miller (19) using $CHCl_3$ and 0.1% SDS to permeate cells.

### S1 nuclease assay

The S1 nuclease assay was performed as described previously (20, 21). Cellular RNAs were isolated from exponentially growing cells harboring plasmids in LB medium. The 278 bp *Eco*RI-*Hin*dIII fragment labeled at its 5′ *Hin*dIII end derived from pUT0 and cellular RNAs were hybridized and treated with S1 nuclease. The reaction products were analyzed on 8% polyacrylamide-9M urea gels.

### DNase I footprinting

The *Aat*II-*Hin*dIII fragments (369 bp − 394 bp) $^{32}$P-labeled at their 5′ ends derived from pUT series plasmids were used for footprinting analysis. The DNA fragments (5 ng) in 100 μl of 50 mM Tris-HCl, pH 7.9, 50 mM NaCl, 3 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM DTT, 100 μg/ml bovine serum albumin and 0.1 mM cAMP were incubated for 5 min at 37°C and then 5 min at 25°C in the presence and absence of CRP. DNase I was added at a concentration of 100 ng/ml and the incubation was continued for 40 s at 25°C. After adding 25 μl of 1.5 M sodium acetate, 20 mM EDTA, 100 μg/ml tRNA, the mixture was treated with phenol and precipitated with ethanol. The products were analyzed on 8% polyacrylamide-9 M urea gels.

## RESULTS

### Construction of spacing mutants

Plasmid pUT0 carries an artificial promoter fused to the *lac* structural genes. Transcription from this promoter is strongly activated by cAMP-CRP. In pUT0 the center of the CRP binding site is located at −71.5 and the distance between the downstream boundary of the CRP site and the upstream boundary of the −35 region is 25 bp. By inserting or deleting nucleotides in the spacer region, we constructed various spacing mutants. The insertions and deletions were designed to be either integral or half-integral turns of the DNA helix. Table 1 summarizes the constructions of the spacing mutants. The structural features of the spacing mutants are shown in Figure 1. Plasmids pUT+21, pUT+11,

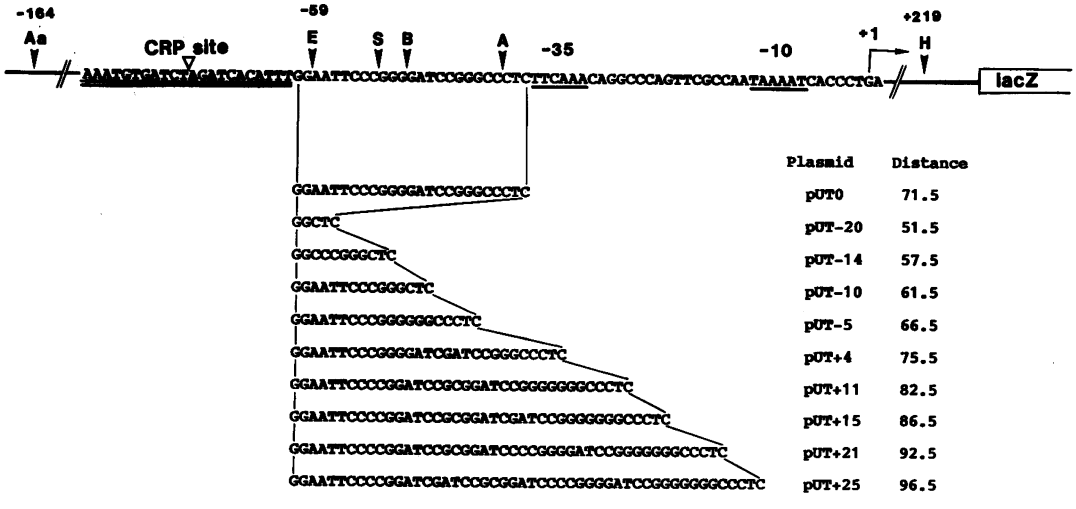


**Figure 1.** Structures of spacing mutants. The upper panel shows the nucleotide sequence along with structural features of the starting promoter in pUT0. The CRP binding site is double-underlined and the center of it is shown by an open triangle. The −35 and −10 sequences are underlined. The start site for transcription is indicated by an arrow and designated +1. The relevant restriction sites are also shown. The abbreviations for restriction sites are: A, *Apa*I; Aa, *Aat*II; B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; S, *Sma*I. The lower panel shows the sequences between the CRP site and the −35 region of a series of promoters. The distance is defined as the length (bp) between the center of the CRP site and the transcription start site.

