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Regulatory effect of a synthetic CRP recognition sequence placed downstream of a promoter

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ABSTRACT

A series of plasmids were constructed in which a promoter was introduced into a lac-based operon fusion vector. A perfectly symmetrical oligonucleotide of 22-bp corresponding to an idealized binding site for cAMP receptor protein (CRP) of E. coli was chemically synthesized. The synthetic CRP site was placed between the promoter and the lacZ structural gene with varying distances from the promoter. Specific binding of cAMP-CRP complex to the synthetic CRP site was shown by a gel retardation and a DNase I footprinting assays. Plasmid constructs were transformed into crp^+ and crp^- cells carrying a chromosomal deletion of the lac genes. The regulatory effect of the inserted CRP site was examined by comparing the β-galactosidase activity and the levels of RNA transcript in two cells harboring the plasmids. We found a strong inhibitory effect of the CRP site in the presence of cAMP and CRP when it was placed close to the promoter. When the CRP site was placed far downstream of the promoter, a moderate repression of transcription was observed.

INTRODUCTION

The cAMP receptor protein (CRP) of Escherichia coli, also known as the catabolite gene activator protein (CAP), is a sequence specific DNA-binding protein which regulates the transcription of a number of genes (1-3). The protein is composed of two identical subunits of molecular weight 23619 (4-6). In the presence of cAMP, CRP elicits a conformational transition (7, 8) and binds to specific sites within or near promoters to modulate transcription. The DNA sites recognized by cAMP-CRP in various genes are about 22-bp , which contain a well conserved pentamer 5'-TGTGA-3' and a less conserved inverted repeat of the pentamer (2, 3).

Although the major role of CRP is to activate transcription of catabolite sensitive operons, in several cases the protein is

known to repress transcription. For example, one of the two overlapping promoters of the gal operon is negatively regulated by cAMP-CRP whereas the other one is regulated positively by the same complex (9, 10). In addition, recently we and others found that the crp (11, 12) and cya (13-16) genes are regulated autogenously at a transcriptional level. In these two operons cAMP-CRP acts only as a negative regulator to repress the transcription. Both genes possess a sequence similar to the consensus CRP site within (in the case of cya) or downstream (in the case of crp) of respective promoter.

These observations prompted us to test whether CRP can generally act as a transcriptional repressor when its binding site is located near or downstream of any promoters. For this we constructed a system where CRP and its binding sequence might be able to exert a functional role to inhibit the transcription. We first synthesized a 22-bp oligonucleotide which corresponds to an idealized CRP recognition sequence and placed the synthetic DNA downstream of a promoter, varying the distance from the promoter. Then we examined the effect of inserted CRP site on the transcription. Our experiments indicate that the synthetic CRP site can be functional to repress transcription in the presence of cAMP and CRP when placed downstream of the promoter. The degree of inhibition varied depending on the distance between the promoter and the CRP site.

MATERIALS AND METHODS

Enzymes and Chemicals

Restriction endonucleases, bacterial alkaline phosphatase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Takara Shuzo. Klenow fragment of E. coli DNA polymerase I, nuclease S1, and nuclease Bal31 were obtained from Bethesda Research Laboratories. [γ-^{32}P]ATP (5000Ci/mmole) was purchased from Amersham. MacConkey-lactose agar was from Difco. cAMP and o-nitro-D-galactopyranoside were from Sigma. CRP was purified by the method of Eilen et al. (8).

Bacterial Strains

The E. coli strains KI70 (araD Δlac thi str spc) and OK6201 (araD Δlac thi str spc crp zhd::Tn10), both are isogenic except

the crp gene and derivatives of MC4100 (17), were used throughout this study. Strain KI70 was obtained from K. Ito (University of Kyoto) and strain OK6201 was from R. Utsumi (University of Kinki).

Plasmids and Oligonucleotides

Plasmid pMS437D is a lac-based cloning vector for operon fusions. It was constructed by ligating the 10-kb HindIII-SacI fragment from pMS437C (15) and the 2-kb HindIII-SacI fragment from pMS42 (18). The construction of operon fusion plasmids (pPxL and pPxSL series) is described in text.

Plasmids were purified by the procedure of Birnboim and Doly (19). For small-scale plasmid preparation, the rapid isolation method of Davis et al. (20) was used. For preparation of DNA fragments, plasmids were digested with appropriate restriction endonucleases, and the resulting fragments were purified by electrophoresis either on polyacrylamide or agarose gels in TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA).

