cAMP–cAMP receptor protein complex: five binding sites in the control region of the *Escherichia coli* mannitol operon

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The control region of the mannitol (*mtl*) operon of *Escherichia coli* has been shown to contain five cAMP receptor protein (CRP) binding sequences, the most yet reported for any operon. A DNA fragment encompassing the entire *mtl* operon regulatory region was generated by PCR, and the binding of the cAMP–CRP complex was studied. Using restriction analysis to separate, delineate and destroy the various putative CRP binding sites, all five sites were shown to be functional for CRP binding *in vitro*. Four of these sites bound the cAMP–CRP complex with high affinity while the fifth site (the most distal relative to the transcriptional start site) bound the complex with lower affinity. Simultaneous binding of cAMP–CRP complexes to several of these sites was demonstrated. The results serve to identify and define five dissimilar CRP binding sites in a single operon of *E. coli*. A model for *mtl* operon transcriptional initiation and repression complexes is presented.

Keywords: cyclic AMP, cyclic AMP receptor protein (CRP), mannitol (*mtl*) operon, CRP binding site (operator), DNA band mobility shift

INTRODUCTION

Adenosine 3',5' cyclic phosphate (cAMP) is known to mediate a principal form of catabolite repression in enteric bacteria such as *Escherichia coli* (Magasanik & Neidhardt, 1987; Saier, 1991; Botsford & Harman, 1992). The mechanism of transcriptional regulation involves the cAMP receptor protein (CRP), which first binds cAMP, alters its conformation in response to cAMP binding, and then binds to the promoter region of a target operon to promote transcriptional initiation (Schultz *et al.*, 1991; Kolb *et al.*, 1993a). The CRP dimer is known to bind to a well-defined 22 bp palindrome on the DNA (Barber & Zhurkin, 1990; see also Fig. 2). The mechanism of CRP activation has been studied in detail and is believed to involve DNA bending, direct interaction with RNA polymerase (Ebright, 1993; Ishihama, 1993; Kolb *et al.*, 1993b; West *et al.*, 1993; Busby *et al.*, 1994; Kumar *et al.*, 1994; Perez-Martin *et al.*, 1994) and interaction with various other transcription factors (Pedersen *et al.*, 1991; Wu *et al.*, 1992).

Many well-characterized CRP-dependent promoters, such as the *lac* and *malT* promoters, contain just one site for

CRP binding. However, other operons have been reported to possess more than one sequence that approximates the CRP consensus sequence (Yamada & Saier, 1987; Gerlach *et al.*, 1991; Pedersen *et al.*, 1991; Fox *et al.*, 1992; Hanamura & Aiba, 1992; Kolb *et al.*, 1993a; Richet & Sogaard-Andersen, 1994). In only a few such cases has the direct binding of CRP to these consensus sequences been demonstrated *in vitro*, and in still fewer cases has the physiological significance of two or more binding sites been established *in vivo*.

In operons where CRP is the sole activator, its binding site is centred at positions –41·5, –61·5 or –71·5 relative to the transcriptional start site, and CRP directly stimulates transcriptional initiation by RNA polymerase. By contrast, when CRP acts together with another transcriptional activator, and CRP is not the primary activator, its binding site is not localized at these positions (Kolb *et al.*, 1993a). An example for the latter scenario is the control region of the *E. coli malK lamB malM* operon, where three CRP binding sites, at positions –132·5, –166·5 and –195·5, are present (Richet & Sogaard-Andersen, 1994). The function of CRP binding to these sites is to bend the DNA and thereby reposition the main activator, MalT, from a set of non-productive binding sites to a nearby set of productive sites (Richet & Sogaard-

Abbreviations: BMR, (DNA) band migration retardation; CBS, CRP binding site.

Andersen, 1994). CRP thus seems to play two fundamentally different roles dependent on the location of its binding site(s) in relation to the transcriptional initiation start site.

