Alterations in the binding site of the cyclic AMP receptor protein at the *Escherichia coli* galactose operon regulatory region

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Gene manipulation techniques have been used to alter the binding site for the cyclic AMP–cyclic AMP receptor protein complex (cAMP–CRP) at the regulatory region of the *Escherichia coli* galactose (*gal*) operon. The effects of these changes on CRP-dependent stimulation of expression from the *galP1* promoter *in vivo* have been measured, and gel binding assays have been used to measure the affinity of cAMP–CRP for the modified sites. Firstly we have deleted progressively longer sequences from upstream of the *gal* CRP site in order to locate the functional limit of the site. A deletion to –49, removing the first base that corresponds to the consensus sequence for a CRP binding site, is sufficient to reduce CRP binding and block CRP-dependent stimulation of *P1*. Secondly, we used synthetic oligonucleotides to invert the asymmetric nucleotide sequence at the *gal* CRP binding site or to make the sequence symmetric. Inversion of the site has little effect on CRP binding, the architecture of open complexes at *P1* revealed by DNAase I footprinting, or the stimulation of transcription from *P1*. Making the site symmetric increases the affinity for CRP by over 50-fold and leads to increased transcription from *P1*, whilst hardly altering the DNAase I footprint of open complexes. Our results confirm that the strength of binding of CRP depends on the nature of the site and show that it is this that principally accounts for differences in CRP-dependent stimulation of transcription.

INTRODUCTION

The cyclic AMP receptor protein (CRP) of *Escherichia coli* allows the regulation of transcription initiation in response to certain stresses, for example, lack of glucose in the growth medium (reviewed by Ullmann & Danchin, 1983). In such growth conditions the intracellular level of cyclic AMP is raised and it binds to CRP, triggering a conformational change in this dimeric protein that allows specific binding to DNA sequences upstream of a number of transcription startpoints, thus regulating initiation at these sites (reviewed by de Crombrughe *et al.*, 1984). Extensive studies of CRP binding to a number of these sites show that the CRP dimer covers at least 22 base pairs, and comparison of the nucleotide sequence of known CRP binding sites suggests the symmetric consensus sequence:

where – represents any nucleotide (Ebright *et al.*, 1984a, 1987; Busby, 1986; Liu-Johnson *et al.*, 1986). Analyses using both X-ray and genetic methods strongly suggest that each of the two subunits of the CRP dimer contacts just one half of the sequence, and that α -helices protruding from each subunit make intimate contacts with bases at either positions 5–8 or 15–18 (Ebright *et al.*, 1984b, 1987; Weber & Steitz, 1984; de Crombrughe *et al.*, 1984; Gent *et al.*, 1987; Jansen *et al.*, 1987).

One of the best characterized cases where CRP acts is the regulatory region of the *E. coli* galactose operon which contains two overlapping promoters, *P1* and *P2* (Musso *et al.*, 1977). In the absence of any transcription activator, *gal* operon expression is due to *P2*; however, increases in intracellular cyclic AMP levels allow CRP to switch transcription from *P2* to *P1* (Aiba *et al.*, 1981). To

study CRP-dependent activation of *P1* in the absence of *P2*, we have exploited point mutations that inactivate *P2*, leaving *P1* as the sole functional promoter (Bingham *et al.*, 1986). Interestingly, in the absence of *P2*, *P1* can function without any activator but transcription initiation is stimulated 4–5-fold by cAMP–CRP (Buc *et al.*, 1986; Busby *et al.*, 1987).

The CRP binding site at *galP1* is located from bp –52 to –31 with respect to the transcription start site (Kolb *et al.*, 1983a,b). Fig. 1(a) compares the nucleotide sequence with the consensus sequence for CRP binding sites. Interestingly, although the downstream half of the sequence corresponds well to the consensus, the upstream half corresponds poorly. The CRP binding site at *galP1* is thus highly asymmetric, in sharp contrast with the consensus sequence, and to sites such as that at the lactose promoter (de Crombrughe *et al.*, 1984). Our first objective was to determine whether the specific nucleotide sequence at the upstream end of the *galP1* CRP site is important for CRP binding and stimulation of transcription initiation at *P1*. Secondly, using synthetic oligonucleotides, we have inverted the *gal* CRP site such that the half corresponding to the consensus is located upstream of the non consensus half. Finally we have replaced the asymmetric CRP binding site at *galP1* with a symmetric nucleotide sequence corresponding to the consensus.