A 22-bp oligonucleotide corresponding to an idealized CRP site (Fig. 1) was chemically synthesized by the β-cyanoethylphosphoramidite method (21) with an automatic DNA synthesizer (Applied Biosystems Inc. Model 380A) followed by purification with an anion exchange HPLC. The sequence of the synthetic oligonucleotide was verified after cloning by DNA

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
A	13	16	9	0	0	1	0	25	5	5	6	8	6	8	5	3	14	11	16	6	12	7	
G	1	3	6	0	26	0	26	1	3	7	4	6	10	6	4	0	6	2	5	6	1	3	frequency of
C	3	1	5	3	0	1	0	0	7	10	4	8	5	5	4	18	4	11	2	3	2	5	appearance of bases
T	9	6	6	23	0	24	0	0	11	4	12	4	5	7	13	5	1	2	3	11	11	10	
5'	A	A	A	T	G	T	G	A	T	C	T	A/C	G	A	T	C	A	C/A	A	T	A/(T)	T	3' consensus sequences
5'	A	A	A	T	G	T	G	A	T	C	T	A	G	A	T	C	A	C	A	T	T	T	3' synthetic sequence

Figure 1. Analysis of CRP binding sites. The sequences of 26 CRP binding sites in various genes listed by Busby (3) were used to obtain the frequency of appearance of each of the four bases at each position. Consensus sequences are comprised of 22-bases which appear most frequently at each position. The T shown in parentheses is the base that appears second most frequently at the 21th position. The synthetic CRP site corresponds to one of the consensus sequences which has a perfect symmetry.

sequence analysis. HindIII linker, d(pCAAGCTTG), was purchased from Takara Shuzo.

Enzyme assays

LB medium containing 50 µg/ml ampicillin was used for the assays of β-galactosidase and β-lactamase. β-Galactosidase activity was determined by the method of Miller (22). β-Lactamase activity was determined according to the method by Sawai et al. (23).

Gel binding assay

DNA fragment (100 ng) and different amount of CRP were incubated for 5 min at 37°C in 30 µl of binding buffer (20 mM Tris-HCl, pH7.9, 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM ditiothreitol, 0.1 mM EDTA, 100 µg/ml bovine serum albumin) containing 100 µM cAMP. Before loading on the gel, 5 µl of 50% glycerol, 0.01% bromphenol blue, 0.01% xylene cyanol in the same buffer was added. The mixtures were electrophoresed on a 6% polyacrylamide gel equilibrated with TBE containing 100 µM cAMP at room temperature.

DNase I footprinting

^{32}P-labeled DNA (50 ng) in 100 µl of binding buffer containing 5 mM $CaCl_2$ was incubated for 5 min at 25°C in the presence and absence of CRP and cAMP. 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 sample was dissolved in 10 µl of 9 M urea, 0.025% bromphenol blue, and 0.025% xylene cyanol in TBE, and electrophoresed on an 8% polyacrylamide-8 M urea gel.

Nuclease S1 Analysis

S1 nuclease assays were carried out essentially as described previously (10, 11). Cellular RNA was isolated from exponentially growing cells harboring plasmids in LB medium at 37°C. DNA fragments were labeled with [γ-^{32}P]ATP at their 5' ends by T4 polynucleotide kinase. The ^{32}P-labeled DNA fragments were further digested with appropriate restriction endonucleases and the resulting fragments were purified by electrophoresis on polyacrylamide gels in TBE to obtain DNA probes.

