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Tandem CRP binding sites in the *deo* operon of *Escherichia coli* K-12

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The locations of DNA binding by the cyclic AMP receptor protein (CRP) in the *deo* operon of *Escherichia coli* have been determined by the DNase I footprinting procedure. Two high affinity sites were found around positions −35 and −90, preceding the second *deo* promoter. *In vitro* data on induction of gene fusions that join different parts of the *deo*P-2 regulatory region to the *lac* genes suggest that: (1) both CRP binding sites are needed for high expression from the *deo*P-2 region; and (2) negative regulation by the *cytR* repressor is accomplished by preventing the cAMP-CRP complex from binding to the second target.

Key words: CRP targets/DNA sequence/footprinting/gene fusions/ gene regulation

Introduction

In bacteria such as *Escherichia coli* and *Salmonella typhimurium*, the complex of adenosine 3',5'-monophosphate (cAMP) and the cAMP receptor protein (CRP) activates the expression of certain genes concerned with transport and utilization of energy sources. The complex of cAMP and CRP binds to a region of the DNA near the beginning of a regulated gene and enhances transcription of the gene. It is suggested that cAMP serves as the key component in a super control system, which indicates to the cell a deficiency in the fuel/carbon supply, and within this control system a hierarchy of promoters exists with different affinities for cAMP/CRP (Alper and Ames, 1978).

Since the levels of cAMP required to induce different genes controlled by cAMP/CRP are not identical, it might be expected that only part of the DNA sequence of different CRP binding sites is conserved. In accordance with this prediction, the CRP target has been found to vary for several genes, not only with respect to the DNA sequences and symmetry characters but also with respect to the distance between the binding site and the start of transcription (Reznikoff and Abelson, 1978; Simpson 1980; Taniguchi *et al.*, 1979; Ogden *et al.*, 1980; Lee *et al.*, 1981; Queen and Rosenberg, 1981; Le Grice *et al.*, 1982). In *lac*, CRP binds between −74 and −50; in *ara* between −106 and −82; and in *gal, cat* and in promoter P-4 on the plasmid pBR322 it binds around −40.

Thus, cAMP/CRP is able to activate transcription from at least three different locations. This variation is further extended by the finding that cAMP/CRP can act not only as a positive effector but also as a negative regulator of gene expression. In the *gal* operon, one of two overlapping promoters is activated; the other is inhibited by the complex (Adhya and Miller, 1979), and expression of the *ompA* gene *in vitro* is likewise inhibited by cAMP/CRP (Movva *et al.*, 1981).

Recently, we have shown that expression of the *deo* operon of *E. coli*, which encodes nucleoside and deoxynucleoside-catabolizing enzymes, occurs from two promoter regions termed P-1 and P-2 located 600 bp apart from each other (Valentin-Hansen *et al.*, 1982a). Initiation of transcription from P-2 is strictly depended on cAMP and negatively controlled by the *cytR* and *deoR* repressors (Hammer-Jespersen and Munch-Petersen, 1975; Jørgensen *et al.*, 1977; Valentin-Hansen *et al.*, 1982a). In this report I demonstrate by using the DNase I 'footprinting method' (Galas and Schmitz, 1978) that two high affinity CRP binding sites are located around −40 (CRP-1) and −95 (CRP-2) base pairs preceding the P-2 transcription initiation site. Evidence is presented, which strongly suggests that both sites are needed for full expression at P-2 and that the *cytR* operator presumably overlaps with CRP site 2.

Results

Footprinting

The binding of proteins to DNA can be detected by the sensitive DNase I footprinting technique described by Galas and Schmitz (1978). End-labeled DNA fragments are partially degraded with DNase I in the presence and absence of the binding protein, and protected regions are visualized by electrophoresis and autoradiography. Since DNase I cleavage involves some sequence specificity, some sites are cleaved more frequently than other sites in the DNA sequence (Bernardi *et al.*, 1975).

Figure 1 shows the 'footprint' of CRP on the P-2 regulatory region. An *Ava*II-*Dde*I restriction fragment (shown at the top of the figure), containing 280 bp before the start site for P-2 and 112 bp after it, was ^{32}P-labeled at the 5' end of the *Ava*II terminus. The nucleotide sequence of the P-2 regulatory region, which has recently been determined (Valentin-Hansen *et al.*, 1982a), is also shown in Figure 1. Aliquots of the labeled fragment were incubated with various concentrations of cAMP and a fixed amount of CRP and partially digested with DNase I. The location of protected regions was then determined on sequencing gels by parallel runs of the purine sequence reaction (Lane 1) of the fragment and the DNase digestions (Lanes 2-4).

Lanes 2-4 show the results of digesting the fragment with DNase I in the presence of CRP (Lane 2); and in the presence of CRP and, respectively, 10 and 20 μM cAMP (Lanes 3-4). cAMP/CRP protects two DNA stretches of ~25 bases against DNase I digestion (around −40 and −90) (Lanes 3-4). Within the protected regions are fragments, whose rate of production is enhanced. This is a common phenomenon in the footprinting procedure and is most likely an effect of protein-induced change in the DNA structure. Specific binding of CRP is completely cAMP-dependent, since CRP without cAMP has no general effect upon the digestion pattern.