In previous publications, we reported the complete sequence of the mannitol (*mtl*) catabolic operon of *E. coli* (Lee & Saier, 1983; Davis *et al.*, 1988; Jiang *et al.*, 1990; Figge *et al.*, 1994). Surprisingly, five striking CRP binding consensus sequences immediately preceding the promoter region of this operon were identified. A similar number of potential binding sites was noted in the control region of the glucitol (*gut*) operon although deviation from the CRP consensus sequence was more pronounced (Yamada & Saier, 1987). The relevance of multiple CRP binding sites to transcriptional initiation of the *mtl* operon has yet to be examined.

In no other operon have so many CRP binding sequences been found. Consequently, we were curious to know whether or not the five putative CRP binding sites in the *mtl* operon control region are of functional significance. In this communication we report the results of *in vitro* CRP-DNA interaction studies which establish that four of the five putative binding sequences bind CRP with high affinity, and that the fifth (the most distal one relative to the transcriptional start site) binds CRP with lower avidity. Restriction analyses coupled with DNA band migration retardation assays have established both the identities and the locations of these sites within the *mtl* operon control region.

METHODS

Materials. Restriction enzymes were obtained from Bethesda Research Laboratories. AmpliTaq DNA polymerase and deoxynucleotide triphosphates were from Perkin Elmer Cetus. [α - 32 P]dATP was purchased from ICN Biochemicals. Oligodeoxynucleotides were obtained from Dr J. Tomich, Kansas State University, Manhattan, KS, USA. QIAquick spin columns were from Qiagen. The nonspecific DNA poly(dI-dC)·poly(dI-dC), used in the DNA band migration retardation experiments at a final concentration of 1 μ g per binding reaction mixture (20 μ l), was from Pharmacia. Other chemicals were obtained from commercial sources and were of the highest purity available.

Oligodeoxynucleotides, polymerase chain reaction and isolation of DNA fragments generated by restriction analyses. Two oligodeoxynucleotides, pmtlA-lacZ #1 (5'-GGGGAATTCCTTTAGAGGTGATGAGTTGCTT-3'; accession number L19044), carrying an *Eco*RI site (underlined), and pmtlA-lacZ #2 (5'-AAAGGATCCCGGATGACATAA-AAACACCCC-3'; accession number V01503), harbouring a *Bam*HI site (underlined), were used in the polymerase chain reaction (PCR) to amplify the 444 bp DNA fragment harbouring the control region of the *E. coli mtl* operon. Chromosomal DNA isolated from *E. coli* strain DH5 α was used as template for the PCR. PCR was performed as described by the manufacturer (Perkin-Elmer Cetus) with a Hybaid thermocycler. DNA fragments were generated by single cycles of 7 min at 95 °C and 5 min at 55 °C followed by 30 cycles of 1 min each at 72, 94 and 55 °C followed by one final cycle of 10 min at 72 °C. PCR-amplified DNA fragments were routinely checked by restriction analyses. For high-specific-activity labelling of the

mtl DNA fragments, the procedure described by Ramsey *et al.* (1993, 1995) was followed. Unincorporated labelled nucleotides were removed by QIAquick spin columns. The labeled *mtl* DNA fragments were digested with restriction enzymes, the digests were loaded onto a 5% (w/v) polyacrylamide gel, and the DNA fragments were separated by allowing the gel to run for 2 h at 200 V. The desired DNA fragments were cut out of the gel and purified as described by Sambrook *et al.* (1989).

DNA band migration retardation (BMR) assays. These assays were performed essentially as described earlier (Ramsey *et al.*, 1993, 1995) using pure CRP generously provided by Dr James C. Lee, University of Texas, Galveston, TX, USA. cAMP was present (i) in the binding reaction and (ii) in the upper gel chamber reservoir during electrophoresis, at final concentrations of 100 μ M and 20 μ M, respectively. The efficacy of our CRP preparation for binding to the *mtl* operon CBSs 2, 3, 4, and 5 proved to be greater than that for binding to the two well-characterized *pckA* CBSs. The efficiency of binding to the *mtl* operon CBS 1, however, was less than that for binding to the *pckA* CBSs as expected from its lack of correspondence to the consensus sequence (Ramsey *et al.*, 1995). Inclusion of the nonspecific DNA poly(dI-dC)·poly(dI-dC) at the concentration of 1 μ g per 20 μ l had no effect on the efficiency of CRP binding. Therefore, the unexpectedly low affinity of CRP binding can be attributed to partial inactivation of the purified protein.