Cellular RNA (30-50 µg) and the DNA probe were mixed

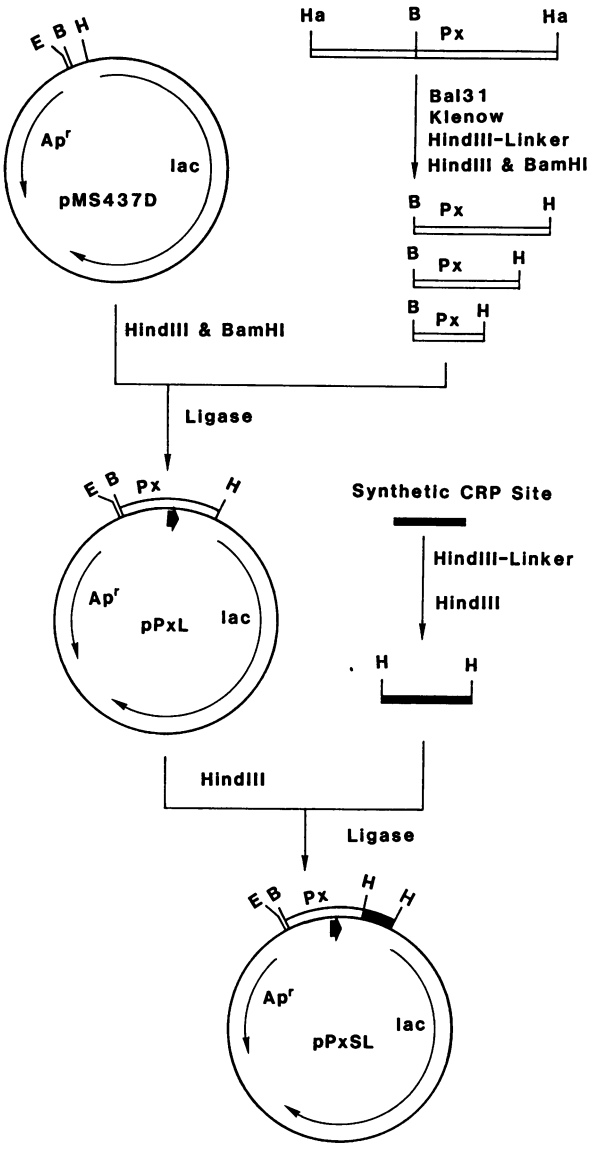


Figure 2. Construction of fusion plasmids carrying the synthetic CRP site. The 140-bp BamHI-HindIII segment of pMS437D was replaced by the BamHI-HindIII fragments carrying Px to construct pPxL series. The synthetic CRP site was inserted into the HindIII site of pPxL to construct pPxSL series. Bold arrows indicate the direction of transcription from Px. Details of construction of plasmids are described in text. The abbreviations for restriction sites are: E, EcoRI; B, BamHI; H, HindIII.

together in 50 µl of hybridization buffer (80% formamide, 20 mM HEPES, pH6.5, 0.4 M NaCl). The mixture was incubated at 75°C for 10 min and cooled gradually to 37°C. The incubation was continued further for 2 h at 37°C. After adding 220 µl of H_2O and 30 µl 10xS1 buffer (0.3 M sodium acetate, pH4.6, 0.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol), the mixture was treated with 10 units of S1 nuclease at 37°C for 10 min. The reaction was terminated by phenol extraction. The reaction products were precipitated with ethanol and dried under vacuum. The precipitate was dissolved in 20 µl of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.025% bromphenol blue and 0.025% xylene cyanol and electrophoresed on a polyacrylamide gel. The gels were exposed to X-ray films.

DNA Sequencing

DNA sequences were determined either by the method of Maxam and Gilbert (24) or the dideoxy method improved by Hattori and Sakaki (25).

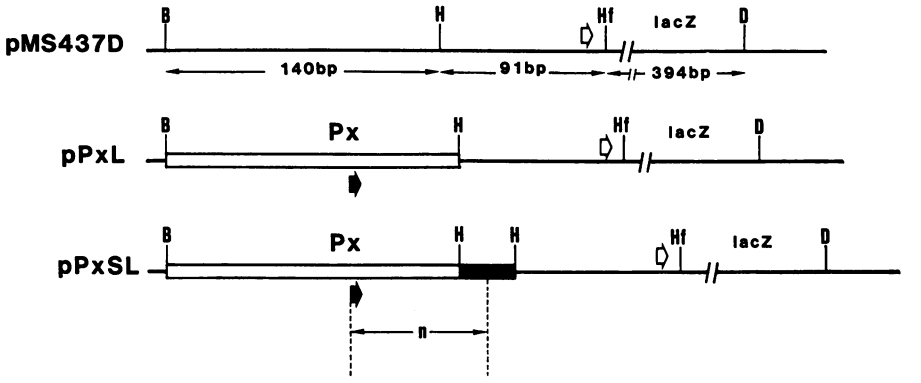
RESULTS

Construction of Plasmids

We constructed a series of plasmids which have the insertion of the synthetic CRP site at various distances downstream of a promoter Px (Fig. 2). Px is a promoter in the *hemC* gene which is located upstream of the *cya* gene (13, 26). The 568-bp HaeIII fragment containing Px was treated with Bal31 and the ends were filled in by Klenow fragment of DNA polymerase I. After joining HindIII linker, the fragments were digested with BamHI and HindIII. The resulting BamHI-HindIII fragments were ligated into *lac*-based operon fusion plasmid pMS437D to construct Px-*lac* fusion plasmids (pPxL series).

HindIII linker was ligated to the 22-bp synthetic CRP site and linkered DNA was digested with HindIII. The resulting DNA was inserted in the HindIII site of pPxL series to construct plasmids carrying the synthetic CRP site (pPxSL series). The exact position of the inserted CRP site was determined by DNA sequence analysis. The characteristics of plasmids including their nucleotide sequences around the promoter are shown in Fig. 3. The distance between the start of transcription and the center of the CRP site ranges between 16-140 nucleotides (nt).