Thus, two 'high' affinity CRP binding sites seem to be present in the *deo*P-2 regulatory region, and the targets show considerable homology with other known CRP sites, both with respect to sequence and position, as will be discussed below.

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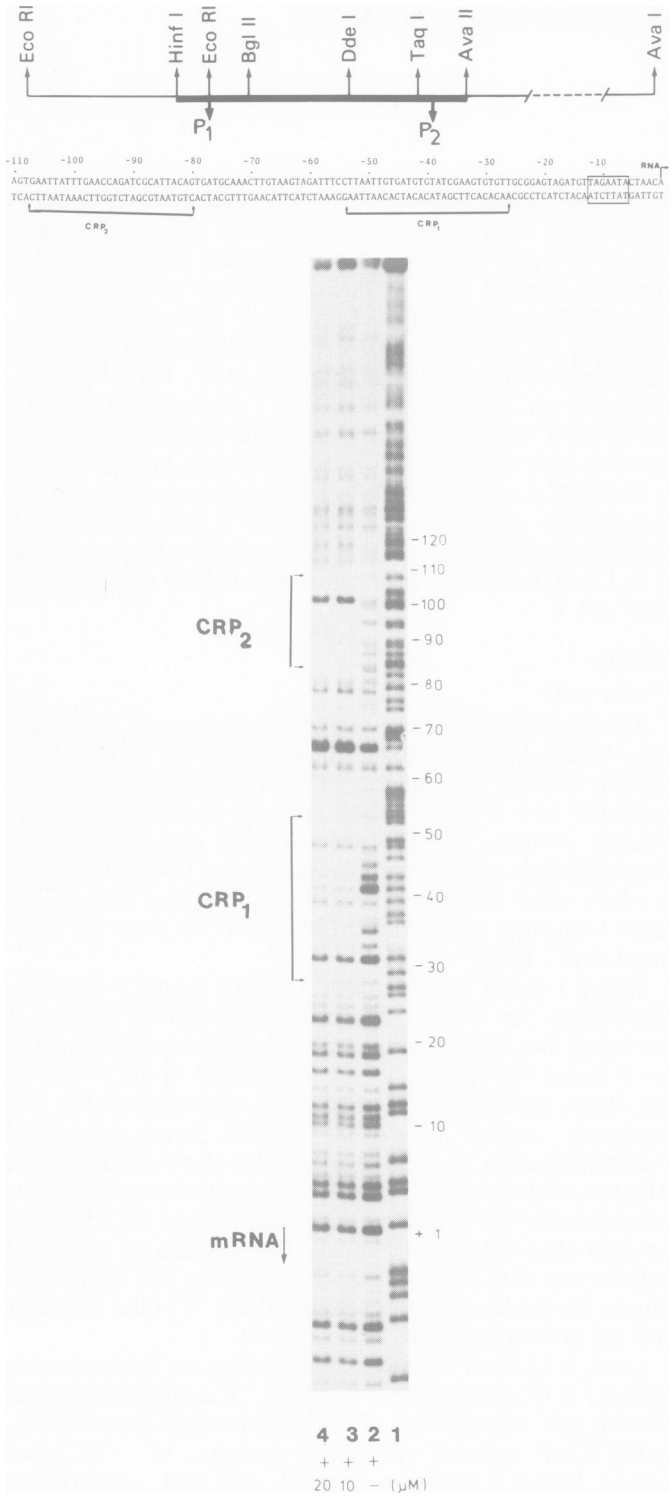

Fig. 1. Protection of the *deo*P-2 regulatory region by CRP. Shown at the top is a partial restriction map of the *deo* DNA present in plasmid pVH17, along with the DNA sequence of the P-2 promoter region (Valentin-Hansen *et al.*, 1982). The 806-bp *Hinf*I-*Ava*II fragment, spanning the *deo* regulatory region, is represented by a thick bar. The *deo* promoters (P-1 and P-2) and the start point for mRNA-2 are indicated by arrows. The autoradiograph shows the effect of CRP (10 μg/ml) and increasing concentrations of cAMP upon digestion by DNase I (13 μg/ml) of the *Ava*II-*Dde*I restriction fragment (~0.2 μg/ml) spanning the P-2 promoter region (5′ labeled with ^{32}P at the *Ava*II end). The fragment was incubated with DNase I for 30 s in the presence of CRP alone (**Lane 2**); CRP and cAMP (**Lanes 3–4**). The nucleotide regions protected by CRP are bracketed, and the sequences are numbered with respect to the P-2 RNA start site (+1). **Lane 1**, purine-specific chemical cleavage of the *Dde*I/*Ava*II [^{32}P]fragment.