RESULTS

Putative CRP binding sites in the *mtl* operon control region

Fig. 1(a) shows the physical map of the control region of the mannitol (*mtl*) operon of *E. coli*. Shown in this figure are: the transcriptional start site (+1; Lee & Saier, 1983); the -10 and -35 regions (open rectangles; Figge *et al.*, 1994); the FruR binding site (closed square; Ramsey *et al.*, 1995); and the five putative CRP binding sites (CBSs) (numbered open rectangles 1-5; Davis *et al.*, 1988; Jiang *et al.*, 1990). All five putative CBSs are upstream of the -35 region. The spacing of these putative binding sites is as follows. The centre of CRP 5 is 58.5 bp from the transcriptional start site or 23.5 bp from the end of the -35 region. The centre-to-centre distances between the CBSs are then 44 bp (between CRP 5 and 4), 73 bp (between CRP 4 and 3), 44 bp (between CRP 3 and 2) and 43 bp (between CRP 2 and 1; see Fig. 1a). Thus, the spacing between CRP sites 1 and 2, 2 and 3, and 4 and 5 are essentially the same. This periodicity of CRP binding site positioning suggests functionality. The fact that a normal helical turn in B-DNA is about 10.5 bp provides theoretical substantiation for this suggestion (see Discussion).

The fragment initially generated by PCR for analysis of CRP binding is shown in Fig. 1(b). This fragment encompasses all five putative CBSs. Restriction sites used in our study are also indicated. Those fragments used in DNA band migration retardation (BMR) assays are shown in Fig. 1(b-e) together with restriction sites that allowed definition of the CBSs.

Fig. 2 shows the CRP binding consensus sequence (top) as well as the five putative CBSs found in the *mtl* operon

Fig. 1. (a) Physical map of the *mtl* regulatory region. Numbers are given with respect to the transcriptional start site (+1). The five CRP binding sites are indicated by rectangular boxes (22 bp) which are numbered 1 through 5. The CRP binding sites 1–5 are centred on positions –58.5, –102.5, –175.5, –219.5 and –262.5, respectively, with respect to the *mtlA* transcription start site. The *FruR* operator site (O) is indicated by a black box (Ramseier et al., 1995). For the nucleotide sequence of the *mtl* operon control region see database accession numbers L19044 and V01503. The DNA fragments used for the BMR experiments are shown below (b–e; drawn to same scale as a). CRP binding sites which were destroyed by restriction enzyme digestion are indicated on the far right († CRP).

control region (middle). At the bottom of the figure, specific residues in CRP that are known to interact with particular bases of the DNA are also indicated. In the consensus sequence, those bases presented in bold print (positions 4–8 in each half site) are of greatest importance for CRP binding. Examination of the putative CBSs present in the *mtl* operon control region reveals near identity with the consensus sequence. Just two exceptions are observed: CRP 1 differs from the consensus sequence in three conserved positions while CRP 4 differs from the consensus sequence in one conserved position (Fig. 2). We therefore predicted that CRP 2, 3 and 5 should exhibit the highest-affinity binding, CRP 4 should exhibit slightly lower-affinity binding, and CRP 1 should exhibit the lowest-affinity binding. These predictions were verified experimentally (see below).

BMR experiments using the 444 bp DNA fragment encompassing the entire control region of the *mtl* operon (see Fig. 1b, top) barely entered the 5% polyacrylamide

gel in the presence of 30 ng CRP. Although multiple bands were seen at lower CRP concentrations, these were poorly resolved (data not shown). Consequently, this large DNA fragment could not be used for detailed analyses.