MATERIALS AND METHODS

Recombinant DNA techniques

Vector plasmid pAA121 and *E. coli* host strain N100 were used for all recombinant DNA work (Kelsall *et al.*, 1985; Bingham *et al.*, 1986). Preparations of plasmid

Fig. 2. DNA used in this study

(a) Schematic diagram of the 144 bp *EcoRI*–*HindIII* fragment carrying *galP1*. The transcription start, S1, is denoted by a wavy arrow that shows the direction of transcription. The position of the –10 hexamer sequence is indicated by a solid box and the location of the p19T mutation that inactivates *galP2* is shown by an arrow. Crosshatched boxes denote the location of the CRP binding site. The bent arrows denote the extent of deletions that replace sequence upstream of –56, –49, –38 and –27 (cf. Fig. 1b). (b) Non-scale diagram of pAA121 denoting the location of the *EcoRI*, *HindIII* and *HaeIII* sites exploited in this study, the location of the 144 bp *galP1* insert (solid), the *galK* gene (crosshatched) and the origin of replication (*ori*) and gene encoding resistance to ampicillin (*Ap^R*). (c) Non-scale diagram of pOM44 showing cloned maltose sequence (solid) encoding the *malT* gene and part of *malP* (P'). The open region denotes the deletion at which *EcoRI* and *HindIII* sites were introduced and the arrow marked 0 denotes the location where a *HindIII* site in pOM41 was removed. *Tc^R* denotes the genes encoding resistance to tetracycline. (d) Diagram of the *malA* region on the chromosome of strains pop2094 and pop2095 (not to scale).

plasmid. The result is a set of pop2094 *crp*⁺ and pop2095 Δ *crp* derivatives carrying a *malQ*–*lacZ* fusion under the control of the *galP1* sequence, originally constructed on a short *EcoRI*–*HindIII* fragment.

The efficiency of *galP1* promoter activity in the different constructions was determined by measuring expression of β -galactosidase using the protocol described by Miller (1972). Cells were grown in minimal media with fructose as a carbon source as described by Busby & Dreyfus (1983): units of expression are as described by Miller (1972). Comparison of expression in pop2094 *crp*⁺ with that in pop2095 Δ *crp* allows an empirical measure of the stimulation of promoter activity by CRP. Additionally the effect of CRP *in vivo* was measured by including 0.1% glucose in the assay with the *crp*⁺ host.

Gel binding assays and footprinting

DNA containing the *galP1* promoter was purified from pAA121 derivatives on a *HaeIII*–*HindIII* fragment (Fig. 2b). The fragment was labelled at the *HindIII* end on the upper strand using [α -³²P]dATP and Klenow enzyme or on the lower strand using [γ -³²P]ATP and T4 polynucleotide kinase (Maniatis *et al.*, 1982). The 203 bp *lac* fragment was isolated and labelled as before (Kolb *et al.*, 1983b).

CRP was purified as previously described (Takahashi *et al.*, 1982). CRP–DNA complexes were formed and separated on polyacrylamide gels which were revealed by autoradiography as before (Kolb *et al.*, 1983a). Free and bound DNA was quantified after scanning autoradio-

Fig. 3. Expression from *galP1* derivatives

The histogram shows the β -galactosidase activities in pop2094 *crp*⁺ (open bars) or pop2095 Δ *crp* (solid bars) after fragments carrying *galP1* sequence to different positions (shown on the x-axis) upstream of the RNA startpoint were incorporated in the chromosome upstream of a *malQ-lacZ* fusion. The level of β -galactosidase made in pop2094 *crp*⁺ grown in medium containing glucose is shown by the hatched bars. Each bar represents the average of three measurements performed on independently obtained colonies (error is less than 10%).

graphs: the relative binding of CRP to different fragments was assessed from these data as described by Kolb *et al.* (1983b).

E. coli RNA polymerase was prepared according to the method of Burgess & Jendrisak (1975) modified as indicated by Lowe *et al.* (1979). Complexes between RNA polymerase and/or cAMP-CRP and labelled fragments carrying *galP1* were formed, and the DNAase I footprint was determined, exactly as described by Spassky *et al.* (1984).