Protein fusions and in vitro regulation

To elucidate the role of the *deo* CRP sites on gene expression, fusions were constructed that join the *lac* structural genes to the transcriptional and translational regulatory signals of the *deo*P-2 region. As detailed in Figure 2, two different *deo* promoter fragments were cloned into the plasmid pMC1403 (Casadaban *et al.*, 1980). This plasmid has unique *Eco*RI, *Sma*I, and *Bam*HI endonuclease cleavage sites adjacent to the eighth codon of *lacZ* but no transcriptional-translational start sites for the *lac* genes. In plasmid pVH1451, the *Ava*II-*Dde*I fragment used in the protection experiment was inserted into the *Sma*I site of pMC1403 by filling in the protruding ends of the *deo* fragment with DNA polymerase I. In plasmid pVH1424, a *Sau*3A fragment extending from position +53 to −93 was joined (see Figure 2) to the *Bam*HI site of pMC1403. Based on the nucleotide sequence and the known start sites for *deo* transcription and translation (Valentin-Hansen *et al*;. 1982a, 1982b), the hybrid β-galactosidase gene fusions should result in the synthesis of two polypeptides consisting of: (A; pVH1424) the first three amino acids of the deoxyriboaldolase (*deoC*) followed by the β-galactosidase polypeptide (minus its first seven amino acids), and (B; pVH1451) the first sixteen amino acids from the *deo*C gene product followed by two amino acids from the *Sam*I-*Bam*HI sequence of pMC1403 and then β-galactosidase. The sequence of the *deo* regulatory region in pVH1451 and pVH1424, and the *Sau*3A-*Bam*HI and *Ava*II-*Sma*I joints

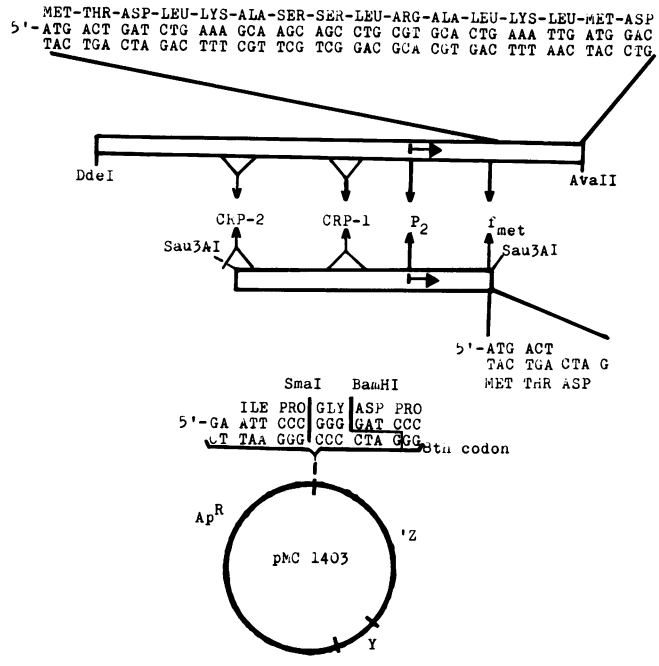

Fig. 2. Construction of fusions between the *deo*P-2 regulatory region and the *lac* operon. Shown at the top are the *deo* restriction fragments inserted into the unique *Sma*I and *Bam*HI cleavage sites of vector pMC1403. The start site for P-2 mRNA synthesis, the start of the *deo*C gene (fmet) and the position of CRP binding sites are indicated by arrows. The nucleotide sequences of the amino-terminal ends of the *deoC* gene product and the chemically synthesized *Eco*RI-*Sam*I-*Bam*HI sequence preceding the 8th codon of the *lacZ* gene are given. The *Dde*I-*Ava*II fragment was inserted into the *Sma*I site of pMC1403, after filling in the protruding ends with DNA polymerase I, to form pVH1451. Plasmid pVH1424 was obtained by ligating the *Sau*3A fragment to *Bam*HI restricted pMC1403 DNA. The nucleotide sequence of the inserted *deo* fragments and the *Ava*II-*Sma*I and *Sau*3A-*Bam*HI joints was directly confirmed by DNA sequence analysis (see Figure 3).

was directly confirmed by DNA sequence analysis (Figure 3 and Materials and methods). Plasmid pVH1451 contains both CRP binding sites, whereas pVH1424 contains only an intact CRP-1 region, since *Sau*3A cuts within the CRP-2 region (Figure 1).

Expression of the *deoA* gene of plasmid pVH11 (the second gene in the *deo* operon) was used as a control system for studying the expression of the *deoC-lacZ* gene fusions in an *in vitro* protein synthesizing system. The plasmid pVH11 contains only an intact *deo*P-2 region and the two first *deo* genes (Jørgensen *et al.*, 1977). Expression of the *deoCA* genes from the P-2 promoter is known to be controlled positively by cAMP/CRP and negatively by the *cytR* repressor, the inducer being cytidine (Jørgensen *et al.*, 1977; Valentin-Hansen *et al.*, 1978). The induction pattern for thymidine phosphorylase, the *deoA* gene product, and the 'β-galactosidases' in a *cytR*$^+$, *deoR*$^+$ S-30 extract is shown in Table I.