A



B

Plasmid	Sequence	Distance(nt)
pPxSL56	5'TGTTAGGATGGACCACGGATGA←116n→CAAGCTTGAAATGTGATCTAGATCACATTT3'	140
pPxSL54	5'TGTTAGGATGGACCACGGATGA← 27n→CAAGCTTGAAATGTGATCTAGATCACATTT3'	51
pPxSL03	5'TGTTAGGATGGACCACGGATGA← 18n→CAAGCTTGAAATGTGATCTAGATCACATTT3'	42
pPxSL21	5'TGTTAGGATGGACCACGGATGA← 13n→CAAGCTTGAAATGTGATCTAGATCACATTT3'	37
pPxSL08	5'TGTTAGGATGGACCACGGATGCAAGCTTGAAATGTGATCTAGATCACATTT3'	23
pPxSL15	5'TGTTAGGATGGACCACGGACAAGCTTGAAATGTGATCTAGATCACATTT3'	21
pPxSL10	5'TGTTAGGATGGACCACGGCAAGCTTGAAATGTGATCTAGATCACATTT3'	20
pPxSL09	5'TGTTAGGATGGACCACAAGCTTGAAATGTGATCTAGATCACATTT3'	17
pPxSL04	5'TGTTAGGATGGACCCAAGCTTGAAATGTGATCTAGATCACATTT3'	16

Figure 3. Structural properties of fusion plasmids. (A) Partial restriction maps of plasmids. The open bars represent DNA fragments carrying Px. The black bar represents the CRP site. The bold arrows show the start point and direction of transcription from Px. The distance between the start of transcription and the center of the CRP site is shown by "n". The open arrows show the start point of translation for trpA-lacZ fusion protein. The abbreviations for restriction sites are: B, BamHI; H, HindIII, Hf, HinfI; D, DdeI. (B) Properties of plasmids carrying the synthetic CRP site. The DNA sequences around Px and the CRP site are shown. The -10 region of Px is boxed. The CRP site is underlined. The center of symmetry of the CRP site is shown by an open triangle. The nucleotide at which transcription starts is shown by an closed triangle. Distance between the start of transcription and the center of the CRP site is given on the right.

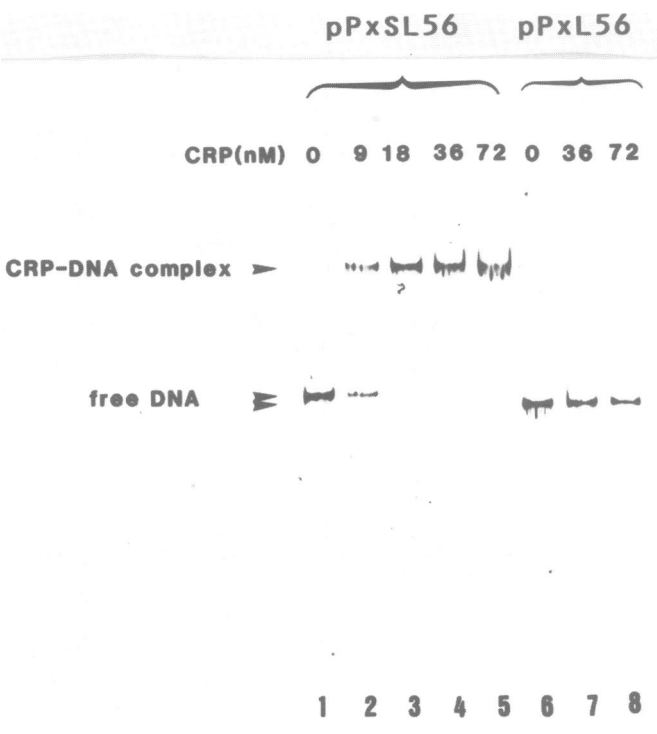


Figure 4. Specific binding of cAMP-CRP to the DNA fragment containing the synthetic CRP site. 100 ng of the 732-bp BamHI-DdeI fragment from pPxSL56(lanes 1-5) or the 702-bp BamHI-DdeI fragment from pPxL56(lanes 6-8) were incubated with indicated amount of CRP and 10^{-4}M cAMP in 30 µl binding buffer. The mixtures were separated on a 6% polyacrylamide gel. DNA bands were visualized by ultraviolet illumination of the gel stained with ethidium bromide.