RESULTS

Deletion analysis of nucleotide sequences essential for CRP-dependent activation of the *galP1* promoter

Fig. 2(a) illustrates the 144 bp fragment carrying the *galP1* promoter which was the starting point of this work: the fragment carries an *EcoRI* linker at -92 and a *HindIII* linker at +45 and contains a G:C to T:A transversion at -19 that specifically inactivates the alternative promoter, *galP2*. The diagram also shows the *P1* transcription startpoint, *SI*, the -10 hexamer sequence, 5' TATGGT 3', and the location of the CRP binding site. The nucleotide sequence of this site, which is located from bp -31 to bp -52, is shown in Fig. 1(a) together with the consensus sequence for CRP binding sites.

To determine the functional upstream limit of DNA sequences essential for CRP binding and stimulation of *P1*, the 144 bp fragment was shortened by removing increasing lengths of *gal* sequence starting at the *EcoRI* end. Linkers carrying an *EcoRI* site were then added to the truncated *gal* sequence (see the Materials and methods section). The result was a series of shortened fragments carrying the *galP1* promoter sequence bounded by a *HindIII* linker at +45 and an *EcoRI* linker at -56, -49, -38 or -27. The nucleotide sequence at the upstream end of each of these fragments is shown in Fig. 1(b): in the case of the deletion to -27, we used a linker that introduced *EcoRI*, *BamHI* and *SphI* sites upstream of the truncated *galP1* sequence. The deletion to -56 does not alter the CRP binding site, the deletion to -49 removes the flanking bases, the deletion to -38 removes half of the site whilst the deletion to -27 replaces the entire CRP binding sequence (Figs. 1a and 1b).

To determine the effects of the deletions on *P1* activity *in vivo*, each fragment was transferred to the chromosome of strains pop2094 *crp*⁺ or pop2095 Δ *crp* at a location just upstream on a *malQ-lacZ* fusion, such that β -

Fig. 4. Binding of CRP to DNA fragments carrying *galP1*

Differing concentrations of CRP, as shown for each lane, were incubated with 0.2–0.5 nM labelled fragment carrying deletions to different positions upstream of the *P1* startpoint as follows: (a) -92, (b) -56, (c) -49 and (d) -38. The Figure shows autoradiograms of gels on which free and bound fragments were separated as indicated.

Fig. 5. Cloning of synthetic CRP sites at *galP1*

(a) Non-scale diagram of pAA121 carrying *galP1* on an *EcoRI*–*HindIII* fragment with a deletion of *gal* sequences upstream of –27 (cf. Fig. 1 and 2). At the site of the deletion a linker containing a *BamHI* and *SphI* site was incorporated that allows the cloning of synthetic DNA carrying *BamHI* and *SphI* cohesive ends. (b) Nucleotide sequence of the top strand of synthetic DNA cloned between the *BamHI* and *SphI* sites carrying four potential CRP binding sites BA', AB', AA' and BB'. For comparison, the wild type *gal* promoter sequences and the consensus for CRP binding is also shown. All sequences are numbered with respect to the *P1* transcription startpoint. A and B sequence elements are underlined and labelled. The shaded arrow head denotes the upstream limit of *gal* nucleotide sequence at –57.

galactosidase expression is dependent on the fragment's promoter activity (see the Materials and methods section, and Fig. 2d). Fig. 3 shows a histogram describing β -galactosidase expression in the *crp*⁺ (open bars) or Δ *crp* strain (solid bars), as a function of the position of the upstream limit of *galP1* promoter sequence. The presence of the *crp*⁺ allele causes a 4–5-fold stimulation in *P1* expression, provided that *P1* sequences up to –56 are present. The deletion to –49 and longer deletions suppress CRP-dependent stimulation of *P1*. As a control for this experiment, the assays in the *crp*⁺ background were repeated in medium containing glucose (Fig. 3; crosshatched bars); in these conditions β -galactosidase expression in the *crp*⁺ background was similar to that found in the Δ *crp* background, confirming that the high levels of expression found with the longer *P1* sequences were due to the action of cAMP–CRP.