The stimulation of β-galactosidase synthesis by cAMP and cytidine is similar to that of thymidine phosphorylase when pVH1451 and pVH11 are used as templates. cAMP stimulates synthesis of both enzymes ~15-fold in the absence of cytidine (repressed situation) and ~200-fold in the presence of cytidine (derepressed situation). The same results have been obtained with a fusion similar to pVH1451, but deleted for the *deo* DNA preceding the second CRP target (data not shown). Thus, the observed regulation of the *lacZ'* gene in pVH1451 agrees well with the regulation of the *deoC* gene in plasmid pVH11. In contrast, when pVH1424 is used as template the expression of 'β-galactosidase' is no longer regulated by the *cytR* repressor. The level of 'β-galactosidase' produced remains about the same whatever cytidine is present or absent in incubations containing cAMP. Furthermore, both the level of 'β-galactosidase' produced and the response to cAMP in the presence of cytidine, are much lower with pVH1424 than with pVH1451 as template (Table I). It should be noted that the low level of 'β-galactosidase' produced from pVH1424 *in vitro* might not solely reflect promoter strength, since only the first three codons from the *deoC* gene are present in this fusion. Recently, it has been proposed that base changes at the beginning of a cistron can change the rate of translational initiation by affecting RNA secondary structure (Iserentant and Fiers, 1980; Schwartz *et al.*, 1981). However, taking into consideration only the response of the low molecular effectors (cAMP/cytidine) and not the actual levels of 'β-galactosidase', the results clearly indicate that the *lacZ'* gene in plasmid pVH1424 is expressed from a *deo*P-2 regulatory

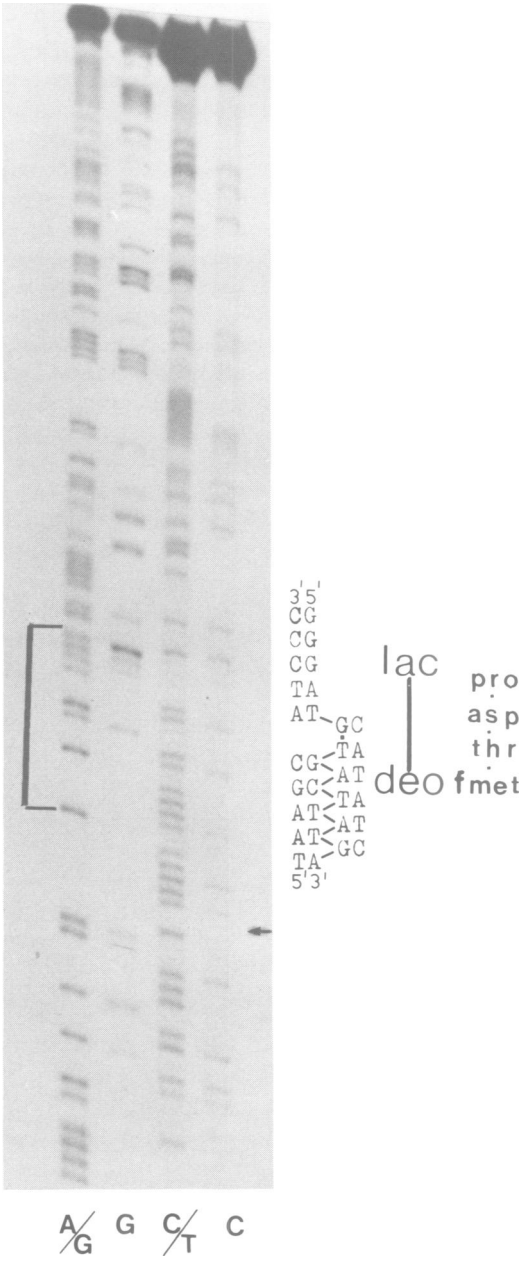

Fig. 3. The nucleotide sequence of the *Sau*3A-*Bam*HI joint of plasmid pVH1424. The plasmid was 3' end labeled by DNA polymerase I and [α-^{32}P]dATP at the unique *Eco*RI restriction site, 10 bp upstream of the *Sau*3A-*Bam*HI joint (Figure 2), and digested with *Pvu*II (a *Pvu*II site is present 78 bp downstream of the 8th codon in *lacZ*). The labeled *Eco*RI-*Pvu*II *deo-lac* fragment was then subjected to the sequencing procedure of Maxam and Gilbert (1980). An *Eco*RII site at position +33 in the *deo* sequence is indicated by an arrow. The nucleotide sequence of the *deoC-lacZ* joint is bracketed, the sequence of the antisense strand is:

5'-TGAAATG ACT GAT CCC-3'

Met- Thr- Asp- Pro- 8th codon of *lacZ*.