Specific Binding of cAMP-CRP to the Synthetic CRP Site

The sequence of the synthetic CRP site is similar but not identical with those of any known CRP binding sites in natural genes. To test whether the synthetic CRP site can be recognized specifically by cAMP-CRP, we first performed a gel retardation assay (27,28) using restriction fragments carrying or missing the synthetic CRP site. The BamHI-DdeI fragments from pPxL56 or pPxSL56 were incubated with increasing amount of CRP in the presence and the absence of cAMP. The reaction mixtures were analyzed by electrophoresis on a 6% polyacrylamide gel. In the presence of cAMP and CRP the DNA fragment from pPxSL56 forms a

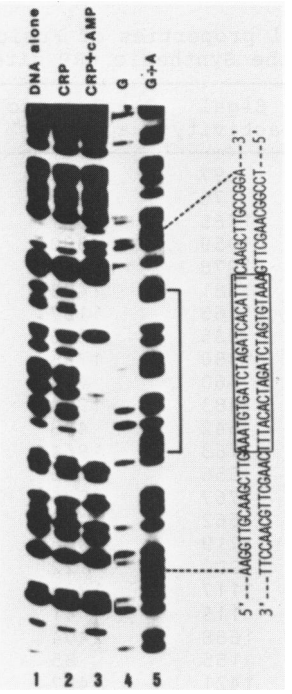


Figure 5. Footprinting analysis of cAMP-CRP binding to the synthetic CRP site. The 338-bp BamHI-HinfI fragment from pPxSL56 ^{32}P-labeled at its HinfI 5' end was treated with DNase I in the presence or absence of cAMP and CRP. The products were fractionated on an 8% polyacrylamide-8M urea gel. Lane 1, no added factors; lane 2, 0.1 µg of CRP added; lane 3, 0.1 µg CRP and 10^{-4}M cAMP added; lane 4, G specific chemical reaction; lane 5, A+G specific chemical reaction. The region protected from DNase I attack by cAMP-CRP is shown by a bracket. The sequence around the CRP site is shown on the right. The synthetic CRP site is boxed.

complex which migrates more slowly than the free DNA (Fig. 4, lanes 1-5). On the contrary, the migration velocity of the DNA fragment from pPxL56 was not affected by cAMP-CRP (Fig. 4, lanes 6-8). These results suggest that cAMP-CRP binds specifically to the synthetic CRP site.

To determine the binding site of CRP precisely, we carried out a DNase I footprinting experiment (29). The BamHI-HinfI fragment of pPxSL56 labeled at its HinfI 5'end was incubated either with or without cAMP and CRP. They were then treated with DNase I and the products were fractionated on a polyacrylamide-8

Table 1. Functional properties of Fusion Plasmids Carrying the Synthetic CRP site

Plasmid	host[1]	color of colony[2]	β-gal activity[3]	β-gal/β-lac activity[4]	repression ratio[5]	distance[6]
pMS437D	crp⁻	w	97	-	-	
	crp⁺	w	76	-	-	
pPxL56	crp⁻	r	1765	1631	1.65	
	crp⁺	r	1059	980		
pPxL04	crp⁻	r	1978	2442	1.64	
	crp⁺	r	1381	1473		
pPxSL56	crp⁻	r	1165	1157	3.25	140
	crp⁺	r	535	356		
pPxSL54	crp⁻	r	1380	1768	4.27	51
	crp⁺	r	460	414		
pPxSL03	crp⁻	r	1483	1904	4.34	42
	crp⁺	r	542	438		
pPxSL21	crp⁻	r	1763	1964	4.41	37
	crp⁺	r	556	445		
pPxSL08	crp⁻	r	1767	2336	5.33	23
	crp⁺	r	562	438		
pPxSL15	crp⁻	r	1719	2008	16.4	21
	crp⁺	r/w	200	122		
pPxSL10	crp⁻	r	1117	1239	30.2	20
	crp⁺	w	115	41		
pPxSL09	crp⁻	r	1688	2404	28.3	17
	crp⁺	w	155	85		
pPxSL04	crp⁻	r	1421	1487	29.1	16
	crp⁺	w	132	52		

[1] The plasmids were transformed into two strains: KI70 (Δlac crp⁺ cya⁺) and OK6201 (Δlac crp⁻ cya⁺).
[2] Determined on MacConkey lactose plates containing 50 μg/ml of ampicillin: w, white; r, red; r/w, red with white annulus.
[3] Activity of β-galactosidase is expressed in Miller units (22).
[4] β-Galactosidase activity corrected for β-lactamase activity, which is an indicator of plasmid copy number, is expressed in arbitrary units. In each case the activity from the vector pMS437D was subtracted.
[5] β-Gal/β-lac in crp⁺ cells to β-gal/β-lac in crp⁻ cells.
[6] Distance between the start of transcription from Px and the center of the CRP site is shown in nucleotides.