We have determined the effects of the deletions on

CRP binding to *galP1* directly using a gel binding assay. *HaeIII*–*HindIII* fragments carrying the truncated *galP1* sequences were purified from pAA121 derivatives, labelled, and incubated with differing concentrations of cAMP–CRP (see the Materials and methods section and Fig. 2b). After gel electrophoresis, free and bound labelled fragment could be distinguished. Figs. 4(a) and 4(b) show that binding of cAMP–CRP is not affected by the deletion of nucleotides from –92 to –56. However binding is reduced 7-fold by the deletion to –49 (Fig. 4c) and abolished by the deletion to –38 (Fig. 4d) or –27 (not shown).

Insertion of synthetic CRP binding sites at *galP1*

Our results show that nucleotide sequences downstream of –56 are sufficient for cAMP–CRP binding to *galP1* and for stimulation of *P1* transcription. Fig. 1(a) shows that this region contains a 22 bp nucleotide

Fig. 6. Expression from *galP1* derivatives carrying synthetic CRP sites

The histogram shows the β -galactosidase levels made in pop2094 *crp*⁺ (open bars) or pop2095 Δ *crp* (solid bars) after fragments containing *galP1* incorporating the BA', AB', BB' or AA' CRP binding sites (indicated on the x-axis) were transferred to the chromosome upstream of a *malQ-lacZ* fusion. The level of β -galactosidase made in pop2094 grown in medium containing glucose is shown by the hatched bars. Each bar represents the average of three independent measurements.

sequence of which the downstream half corresponds well to the consensus for CRP binding sites, whereas the upstream half corresponds poorly. To investigate the importance of this sequence we have made a number of synthetic CRP-binding nucleotide sequences and inserted them at the same distance from the *galP1* transcription start site. To do this we exploited the longest deletion to -27 which introduced an *SphI* site from -31 to -26 just downstream of *BamHI* and *EcoRI* sites (Fig. 1b). Pairs of synthetic oligonucleotides which generated *BamHI* and *SphI* cohesive ends were then synthesized and inserted between the *BamHI* and *SphI* sites of the plasmid carrying this deletion (Fig. 5). The result was a series of *EcoRI-HindIII* fragments carrying different CRP binding sites and *gal* sequence up to -57 (the upstream limit was chosen in view of the results in Figs. 3 and 4).

The dramatic effects of deletions to -49 and -38 on the stimulation of *P1* by cAMP-CRP show that the nucleotide sequence of the upstream half of the *P1* CRP binding site is essential. The published models discussed in the Introduction suggest that the two subunits of the CRP dimer make the strongest contacts at *galP1* with the sequence from -33 to -38, which agrees with the consensus and which we label the 'A' sequence, and with the nucleotides from -49 to -45 which varies from the consensus and which we label the 'B' sequence. The *gal* CRP binding site is thus asymmetric (Fig. 5). To test the importance (if any) of this asymmetry, we have altered the *gal* CRP site such that the *gal* sequence from -33 to -38 is replaced by the sequence from -49 to -45 and vice versa. Thus an 'A' sequence, that corresponds to the consensus, appears between -49 and -45, with the non-consensus 'B' sequence downstream from -33 to -38 (Fig. 5). Essentially this manipulation reverses the orientation of the *gal* CRP binding site changing the wild type arrangement, BA', to a new arrangement, AB' (in

Fig. 7. Binding of CRP to different DNA fragments

(a) 4 nM (lanes a-d) or 50 nM (lanes e-h) CRP was incubated with a labelled fragment carrying *galP1* containing different CRP sites as follows: BA' (lanes a, e); AA' (lanes b, f); AB' (lanes c, g); BB' (lanes d, h). The Figures show autoradiograms of gels on which free and bound fragments were separated as indicated. (b) Approximately equimolar quantities (0.2-0.5 nM) of labelled fragments carrying the *lac* promoter or *galP1* promoter incorporating the AA' CRP binding site were incubated with different concentrations of CRP as shown. Free and bound *lac* and *gal* fragments were separated by gel electrophoresis as shown in the Figure.