Table I. Induction pattern of thymidine phosphorylase and β-galactosidase *in vitro* using pVH11, pVH1451, and pVH1424 as templates in wild-type S-30 extracts (*cyt*R$^+$, *deo*R$^+$)

DNA template	pVH11	pVH1451	pVH1424
Enzyme synthesized	Thymidine phosphorylase	β-galactosidase	
Effector added			
–	5	0.06	0.0008
Cytidine	7	0.08	0.0010
cAMP	80	1.0	0.016
cAMP + cytidine	950	9.0	0.020

Thymidine phosphorylase and β-galactosidase are synthesized from different templates using an S-30 extract from S0928 (*cyt*R$^+$, *deo*R$^+$, Δ*lac*, Δ*deo*) in the presence of various combinations of effectors. See Materials and methods for conditions for protein synthesis and measurements of enzyme activities.

The amount of thymidine phosphorylase is expressed as units enzyme-synthesized for 40 min per 100 μl *in vitro* synthesis mixture, whereas β-galactosidase is expressed as ΔOD_{420}/10 min according to Zubay (1973). The actual DNA concentrations are: pVH11, 0.2 μg/ml; pVH1424 and pVH1451, 0.5 μg/ml.

The concentration of effectors is: cytidine, 100 μM; cAMP, 250 μM. Tetrahydrouridine (200 μM) is added to prevent deamination of cytidine when cytidine is present (Hanze, 1967).

region missing an active *cytR* operator and partially defective in its cAMP/CRP control system. The results obtained with plasmid pVH1424 further suggest that the CRP-1 target also contributes to enhance expression, since more than half of the CRP-2 site has been deleted in this fusion. Thus, the *in vitro* data confirm the functional importance of both regions identified by 'footprinting' and suggest that a crucial role of both is to determine the rate at which RNA polymerase initiates transcription.

The control region of P-2

So far, the operator site for the *cyt*R repressor has not been identified. However, in light of the information described above, we now feel that the repression of P-2 transcription by the *cytR* product can be described by a model in which the repressor affects binding of the cAMP-CRP complex at CRP-2. Examination of the DNA sequence shows that a region with high degree of symmetry overlaps partially with CRP-2 from the base pairs −96 to −66 (ACCAGATCGCATTACAGTGATGCAAACTTGT). Thus, it is tempting to hypothesize that this region represents the *cytR* operator, and the repressor might act simply as an antagonist to binding of cAMP/CRP at site-2 (Figure 4), or it may interfere with RNA polymerase if tandem binding sites are present for this protein.

Recently we have shown that the regulation of expression from the P-2 region is even more complex, since initiation of transcription is controlled negatively, not only by the *cytR* repressor but also by the *deoR* repressor, the target being the near perfect repeat of a 16-bp palindrome, which encompasses the Pribnow box sequence. The *deoR* gene product controls transcription from both *deo* promoters (Valentin-Hansen *et al.*, 1982a). However, repression at P-2 by the *deo*R repressor both *in vivo* and *in vitro* is 'relatively' weak (manuscript in preparation). The model for *deo*P-2 regulation is outlined in Figure 4.

Discussion

One approach to extracting the relevant information of CRP binding sites is to compare the different targets for conserved sequences and adjust the obtained information with known mutations and enzymatic and chemical protection studies. This approach has led to several models for how CRP might interact and for the structure of the interaction sites (Gilbert, 1976, O'Neill *et al.*, 1981; McKay and Steitz, 1981; Queen and Rosenberg, 1981; Ebright, 1982). Ebright has proposed a 10-bp consensus: 5′-AANTGTGANNTNNNNCA-3′, as the recognition sequence for the CRP protein. This consensus sequence includes and extends the model for the CRP binding site proposed by O'Neill *et al.* (1981) in which it is postulated that the two subunits of the CRP dimer recognize a core of two inverted repeats, which overlap each other; the core recognition sequence being:

5′-TGTCACA-3′
3′-ACAGTGT-5′

The two CRP binding sites in *deo* are in nearly perfect agreement with the 10-bp consensus proposed by Ebright, i.e., a fit of 9 out of 10 for the proximal site (from −51 to −35) and 8 out of 10 for the distal site (from −83 to −99) (see Figure 5). However, comparison of the *deo* sites with the other known CRP sites (Figure 5) suggests that the cAMP-CRP complex recognizes not only the consensus described by Ebright but two specific 8 bp regions separated by a region of 6 bp. The evidence for the preceding statement may be summarized as follows.

There is a striking similarity between part of the sequences of the different binding sites. In *ara* and *gal* the sequence GTCACACTTT is located in the right-hand site of the protected regions – when oriented with respect to the Pribnow box – whereas the sequence ATTATTTG$^{A}/_{C}$AC is present in the left part of the *ara* and *deo* P-2 sites. The highly conserved regions in *ara* and *deo* site 2 are located exactly in the same position relatively to the start sites of *ara* BAD and *deo* P-2 RNA synthesis. These data strongly suggest that two separate regions on the DNA helix are recognized by CRP, and this assumption is further supported when the known CRP binding sites are compared. In Figure 5, these sites are aligned with respect to the conserved regions and the frequency with which a given base appears in the conserved regions is tabulated.