**Table 1.** Construction of plasmids containing spacing mutants

| Plasmid | Derivation |
|---|---|
| pUT0 | Deleted one of the two CRP binding sites of pRCI3 |
| pUT+4 | Cleaved pUT0 with *Bam*HI, filled in with the Klenow fragment |
| pUT−5 | Cleaved pUT0 with *Bam*HI, treated with S1 nuclease |
| pUT+11 | Cleaved pUT−5 with *Sma*I, inserted two 8-bp *Bam*HI linkers |
| pUT+15 | Cleaved pUT+11 with *Bam*HI (partial), filled in with the Klenow fragment |
| pUT+21 | Cleaved pUT+11 with *Bam*HI (partial), filled in with the Klenow fragment, inserted 6-bp *Sma*I linker |
| pUT+25 | Cleaved pUT+21 with *Bam*HI (partial), filled in with the Klenow fragment |
| pUT−10 | Cleaved pUT0 with *Bam*HI and *Apa*I, treated with S1 nuclease |
| pUT−14 | Cleaved pUT−10 with *Eco*RI (partial), treated with S1 nuclease |
| pUT−20 | Cleaved pUT0 with *Eco*RI (partial) and *Apa*I, treated with S1 nuclease |

pUT−10, and pUT−20 contain either insertions or deletions of integral turns of the DNA helix. These mutants are referred to as in-phase mutants, since the phasing of the CRP site and the −35 region is preserved as in the original. On the other hand plasmids pUT+25, pUT+15, pUT+4, pUT−5, and pUT−14 are out-of-phase mutants, since half-integral turns of the DNA helix were added or deleted in these mutants.

### Binding of CRP to spacing mutants

We previously reported that cAMP-CRP strongly binds to the synthetic consensus CRP site (11). More quantitative binding assay by Ebright *et al.* (13) revealed that CRP exhibited a 450-fold higher affinity for the consensus CRP site than the *lac* CRP site. To examine the binding properties of CRP to the CRP sites in various spacing mutants, we performed DNase I footprinting assay using the *Aat*II-*Hin*dIII fragments labeled with $^{32}P$ at their 5′ *Hin*dIII ends. The results are shown in Figure 2. With all of the DNA fragments used about 26-bp region containing the CRP site is protected by cAMP-CRP from DNase I attack. It is apparent that the binding pattern and affinity of CRP to the CRP site are essentially the same among promoter mutants.

### Effect of the distance between the CRP site and the −35 region on promoter activity

In order to examine the effect of the spacer length on CRP activation, we introduced plasmid constructs into *crp*$^+$ and Δ*crp* cells, and measured the expression of the *lacZ* gene in cells. First, the promoter of the parental plasmid pUT0 was characterized. The β-galactosidase activity of *crp*$^+$ cells harboring pUT0 is 16-fold higher than that of Δ*crp* cells harboring the same plasmid (Table 2 and Figure 3). Thus the transcription of the promoter in pUT0 is highly dependent on CRP as in the case of pRCI3. Then we determined β-galactosidase activity both in *crp*$^+$ and Δ*crp* cells harboring a series of spacing mutants. The results are shown in Table 2. Inserting or deleting DNA of non-integral helical turns (+4 bp, +15 bp, +25 bp, −5 bp, and −14 bp) between the CRP site and the −35 region eliminated drastically the β-galactosidase activity in *crp*$^+$ cells. On the other hand, the 11-bp and 21-bp insertions gave β-galactosidase levels of 50% and 20% of the parent promoter, respectively. Interestingly, the introduction of 10-bp deletion markedly promoted the activity (189% of the original activity). However the 20-bp deletion resulted in a considerable decrease in the activity. In Δ*crp* cells all the spacing mutants exhibited low levels of β-galactosidase activity. Thus the promoter activities of in-phase mutants are dependent on CRP as in the parental plasmid pUT0. The stimulation ratio (the activity of *crp*$^+$ cells to that of Δ*crp* cells) was plotted as a function of the distance between the CRP site and the transcription start site (Figure 3). This clearly indicates that transcription activation by CRP is dependent on the helical phase between the CRP site and the −35 region.