Fig. 8. Footprint analysis of complexes between *galP1*, cAMP-CRP and RNA polymerase

The Figure shows a gel analysis of the pattern of DNAase I attack of the lower strand of *galP1* incorporating the four synthetic CRP binding sequences BA', AB', AA', and BB' as indicated. The addition of cAMP-CRP and RNA polymerase (POL) is indicated in each case: the numbers 1 and 2 denote the order of addition. The gel was calibrated with respect to S1 using the markers run in lanes a, g, m and s.

this terminology A' is complementary to the 'A' sequence and B' is complementary to 'B'). Additionally we replaced both zones from -49 to -45 and from -33 to -38 with B sequences to give the BB' sequence, and with A sequences to give the AA' sequence, which agrees almost perfectly with the consensus. Sequence analysis of different CRP binding sites shows that there is little consensus in the nucleotide sequence of the central 6 bp of the 22 bp site, which in the case of *galP1* are located from -44 to -39. During the syntheses, we therefore altered the sequence of this region, 5' TCCATG 3', to an *Xba*I site, 5' TCTAGA 3'.

*Eco*RI-*Hind*III fragments carrying *galP1*, incorporating the four synthetic CRP sites, BA', AB', BB' and AA', were transferred to the chromosome of strains pop2094 *crp*⁺ and pop2095 Δ *crp* as described in the Materials and methods section. Comparison of the levels of β -galactosidase expression in these two backgrounds

(Fig. 6) shows that the stimulation of expression from *P1* by cAMP-CRP is similar with both the BA' or AB' synthetic binding site. Significantly the 2-3-fold stimulation is less than the 4-5-fold found with the wild type *gal* sequence and values with BA' are reproducibly lower than with AB'. Inclusion of glucose in the growth medium depresses the level of β -galactosidase expression with both BA' and AB' to the level found in the Δ *crp* background.

Fig. 6 shows that there is no CRP-dependent stimulation of *P1* activity when the BB' synthetic site is incorporated. In sharp contrast, CRP-dependent stimulation of *P1* is increased to 7-8-fold when the AA' site is present. The magnitude of the effect is probably masked by an unexpected increase in the CRP-independent level of β -galactosidase expression seen in the Δ *crp* background. Interestingly, in contrast with the previous cases, the elevated level of expression obtained with the AA'

Fig. 9. Gel analysis of the pattern of DNAase I attack of the upper strand of *galP1* incorporating four different CRP binding sequences

Conditions were exactly as in Fig. 8.

site in the *crp*⁺ background is hardly repressed by the inclusion of glucose in the growth medium.

To measure the fixation of cAMP–CRP directly to the four synthetic CRP sites, we performed gel binding assays on labelled *Hae*III–*Hind*III fragments exactly as before. The results in Fig. 7(a) show that cAMP–CRP binding to the *galP1* promoter carrying the BB' site is undetectable (lanes *d* and *h*). However, CRP binds to both the BA' and AB' sites with a similar affinity (lanes *a*, *c*, *e*, and *g*). A detailed comparison of the binding to the AB' and BA' sites reveals that binding to AB' is slightly favoured: binding to AB' is 2–3-fold weaker than binding to the wild type sequence (not shown). In contrast, cAMP–CRP binding to the AA' site is clearly much tighter, and no free fragment remains even at the lower CRP concentration (lanes *b* and *f*). Fig. 7(b) shows a competition experiment where the fragment carrying the AA' site was mixed with a 203 bp fragment containing the almost symmetric *lac* CRP site. The results show that binding to AA' is tighter than to the *lac* CRP site by a factor of greater than 50.

DNAase footprint analysis

We have analysed complexes between RNA polymerase and DNA fragments carrying *P1* with the four synthetic sites, in the presence and absence of cAMP–CRP, using DNAase footprinting. Results with the lower and upper strands are shown in Figs. 8 and 9

respectively. In the presence of RNA polymerase alone (lanes *f*, *l*, *r* and *x*), a clear footprint is seen from around +20 to –30 and some protected bases are seen upstream of –40. The similarity of the footprints in the four cases confirms that the overall architecture of open complexes at *galP1* formed in the absence of CRP is largely independent of the exact sequence upstream of –27 (Busby *et al.*, 1987). In the presence of cAMP–CRP alone (lanes *c*, *i*, *o* and *u*) clear footprints at the CRP binding sites are seen, save with the BB' site. Moreover the footprint is stronger with the AA' site confirming the gel binding analysis.