All sites consist of the sequence $^{T}/_{A}$ $^{T}/_{A}$ $^{A}/_{T}$ T $^{T}/_{G}$ T G $^{A}/_{C}$ N_6 T C $^{G}/_{A}$ C A C T $^{T}/_{G}$ or a sequence differing by two bases. The two conserved regions are arranged around a center of symmetry (Figure 5) – suggesting that the symmetric elements may correspond to symmetric features of the CRP dimer. Also the CRP target of the *omp*A promoter shows this sequence homology (Movva *et al.*, 1981), whereas the right hand site of the *cat* target contains four changes from the consensus (Le Grice *et al.*, 1982). Each CRP-binding site includes (on one side) the consensus sequence 5′-AANTGTGANNTNNNNCA-3′ (Ebright, 1982), or a sequence differing by two bases, plus (on the other side) a related but more variable sequence, differing by two to five bases.

Chemical protection studies of the *ara* and *lac* sites as well as mutational data suggest that cAMP/CRP recognizes two regions on the DNA helix. Four CRP target mutations in *lac* and *gal* have been sequenced (the bases changed are boxed in Figure 5). All of them change highly conserved bases in the consensus sequence (Dickson *et al.*, 1977; Reznikoff and Abelson, 1978; Busby *et al.*, 1982).

Comparison of the different cAMP/CRP targets has, as discussed, revealed certain structural homologies; however,

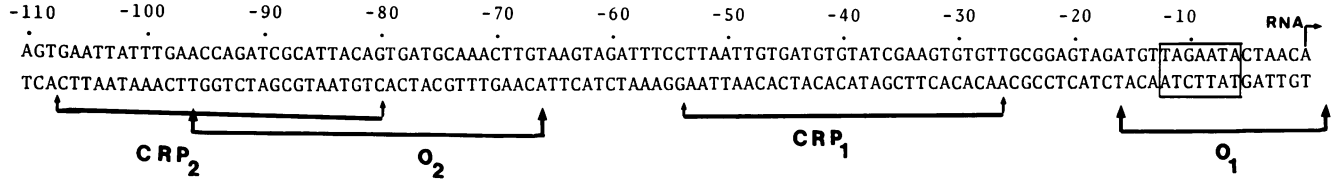

Fig. 4. The DNA sequence, the operator sites, the CRP binding sites and the transcription start point in the *deo*P-2 regulatory region. O_1 is the site recognized by the *deo*R repressor, CRP_1 and CRP_2 the cAMP/CRP targets, and we presume that O_2 is the *cytR* operator. The Pribnow heptamer is boxed, and +1 corresponds to the start site of P-2 mRNA.

how sequence-specific recognition occurs and how cAMP/CRP modulates the rate of promoter utilization is still far from clear. The difficulties in developing a general model for binding and function of cAMP/CRP seems mainly to be due to the structural and regulatory differences which occur among regulatory regions. Any comparison is limited by considerations such as the fact that some regulatory regions also contain recognition sites for other sequence-specific proteins than CRP and RNA polymerase; the presence of multiple promoters; and that cAMP/CRP can influence initiation of transcription positively or negatively. In *gal*, for example, one of two overlapping promoters is inhibited by cAMP/CRP, the other activated; whereas in *ara* two divergently oriented promoters are stimulated by cAMP/CRP from a single target and activation of one of the promoters takes place only in the presence of the *ara*C protein in its inducing conformation. Thus, it remains extremely difficult to correlate the structural differences or similarities between these targets, and it is clear that modulation of transcription by cAMP/CRP is a much more subtle and intricate process than would be expected.

In summary, I have shown by the footprinting procedure that tandem high affinity cAMP/CRP binding sites are located in the *deo*P-2 regulatory region. *In vitro* data on gene fusions as well as homology with other known CRP sites, both with respect to DNA sequence and position, strongly indicate that both CRP target sites are needed for high expression from the P-2 promoter. The presence of two distinct CRP targets in the promoter region of *lac* (Schmitz, 1981), *ara* (Lee *et al.*, 1981), and *cat* (Le Grice *et al.*, 1982) has been described. However, both in *ara* and *lac*, one of the sites is a non functional low-affinity target and *in vitro* data suggest that the two CRP targets in *cat* regulate two distinct promoters. To what extent each of the CRP targets in the *deo*P-2 regulatory region contributes to enhance transcription is still unknown, but experiments to define the features of the *deo*P-2 regulatory region are currently in progress.