M urea gel. The results are shown in Fig. 5. Lane 1 shows the result of digesting the fragment with DNase I in the absence of any added factors. The addition of CRP alone (lane 2) did not affect the digestion pattern. When CRP along with cAMP is added in the reaction mixture, a distinct DNA region is strongly protected from DNase I digestion (lane 3). This region exactly corresponds to the synthetic CRP site. We conclude that the synthetic CRP site can be specifically recognized by cAMP-CRP complex as in the case of CRP sites in the natural genes.

Regulatory Effect of the Synthetic CRP Site

To analyze the regulatory effect of the CRP site on transcription, plasmid constructs were transformed into two lac-deleted strains, KI70(Δlac crp$^+$ cya$^+$) and OK6201(Δlac crp$^-$ cya$^+$). The lactose utilizing phenotype of cells harboring each plasmid was monitored on MacConkey-lactose indicator plates. The results are shown in Table 1. Both crp$^+$ and crp$^-$ cells harboring any pPxL plasmids, which have no CRP site, gave rise to red (Lac$^+$) colonies. On the other hand several pPxSL plasmids, in which the distance between the start of transcription and the CRP site is less than 23-nt, conferred Lac$^-$ phenotype to crp$^+$ cells, while it did Lac$^+$ to crp$^-$ cells. This result argues that the synthetic CRP site could mediate inhibition of transcription by cAMP-CRP complex when placed just downstream from Px promoter. When the distance is longer than 23-nt, pPxSL plasmids conferred Lac$^+$ to both strains as with pPxL plasmids.

To examine the inhibitory effect of CRP site quantitatively, we measured the β-galactosidase activity in crp$^+$ and crp$^-$ cells harboring plasmids. We also assayed the β-lactamase activity of cells as a plasmid copy number indicator. Then we compared the normalized β-galactosidase activity (β-gal/β-lac) in crp$^+$ and crp$^-$ cells. The results are shown in Table 1. The ratio of the value of β-gal/β-lac in the crp$^-$ cells to that in the crp$^+$ cells represents the degree of the repression by cAMP-CRP bound to the CRP site. In these assays the crp$^-$ cells produced a slightly higher activity of β-galactosidase compared to the crp$^+$ cells for unknown reasons. As a result the ratios are around 1.6 even for pPxL plasmids which have no CRP site. However, it is notable that the repression ratio with pPxSL plasmids are consistently higher than those for pPxL plasmids. In addition, the ratios decrease with increasing the distance between Px and the CRP site. When the distance is less than 23-nt, the ratios are 16 to 30, indicating a strong repression of transcription by cAMP-CRP. However when the distance is longer than 23-nt, the ratios is between 3 and 5. These results imply that the synthetic CRP signal inserted downstream of promoter exerts a moderate but significant regulatory function to inhibit the transcription in

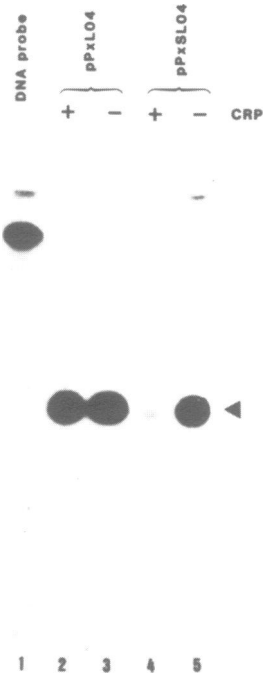


Figure 6. S1 analysis of Px RNA in cells harboring pPxL04 or pPxSL04. The 308-bp BamHI-HinfI fragment from pPxL56 labeled at its HinfI 5' end was used as a DNA probe to determine the levels of Px RNA. The DNA probe was hybridized to 50 μg of RNA from the following strains: lane 2, KI70(crp$^+$)/pPxL04; lane 3, OK6201(crp$^-$)/pPxL04; lane 4, KI70/pPxSL04; lane 5, OK6201/pPxSL04. The hybrids were treated by 10 units of S1 nuclease for 10 min and the products were fractionated on an 8% polyacrylamide-8M urea gel. Lane 1 is a DNA probe without S1 treatment. A closed triangle represents DNA bands protected from S1 digestion by Px RNA.

the presence of cAMP-CRP even when it is placed distal to the promoter.