The 444 bp DNA fragment was digested either with *Hinfi* (Figs 1b and 3) or with *MboI* (Fig. 1b). Using *Hinfi*, four restriction fragments resulted, and the CRP 2 site was destroyed. DNA fragments of 15 bp (not seen on the gel in Fig. 3) and of 133 bp (see Fig. 3) contain no putative CBS and were not retarded in the presence of increasing amounts of CRP. The 109 bp DNA fragment contained only CRP 1, and it was retarded only at a very high CRP concentration (200 ng) as indicated by the arrow in Fig. 3 (right). The 187 bp DNA fragment contained CRP 3, CRP 4 and CRP 5, and this fragment showed three retarded bands, dependent on the CRP concentration employed (Fig. 3). These three bands presumably correspond to the 187 bp DNA fragment with 1, 2 and 3 CRP

	1 2 3	4 5 6 7 8	9 10 11	11 10 9	8 7 6 5 4	3 2 1		
Consensus:	A A A	T G T G A	T C T	A G A	T C A C A	T T T	Conserved:	
CRP 1	T A A	C A T G C	T G T	A G A	T C A C A	T C A	7	8
CRP 2	T C T	T G T G A	T T C	A G A	T C A C A	A A G	10	4
CRP 3	A A A	T G T G A	C A C	T A C	T C A C A	T T T	10	6
CRP 4	T T T	T G T G A	T G A	A C G	T C A C G	T C A	9	3
CRP 5	T T A	T G T G A	T T G	A T A	T C A C A	C A A	10	4
		▲▲▲ Arg Glu Arg 180 181 185		▲▲▲ Arg Glu Arg 185 181 180				

Fig. 2. Alignment of the five CRP binding sequences from the control region of the *E. coli mtl* operon. The CRP binding consensus sequence, a perfect palindrome with a half-site of 11 nucleotides, is shown at the top. The most conserved nucleotides (positions 4–8 in each half-site) are highlighted in bold. Amino acids of the helix-turn-helix motifs of the CRP dimer which contact the DNA base-pairs are indicated below the aligned sequences. The numbers of nucleotides conserved within the five CRP binding sequences are indicated on the right (bold figures, conserved at positions 4–8; normal figures, conserved at positions 1–3 and 9–11).

Fig. 3. BMR assays using CRP and the 444 bp DNA fragment harbouring the control region of the *mtl* operon which was digested with *HinfI*. The resulting 187, 133 and 109 bp DNA fragments were titrated with increasing amounts of CRP. The 187 bp DNA fragment contains the three CRP binding sequences CRP 3, CRP 4 and CRP 5 (see Fig. 1). The 109 bp DNA fragment contains CRP 1. CRP binding site 2 was destroyed by *HinfI* digestion. The experiment was conducted as described in Methods.

Fig. 4. BMR assays using CRP and the two isolated *DraI* DNA fragments. Titration of (a) the 168 bp DNA fragment containing CRP 1 and CRP 2, and (b) the 276 bp DNA fragment containing CRP 4 and CRP 5, with increasing amounts of CRP. The experiment was conducted as described in Methods.

dimers bound, respectively (Ramseier *et al.*, 1993, 1995). Only at the highest CRP concentration was the fragment maximally retarded.

Similar results were obtained for the *MboI* restriction digest, which destroyed CRP 1 and CRP 2 (see Fig. 1b). Neither the resulting 71 bp DNA fragment nor the 43 bp fragment contained a putative CBS, and neither of these two fragments was retarded, even at a very high CRP concentration (200 ng; data not shown). The 322 bp DNA fragment containing CRP 3, CRP 4 and CRP 5 was retarded like the 187 bp *HinfI* DNA fragment (see Fig. 3), showing three retarded bands dependent on the CRP concentration employed (data not shown).

Characterization of CRP 1 and CRP 2

As shown in Fig. 1(c), a 168 bp *DraI* DNA fragment contains CRP 1 and CRP 2 but lacks all other recognized protein binding sites in the *mtl* operon control region. This DNA fragment was isolated as described in Methods. BMR studies using this fragment (Fig. 4a) confirmed the presence within the 168 bp *DraI* DNA fragment of two

Fig. 5. BMR assays using CRP and the isolated 276 bp *DnaI* DNA fragment digested with restriction enzymes to destroy the CRP binding sites. (a) *EcoRV* digestion and titration of the resulting 168 and 108 bp DNA fragments with increasing amounts of CRP. The 108 bp DNA fragment contains CRP 4. CRP binding site 5 was destroyed by the *EcoRV* digestion. (b) *MaeIII* digestion and titration of the resulting 212 and 64 bp DNA fragments with increasing amounts of CRP. The 212 bp DNA fragment contains CRP 5. CRP binding site 4 was destroyed by the *MaeIII* digestion. The experiment was conducted as described in Methods.

functional CBSs, one of high affinity (CRP 2) and the other of low affinity (CRP 1) (see also Fig. 3). CRP 2 was titrated with CRP in the range 10–90 ng while CRP 1 was titrated only when the CRP range exceeded 100 ng.