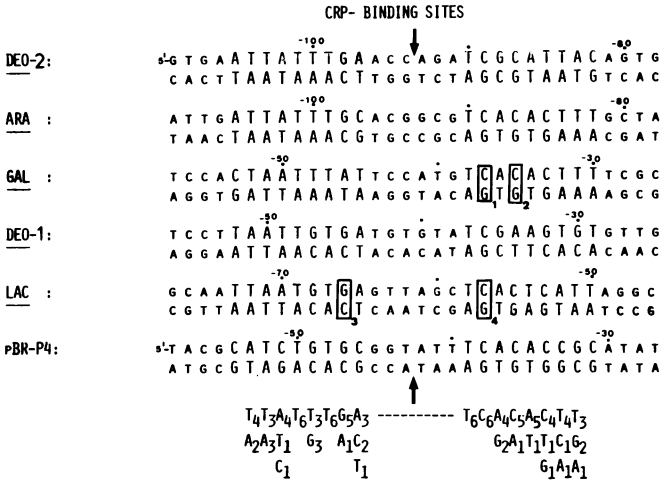

Fig. 5. DNA sequences of the six known cAMP/CRP targets. Numbers given on the anti-sense strands refer to nucleotide positions relative to the start sites of CRP-dependent mRNA synthesis. The sequences have been oriented to reveal the similarities of the conserved regions. The arrows indicate the axis of partial two-fold symmetry in each site. The frequency with which a given base appears in the conserved regions is tabulated. Base pairs changed by mutations that decrease repressor affinity are boxed. The subscript corresponds to the following base changes (see text): (1) $^{C}/_{G} \rightarrow {}^{T}/_{A}$; (2) $^{C}/_{G} \rightarrow {}^{G}/_{C}$; (3) $^{G}/_{C} \rightarrow {}^{A}/_{T}$; (4) $^{C}/_{G} \rightarrow {}^{T}/_{A}$.

Materials and methods

Restriction endonucleases were either purchased from commercial suppliers (BRL, New England Biolabs) or prepared by published procedures. Conditions for restriction endonuclease digestions were those recommended by commercial suppliers. T4 DNA ligase, *E. coli* DNA polymerase I (large fragment), bacterial alkaline phosphatase and T4 polynucleotide kinase were obtained from BRL or New England Biolabs, and γ-^{32}P- and α-^{32}P-labeled nucleotide triphosphates from I.C.N. Corp. CRP was a generous gift from Dr. J. Krakow.

Isolation of plasmid DNA, cloning, transformation of *E. coli* with plasmid DNA, and gel analysis of recombinant plasmids have been described previously (Jørgensen *et al.*, 1977). Growth of cells and preparation of S-30 extracts, conditions for cell-free synthesis and assay of enzyme activities were described in Valentin-Hansen *et al.*, 1979.

Strain *E. coli* SO063, deleted for the *deoC* gene, was used for plasmid transformation and DNA cloning. Only the *deoC* (deoxyriboaldolase) gene needs to be introduced and expressed for the utilization of deoxyribonucleosides. Strains MC1000 or CSH50, deleted for the *lac* genes, were used for cloning of *lacZ* gene fusions.

Construction of plasmids

Plasmid pVH11 was constructed as follows: a total of 2 μg each of pBR322 and λ*ddeo* CABD (Jørgensen *et al.*, 1977) were digested with restriction endonucleases *Eco*RI and *Hind*III and then heated for 10 min at 70°C. The sample was ligated with T4 ligase and transformed into *E. coli* strain SO063 (*deoC*$^-$). Plasmid pVH11 was isolated from ampicillin-resistant *deoC*$^+$ transformants selected on AB minimal medium supplemented with 0.1% thymidine as carbon source.

Plasmids pVH1424 and pVH1425 were constructed in the following way: a purified *Bgl*II-*Ava*II fragment, spanning the *deo*P-2 regulatory region (see below), was digested with *Dde*I or *Sau*3A. The *Sau*3A fragments were ligated with *Bam*HI restricted plasmid pMC1403 (Casadaban *et al.*, 1980), whereas the *Ava*II-*Dde*I fragment, after filling in the protruding ends with DNA polymerase I (*E. coli* large fragment), was ligated with *Sma*I restricted plasmid pMC1403. The ligated DNA was transformed into *lac* deletion strains. Cells harbouring the resulting plasmids, pVH1424 and pVH1451, were selected as Lac$^+$Ap^r colonies using lactose-McConkey indicator agar containing ampicillin, 50 μg/ml. Cells containing these plasmids appeared bright red on the indicator plates; however, cells containing pVH1451 make considerably more β-galactosidase than cells harbouring pVH1424.

DNA sequencing, labeling procedures, and DNase I protection

The chemical method of Maxam and Gilbert (1980) was used with the modifications described in Valentin-Hansen *et al.* (1982a). DNA fragments for sequencing were either labeled at the 5′ end of strands by polynucleotide kinase or at the 3′ end of strands by DNA polymerase (*E. coli* large fragment). Plasmid pVH1451 was labeled at the unique *Eco*RI or *Bam*HI sites flanking the inserted *deo* fragment. The labeled *Eco*RI-*deo*-*Bam*HI fragment was then isolated from polyacrylamide gels. Plasmid pVH1424 was labeled as described in Results. The labeled fragment used in the footprinting procedure was obtained from a purified *Bgl*II-*Ava*II *deo* fragment (see below). This fragment was labeled at its 5′ ends, digested with *Dde*I, and applied to a 6% polyacrylamide gel. The uniquely 5′ end labeled *Dde*I-*Ava*II fragment was purified from gel slices by electroelution. DNase I studies were carried out by the method Galas and Schmitz (1978).