### S1 analysis of promoter activity

Although the −35 and −10 sequences of the spacing mutants are the same as those of pUT0, we can not exclude the possibility that insertions or deletions of nucleotides in the spacer region alter the start site for transcription in the original promoter. To examine this and to characterize further promoter activity, we conducted a quantitative S1 assay. Cellular RNAs from *crp*$^+$ and Δ*crp* cells harboring plasmids were hybridized to the *Eco*RI-*Hin*dIII fragment derived from pUT0 $^{32}P$-labeled at its *Hin*dIII 5′ end. In this assay RNA from cells harboring the parent plasmid pUT0 gives an S1 resistant DNA band of about 220 bases (Figure 4). The same S1 resistant DNA bands in size were also produced by RNAs from all the spacing mutants. This indicates that the start sites for transcription in mutant promoters are the same as that of pUT0. In addition the variation of the intensity of the S1 resistant DNA bands clearly indicates that the promoter activity varies periodically depending on the helical phase between the

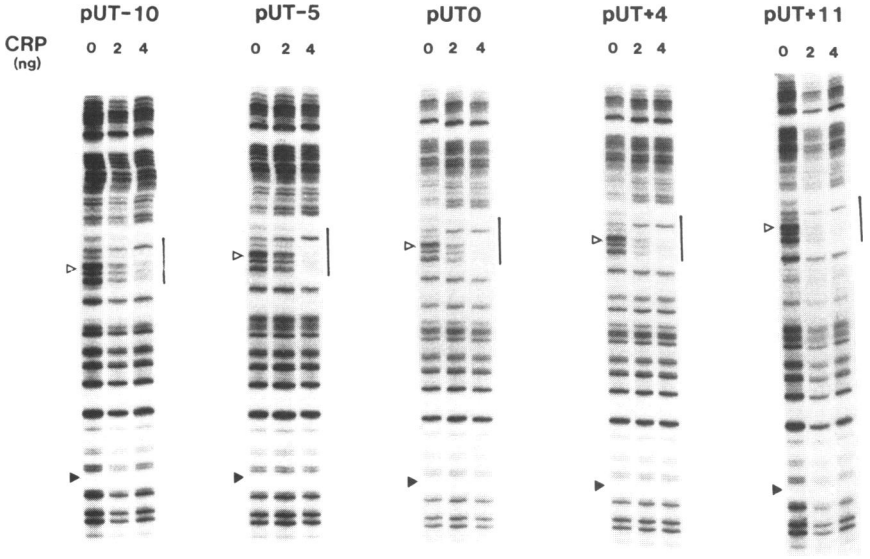


**Figure 2.** Footprinting analysis of cAMP-CRP binding to the spacing mutants. The *Aat*II-*Hin*dIII fragments from, pUT−10, pUT−5, pUT0, pUT+4, and pUT+11 labeled with $^{32}P$ at their 5′ *Hin*dIII ends were treated with DNase I in the presence of 100 μM cAMP and indicated amount of CRP. The products were fractionated on 8% polyacrylamide-9M urea gels. The regions protected from DNase I digestion by cAMP-CRP, shown by vertical lines, are from −50 to −75 in pUT−10, from −55 to −80 in pUT−5, from −60 to −85 in pUT0, from −64 to −89 in pUT+4, and from −71 to −96 in pUT+11. The closed and open triangles represent the transcription start site and the center of the CRP site, respectively.

binding sites for CRP and RNA polymerase as seen in the β-galactosidase assay. The results of S1 assay are fully consistent with those of β-galactosidase assay, indicating again that the activation of transcription by CRP is strictly dependent on stereospecific alignment between the CRP and RNA polymerase binding sites.

## DISCUSSION

A prevailing view concerning the mechanism of transcriptional activation both in prokaryotes and eukaryotes is that the activation is mediated by direct interaction between activator protein(s) and transcriptional machinery (22–24). In the case of CRP, several lines of evidence suggest that CRP activates transcription by contacting RNA polymerase. First, evidences for the interaction between two proteins were obtained from fluorescence (25, 26), ultracentrifugation (27), and immunological (28) studies. Second, cooperative binding to the *lac* promoter region of CRP and RNA polymerase was demonstrated by protection experiments such as DNase I footprinting (16, 29, 30). CRP and RNA polymerase are also shown to bind cooperatively to *gal* promoter region (29, 31). Third, the importance of the CRP-RNA polymerase interaction is supported by the isolation of CRP mutants that are deficient in the gene activation, but are able to bind DNA (32).

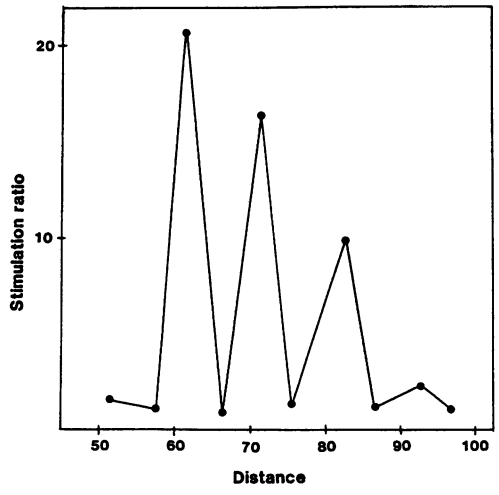


**Figure 3.** Effect of the distance between the CRP site and the start site on activation by cAMP-CRP. Stimulation ratio is the relative β-galactosidase activity of each promoter in *crp*$^+$ background to that in Δ*crp* background. The values for β-galactosidase activity were taken from Table 2.