RNA analyses by quantitative S1 mapping

To examine directly the regulatory effect of the CRP site on transcription, we conducted an S1 assay. First the 308-bp BamHI-HinfI fragment from pPxL56 ^{32}P-labeled at its 5' HinfI end was used as a DNA probe. Cellular RNA from cells carrying either pPxL04 or pPxSL04 was hybridized to the ^{32}P-labeled DNA probe and the hybridization mixture was treated with S1 nuclease. The

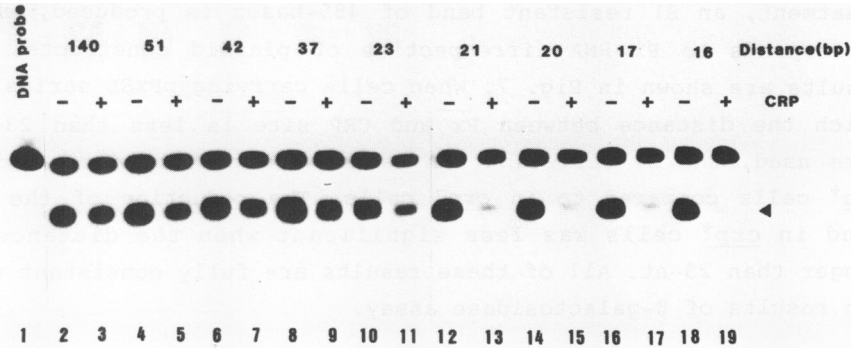


Figure 7. S1 analysis of Px RNA in cells harboring plasmids carrying the CRP site at various distances downstream from the start of transcription. The levels of Px RNA in crp$^+$ and crp$^-$ cells carrying the pPxSL series were determined by S1 analysis. The 625-bp BamHI-DdeI fragment from pMS437D labeled at its DdeI 5' end was used as a DNA probe. The DNA probe was hybridized to 30 µg of RNA from the following strains: lane 2, OK6201/pPxSL56; lane 3, KI70/pPxSL56; lane 4, OK6201/pPxSL54; lane 5, KI70/pPxSL54; lane 6, OK6201/pPxSL03; lane 7, KI70/pPxSL03; lane 8, OK6201/pPxSL21; lane 9, KI70/pPxSL21; lane 10, OK6201/pPxSL08; lane 11, KI70/pPxSL08; lane 12, OK6201/pPxSL15; lane 13, KI70/pPxSL15; lane 14, OK6201/pPxSL10; lane 15, KI70/pPxSL10; lane 16, OK6201/pPxSL09; lane 17, KI70/pPxSL09; lane 18, OK6201/pPxSL04, lane 19, KI70/pPxSL04. The hybrids were treated by 10 units of S1 nuclease for 10 min and the products were fractionated on a 6% polyacrylamide-8M urea gel. Lane 1 is a DNA probe without S1 treatment. A closed triangle represents DNA bands protected from S1 digestion by Px RNA.

products were fractionated on a 6% polyacrylamide-8 M urea gel. In this assay an S1-resistant DNA band of 95-bases, which corresponds to RNA derived from Px, is produced after S1 treatment. The intensity of DNA band which is protected against S1 digestion reflects the levels of Px RNA in cells. As shown in Fig. 6, the levels of Px RNA derived from pPxSL04 are markedly reduced in crp$^+$ compared to crp$^-$ backgrounds. On the other hand the levels of Px RNA from pPxL04 were not affected by the presence of CRP.

Next we conducted a systematic S1 assay by using the 625-bp BamHI-DdeI fragment from pMS437D ^{32}P-labeled at its DdeI 5' end and cellular RNA prepared from crp$^+$ and crp$^-$ cells carrying a series of pPxSL plasmids. In these experiments, after S1

treatment, an S1 resistant band of 485-bases is produced, which corresponds to Px RNA, irrespective of plasmid constructs. The results are shown in Fig. 7. When cells carrying pPxSL series, in which the distance between Px and CRP site is less than 23-nt, were used, the S1 resistant DNA bands were greatly decreased in *crp*$^+$ cells compared to in *crp*$^-$ cells. The reduction of the DNA band in *crp*$^+$ cells was less significant when the distance is longer than 23-nt. All of these results are fully consistent with the results of β-galactosidase assay.