With both cAMP–CRP and RNA polymerase present, similar effects are found with the fragments carrying the BA', AB' and AA' sites: the footprint of RNA polymerase is extended upstream to around –60 whilst there is no change at the downstream end of the footprint. In contrast, with the fragment carrying the BB' site, the footprint due to polymerase alone is unchanged by the inclusion of cAMP–CRP. A detailed analysis of these footprints and a comparison with results at the wild type *gal* sequence will be published elsewhere.

DISCUSSION

Although the consensus sequence for CRP binding sites displays 2-fold symmetry, in many cases, the actual

nucleotide sequence at a site displays little symmetry (de Crombrughe *et al.*, 1984; Ebright *et al.*, 1984). Variations in the sequence lead to differences in affinity for cAMP-CRP that affect the level of intracellular cyclic AMP at which expression can be triggered from that site. Results reported by Kolb *et al.* (1983b), Jansen *et al.* (1987), and this study have clearly shown that, to a first approximation, the affinity of CRP binding to a site correlates with the deviation of the nucleotide sequence from the symmetric consensus sequence.

The CRP binding site at the *galP1* promoter is highly asymmetric, the upstream half corresponding poorly with the consensus. The deletion analysis reported here shows that specific nucleotides in this region of poor homology are crucial for CRP binding. Additionally, bases upstream of -49, that do not appear to be involved in direct contacts with the helix-turn-helix motif of the protein (see the Introduction) are vital. At *galP1*, cAMP-CRP stimulates transcription by making an interaction with RNA polymerase that accelerates initial recognition of the promoter by polymerase (reviewed by Buc *et al.*, 1986; Busby & Buc, 1987). We have considered that asymmetry in the CRP-DNA complex imposed by the asymmetric binding site may be important in making this interaction. Our observation that CRP binding and stimulation of *P1* expression is largely independent of the orientation of the asymmetric *gal* CRP site clearly shows that this is not the case here: the simplest explanation for our results is that although the binding site is asymmetric, the final complex between dimeric CRP and DNA is symmetric. In this case the asymmetry in the binding site therefore only serves to set the affinity for CRP.

The results in Figs. 3 and 6 show that, in our conditions, in a *crp*⁺ background, expression from *P1* is directly related to the affinity of CRP binding to its site. The small differences in affinity between the AB', BA' and wild type sites are reflected in small differences in CRP-dependent stimulation of expression. Most probably these differences are due to subtle sequence changes, e.g. at bp -31 (Fig. 5), introduced by the cloning, that have small effects on CRP binding.

Binding of cAMP-CRP to the AA' site was over 50 times stronger than to the *lac* site. To our knowledge this is the tightest binding CRP site that has been reported to date: we estimate the dissociation constant to be between 10⁻¹¹ and 10⁻¹² M. At first sight this result is in conflict with that of Jansen *et al.* (1987) who reported that if the *lac* CRP site is made symmetric the affinity for CRP is improved by only 3-fold. Clearly this difference must be due to elements in the sequence in which the consensus site is 'embedded'. In particular the *Xba*I recognition sequence 5' TCTAGA 3' located between the sequences 5' TGTCA 3' and 5' TCACA 3' at the AA' site (Fig. 5) may contribute to increased CRP binding: in a survey of the 6 bp sequence at the centre of all known CRP sites one of us (K. Gaston, unpublished work) noted that there was a weak preference for the symmetric sequence 5' TCTAGA 3'.

In the *crp*⁺ background we observed increased CRP-dependent expression from the *P1* construction carrying the AA' site. However, although this increased expression was dependent on cyclic AMP and CRP, it was not repressed by glucose. We suggest that this is because CRP binding to the AA' site is so tight that it is triggered by levels of cyclic AMP lower than those reached in cells

grown in our conditions. Thus, although the improvement in the CRP binding causes an increase in expression, it reduces the sensitivity of the system to physiological fluctuations in cyclic AMP concentrations. We have noted that the tight-binding AA' sequence has not been found at any *E. coli* promoter sequence. Presumably this is because such a site, whilst ensuring high levels of transcription initiation, would be useless in mediating fluctuations in transcriptional activity in response to changes in environmental stress.

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