Further evidence supporting CRP-RNA polymerase interaction came from phasing experiments (15, 16). Initially, Mandecki and Caruthers (15) characterized the *lac* promoter mutants with varying lengths of the spacer between the CRP binding site and the −35 region. They found that the insertion of 5 bp between two sites eliminated CRP activation, while the activation was partially restored with an 11-bp insert. More recently, Straney *et al.* (16) have shown by using similar spacing mutants that stabilization of CRP binding to the *lac* DNA by RNA polymerase occur in parallel with CRP activation. These data support the view that the interaction between CRP and RNA polymerase bound on the same side of DNA helix is important for CRP action. However in the previous phasing experiments the variation in the spacer length was limited within only one turn of DNA helix. In addition the start sites for transcription in spacer mutants were not analyzed. This is important, since it is possible that changing the spacer length could alter the transcription start site.

In this paper we carried out a more extensive phasing experiment by using a synthetic CRP-dependent promoter. We constructed a set of spacing mutants in which the distance between the CRP site and the −35 region varies over 4 turns of DNA helix. Then we analyzed the transcriptional activity of spacing mutants *in vivo* by β-galactosidase and S1 assays. All in-phase mutants, which preserve the integral helical spacing between the CRP site and the −35 region, have been shown to retain more or less the ability to be activated by CRP. Whereas out-of-phase mutants, which have insertion or deletion of non-integral helical turns, can no longer respond to CRP. The observation that the periodicity of the stimulation by CRP is around 10.5 bp (one helical turn of B-DNA) is consistent with those of the previous

**Table 2.** Influence of variation of the distance between the CRP binding site and the −35 sequence on promoter activity

| Plasmid | Spacer length[a)] | Distance[b)] | β-Galactosidase Activity[c)] (units) | |
|---|---|---|---|---|
| | | | *crp*$^+$ | Δ*crp* |
| pUT−20 | 5 | 51.5 | 286 | 184 |
| pUT−14 | 11 | 57.5 | 156 | 152 |
| pUT−10 | 15 | 61.5 | 2499 | 121 |
| pUT−5 | 20 | 66.5 | 112 | 132 |
| pUT0 | 25 | 71.5 | 1325 | 81 |
| pUT+4 | 29 | 75.5 | 154 | 123 |
| pUT+11 | 36 | 82.5 | 678 | 97 |
| pUT+15 | 40 | 86.5 | 125 | 104 |
| pUT+21 | 46 | 92.5 | 226 | 98 |
| pUT+25 | 50 | 96.5 | 101 | 115 |

a) Defined as the length (bp) between the downstream boundary of the CRP binding site and the upstream edge of the −35 region.
b) Defined as the length (bp) between the center of the CRP binding site and the transcription start site.
c) The β-galactosidase assay was performed with strains TB100 (Δ*lac crp*$^+$ *cya*$^+$) and TB102 (Δ*lac* Δ*crp cya*$^+$) harboring plasmids. The activity is expressed in Miller units (19). The values are averages from three assays on independently grown bacterial cultures. The relative amount of plasmid in cells was estimated by an electrophoretic analysis of cell extracts. No significant variation in the plasmid copy number was observed for these plasmids in both strains.

phasing experiments on the *lac* promoter (15, 16). Since we observed no significant differences in binding of cAMP-CRP to DNA among the spacing mutants, the periodical variation of promoter activity strongly suggests that the positioning of the CRP and RNA polymerase binding sites on the same face of DNA helix is strictly required for CRP action. The helical phase dependence of CRP action supports the model that CRP activates transcription by touching with RNA polymerase. The importance of stereospecific positioning of the binding sites for an activator and RNA polymerase has also been shown in several other promoters (33−36) by analogous phasing experiments. Among these the OmpR-mediated activation of the *ompC* promoter (34) is quite similar to that of CRP dependent promoter.