DISCUSSION

Based on comparative analyses of DNA sequences known or thought to be specifically recognized by cAMP-CRP, consensus sequences for CRP binding site have been proposed by several investigators. For example, Ebright (30) reported a consensus sequence of 5'-AA-TGTGA--T----CA-3' by analyzing 11 CRP sites statistically. An improved consensus sequence of 21-bp, 5'-AA-TGTGA--T----CA-ATT-3', was given by de Crombrugghe *et al.* (2), that contains a well conserved pentamer 5'-TGTGA-3' and a less well conserved inversion of the pentamer. A recent version of the consensus sequence for CRP site was reported by Busby (3). He analyzed 26 binding sites of CRP to obtain a 22-bp consensus sequence of 5'-AA-TGTGA--T-G-TCAAATAT-3'. In addition, Berg and von Hippel have just reported a new consensus CRP sequence by analyzing 23 CRP recognition sequences and their inversions (31).

In the present study, we have re-evaluated the CRP binding sites documented by Busby. When the frequency of each of the four bases at each position was summarized, new consensus sequences, which consist of a perfect or nearly perfect palindromic 22-mer, emerged. We synthesized one of these sequences that has a perfect dyad symmetry. It is worth noting that the synthetic sequence is completely identical to the consensus sequence obtained by Berg and von Hippel (31). The synthetic CRP site was shown to be specifically recognized by cAMP-CRP *in vitro* when it was inserted into plasmids. We have observed by a gel retardation assay that the synthetic CRP site binds cAMP-CRP more tightly than do CRP sites in the natural genes such as *lac* and *cya* (unpublished results). The importance of symmetry in the sequence at CRP

binding sites for CRP binding was suggested by Kolb et al. (32, 33). More recently, Jansen et al. has shown that a completely symmetrical altered lac CRP site binds CRP better than the natural lac CRP site (34). Our observation is consistent with these previous reports and provides a further evidence for the correlation with the degree of symmetry in the CRP site and the strength of CRP binding. It is also interesting to note that a perfectly symmetric lac operator binds the lac repressor more tightly than the natural lactose operator sequence (35).

Our experiments clearly show that the CRP site placed downstream of promoter is functional in the presence of cAMP-CRP to block the transcription. The level of repression decreases with increasing distance of the CRP site from promoter. When the CRP site is placed immediately downstream from promoter (within 23-nt from the transcription start site), it exerts a strong effect. The repression level by the synthetic CRP site is enough to confer cells containing Px-lacZ fusion plasmids Lac^- phenotype. The DNase I footprinting studies show that binding of cAMP-CRP to the inserted CRP site prevent the binding of RNA polymerase to Px promoter (unpublished results). Thus in this case CRP may simply block the initial binding of RNA polymerase to the promoter resulting in inhibition of transcription. When the distance between CRP site and Px is longer than 23-nt, the inhibitory effect of CRP site reduces markedly. This means that cAMP-CRP bound to the CRP site distal to a promoter can only moderately block the action of elongating RNA polymerase.

It has been reported that the repression effect of the lac operator placed downstream of a promoter decreases with increasing distance between operator and promoter just as in our CRP system (36, 37). However, the lac operator is known to mediate efficiently transcription repression through lac repressor even when placed far downstream from a promoter (37-40). The significant difference in repression effect on ongoing RNA polymerase between two systems may be explained by the higher affinity of lac repressor to its operator than that of CRP to its binding site.

In any case, the present study indicates that cAMP-CRP can generally act as a negative regulator when bound to its DNA

target placed downstream from promoter. As we have shown previously, crp and cya genes are typical examples which transcription is regulated negatively by cAMP-CRP and its binding site situated downstream of promoter. It is reasonable to speculate that some other genes adopt the same regulatory mechanism, since it is known that cAMP and CRP affect negatively the synthesis of several proteins (41). We are currently trying to clone systematically such genes.

Recently several artificial regulatory systems have been constructed in which a DNA binding protein and its cognate DNA sequence is functional to repress transcription. For example, it was repeatedly shown that either natural or synthetic lac operators can mediate transcription repression through lac repressor when placed downstream from several promoters (36-40). Second, gal4 protein in yeast, that is known as a transcriptional activator, was shown to repress the lacZ expression in E. coli when the lac operator site is replaced by the gal4 binding site (42). Similarly, it was shown that the GC box placed just downstream of the promoter can efficiently block the transcription in E. coli cells when the GC box binding protein Sp1 is produced in the same cells (43). On the other hand, it has been shown that suitably engineered lac repressor-operator systems are functional in mammalian cells just as in E. coli (44-46). Likewise, the Tn10 encoded tet repressor-operator system has been successfully used to regulate transcription in plant cells (47). These imply that any sequence specific DNA-binding proteins, including those known as activators, could act as a repressor when their target sites are located around or downstream of a promoter in both prokaryote and eukaryote cells.

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