Isolation of DNA fragments

All fragments used for DNase protection and cloning were obtained from plasmid pVH17 (Valentin-Hansen *et al.*, 1982a). The plasmid was digested with *Bgl*II and *Ava*II and applied to a 5% polyacrylamide gel. The appropriate *Bgl*II-*Ava*II fragment, which spans the *deo*P-2 regulatory region (Valentin-Hansen *et al.*, 1982a), was electroeluted from the gel slices and applied to a 100 μl DEAE-52 (Whatman) column equilibrated with 20 mM Tris/HCl, pH 7.6, 250 mM NaCl and 5 mM EDTA. DNA was eluted with 1 ml 1 M NaCl, 15% EtOH and precipitated with 96% EtOH. This purified fragment was used in the cloning and DNase I protection procedures. All DNA fragments used for DNA sequencing were also electroeluted from gels and further purified on small column (100 μl) of BND cellulose (Serva) using the buffers described for DEAE-52 columns.

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References

Adhya,S., and Miller,W. (1979) *Nature,* **279**, 492-494.
Alper,M.D., and Ames,B.N. (1978) *J. Bacteriol.,* **133**, 149-157.
Bernardi,A., Gaillard,C., and Bernardi,G. (1975) *Eur. J. Biochem.,* **52**, 451-457.
Busby,S.J.W., Aiba,H., and de Crombrugghe,B. (1982) *J. Mol. Biol.,* **154**, 197-209.
Casadaban,M.J., Chou,J., and Cohen,S.N. (1980) *J. Bacteriol.,* **143**, 971-980.
Dickson,R.C., Abelson,J., Johnson,R., Reznikoff,W.S., and Barnes,W.M. (1977) *J. Mol. Biol.,* **111**, 65-75.
Ebright,R.H. (1982) in Griffer,J., and Duax,W. (eds.), *Molecular Structure and Biological Function,* North Holland Elsevier, NY, in press.
Galas,D.J., and Schmitz,A. (1978) *Nucleic Acids Res.,* **5**, 3157-3170.
Gilbert,W. (1976) in Losick,R., and Chamberlin,M. (eds.), *RNA polymerase,* Cold Spring Harbor Laboratory Press, NY, pp. 193-205.
Hammer-Jespersen,K., and Munch-Petersen,A. (1975) *Mol. Gen. Genet.,* **137**, 327-335.
Hanze,A.R. (1967) *J. Am. Chem. Soc.,* **89**, 6720-6725.
Iserentant,D., and Fiers,W. (1980) *Gene,* **9**, 1-12.
Jørgensen,P., Collins,J., and Valentin-Hansen,P. (1977) *Mol. Gen. Genet.,* **155**, 93-102.
Lee,N., Geilow,W.O., and Wallace,R.G. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 752-756.
Le Grice,F.J., Matzura,H., Marcoli,R., Iida,S., and Bickle,T.A. (1982) *J. Bacteriol.,* **150**, 312-318.
Maxam,A., and Gilbert,W. (1980) in Grossman,L., and Moldave,K. (eds.), *Methods in Enzymbology,* vol. **65**, Academic Press, NY, pp. 499-560.
McKay,D.B., and Steitz,T.A. (1981) *Nature,* **290**, 744-749.
Movva,R., Green,P., Nakamura,K., and Inouye,M. (1981) *FEBS Lett.,* **128**, 186-190.
Ogden,S., Haggerty,D., Stoner,C.M., Kolodrubetz,D., and Schleif,R. (1980) *Proc. Natl. Acad. Sci. USA,* **77**, 3346-3350.
O'Neill,M.C., Amass,K., and de Crombrugghe,B. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 2213-2217.
Queen,C., and Rosenberg,M. (1981) *Nucleic Acids Res.,* **9**, 3365-3377.
Reznikoff,W.S., and Abelson,J. (1978) in Miller,J.H., and Reznikoff,W.S. (eds.), *The Operon,* Cold Spring Harbor Laboratory Press, NY, pp. 221-244.
Schmitz,A. (1981) *Nucleic Acids Res.,* **9**, 277-292.
Schwartz,M., Roa,M., and Débarbouillé,M. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 2937-2941.
Simpson,R. (1980) *Nucleic Acids Res.,* **8**, 759-767.
Taniguchi,T., O'Neill,M., and de Crombrugghe,B. (1979) *Proc. Natl. Acad. Sci. USA,* **76**, 5090-5094.
Valentin-Hansen,P., Svenningsen,B., Munch-Petersen,A., and Hammer-Jespersen,K. (1978) *Mol. Gen. Genet.,* **159**, 191-202.
Valentin-Hansen,P., Hammer-Jespersen,K., and Buxton,R.S. (1979) *J. Mol. Biol.,* **133**, 1-17.
Valentin-Hansen,P., Aiba,H., and Schümperli,D. (1982a) *EMBO J.,* **1**, 317-322.
Valentin-Hansen,P., Boëtius,F., Hammer-Jespersen,K., and Svendsen,I. (1982b) *Eur. J. Biochem.,* **125**, 561-566.
Zubay,G. (1973) *Annu. Rev. Genet.,* **7**, 267-287.