Our data indicate that CRP can most efficiently activate transcription when the center of its binding site is positioned 61.5 bp upstream from the start site. This location is exactly the same as that in *lac* (37) and *tna* (38). In addition it has been shown that the placement of the CRP site at −61.5 in the *lac-gal* hybrid promoter is also very effective for CRP activation (39). Thus in several different promoters CRP can activate transcription very efficiently at −61.5. This position is apparently optimum for CRP to interact with RNA polymerase in type I CRP-dependent promoter (17) in which the CRP site is separated from the −35 region. The activation by CRP decreases rapidly with the deviation from the optimum spacer length. However we still observed a significant activation when the CRP site was placed 92.5 bp upstream from the start site. In naturally occurring promoters, where CRP acts as a sole activator, the greatest distance between the CRP site and the transcription start site is 70.5 bp as seen in *malT* (40). The CRP sites are located further upstream in *araBAD* (41) and *malE-malK* (42) operons. In these cases however additional proteins are required for CRP action (43, 44). Recently, it has been found that the CRP site at *papB* is separated more than 200 bp from the start site (45). It remains to be seen if CRP acts alone or with additional factors in this operon.

It is interesting to note that deleting one turn of the helix in the spacer region of the optimum promoter (from pUT−10 to pUT−20) reduces dramatically CRP activation. In this case the CRP site is probably too close to the −35 region for an effective interaction between CRP and RNA polymerase. Aside from the present results, CRP is also known to activate transcription effectively when its binding site is placed around −40 as seen in *gal* (46), pBRP4 (47) and two artificial promoters (17). In these promoters, which we call type II CRP-dependent promoter (17), the CRP site overlap the −35 region. It remains to be seen whether the mode of interaction between CRP and RNA polymerase is different from that in type I promoter.

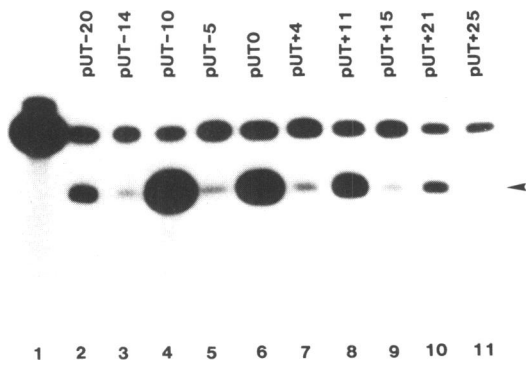


**Figure 4.** S1 analysis of the transcripts in cells with spacing mutants. The labeled DNA probe was hybridized to 50 μg of RNAs from *crp*$^+$ cells harboring following plasmid: pUT−20 (lane 2), pUT−14 (lane 3), pUT−10 (lane 4), pUT−5 (lane 5), pUT0 (lane 6), pUT+4 (lane 7), pUT+11 (lane 8), pUT+15 (lane 9), pUT+21 (lane 10), pUT+25 (lane 11). The hybrids were treated with S1 nuclease and the products were analyzed on an 8% polyacrylamide-9M urea gel. Lane 1 shows the DNA probe without S1 treatment. An arrowhead represents the position of S1 resistant DNA bands.

The contact between two proteins at variable distances should require structural distortions in DNA and/or proteins. We have observed (unpublished results) that CRP induces a bending upon binding to the promoter fragments derived from pUT series plasmids as in the case of the *lac* promoter (48). However, this bending alone is probably not sufficient to account for the long-range CRP-RNA polymerase interaction, since the CRP-induced bend is thought to occur around the center of the CRP site (49). Additional bending or looping out in the spacer region would be needed to accommodate the protein-protein interaction.

DNA looping mediated by protein-protein interaction between two proteins bound to specific DNA sequences at distant locations, originally proposed in the *gal* operon (50), has been implicated widely in gene transcription, replication, and recombinations. The well characterized examples for this include λ (51), *lac* (52), and *ara* (53) repressors. In these cases, several lines of evidence strongly indicate that the interaction between the homologous repressor proteins bring the two operator sites together via loop formation. Although loop formation involving an activator protein and RNA polymerase has been previously proposed based on phasing experiments in several systems (16, 33−36) and the present results support this view further, more direct evidences are required to confirm the looping mechanism in transcription activation. The spacing mutants described here are providing a useful model system for studying possible structural distortions in DNA and proteins caused by CRP-RNA polymerase interaction.

After completing this work, we have learned that Gaston *et al.* (54) have characterized a set of mutants with varying lengths between the CRP site and the −10 region of *melR* promoter. They showed that the activation by CRP required a stringent spacing between two sites. Moreover they found that the distances at which CRP strongly activates transcription correspond exactly to those at the *galP1* (type II) and *lacP1* (type I), proposing a two-position model for the interaction of CRP and RNA polymerase.

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