Characterization of CRP 4 and CRP 5

A 276 bp *DraI* DNA fragment containing CRP 4 and CRP 5 but lacking all other recognized CBSs (Fig. 1d) was isolated and used for BMR studies (Fig. 4b). As expected, two bands resulted from the titration of this DNA fragment with CRP, and the range of CRP concentrations giving rise to BMR (10–90 ng) suggested that both sites bind CRP with high affinity. This result is in agreement with the observation, noted in Fig. 2, that CRP 4 and CRP 5 exhibit sequences which closely approximate the CRP binding consensus sequence.

Restriction sites were found within CRP 4 and CRP 5, contained within the 276 bp *DraI* DNA fragment. Thus, as shown in Figs 1(d) and 5(a), treatment of the 276 bp DNA fragment with *EcoRV* gave a 168 bp DNA fragment and a 108 bp DNA fragment. The latter should contain CRP 4, and as shown in Fig. 5, this fragment was retarded with low concentrations of CRP (10–90 ng). The 168 bp

Fig. 6. BMR assays using CRP and the isolated 322 bp *Sau3AI* DNA fragment digested with restriction enzymes to destroy the CRP binding sites. (a) *EcoRV* digestion and titration of the resulting 162 and 160 bp DNA fragments with increasing amounts of CRP. The 162 bp DNA fragment contains CRP 3 and CRP 4. CRP binding site 5 was destroyed by the *EcoRV* digestion. (b) *MaeIII* digestion and titration of the resulting 204, 82 and 36 bp DNA fragments with increasing amounts of CRP. The 36 bp DNA fragment is not visible. The 204 bp DNA fragment contains CRP 5. CRP binding sites 3 and 4 were destroyed by the *MaeIII* digestion. The experiment was conducted as described in Methods.

DNA fragment was not retarded as expected assuming that CRP 5 was destroyed.

Treatment of the 276 bp DNA fragment with *MaeIII* gave a 212 bp DNA fragment and a 64 bp DNA fragment with expected destruction of CRP 4. The former DNA fragment, but not the latter one, was retarded by low CRP concentrations. It should be noted that, as expected, based on the alignment with the CRP binding consensus sequence shown in Fig. 2, CRP 5 (Fig. 5b) was titrated with slightly lower CRP concentrations than was CRP 4.

Characterization of CRP 3

In order to unequivocally establish that CRP binds to CRP 3, the 322 bp *Sau3AI* restriction fragment was purified on a polyacrylamide gel as described in Methods, and it was then digested with *EcoRV* and *MaeIII* (see Fig.

1e). Treatment of the 322 bp DNA fragment with *EcoRV* gave two DNA fragments of nearly equal size (160 and 162 bp) which migrated together in the polyacrylamide gel. As shown in Fig. 6(a), one of these fragments, probably the larger more slowly migrating DNA fragment, was retarded at low CRP concentrations with two resultant band shifts as expected for a DNA fragment containing two high-affinity CRP sites. When the 322 bp DNA fragment was treated with *MaeIII*, three fragments (36, 82 and 204 bp) were generated, and only the last of these was retarded. These observations are consistent with the prediction that *MaeIII* should destroy CRP 3 and CRP 4 but not CRP 5.

DISCUSSION

In the research described in this paper we isolated DNA fragments from the control region of the *mtl* operon and used these fragments to establish that the five putative CBSs are all functional for binding of the cAMP–CRP complex. This was accomplished using BMR assays coupled to restriction analyses with a variety of enzymes. In this way we were able to specifically destroy sites and thereby establish their positions as well as their relative affinities for CRP. Analyses of the five CBSs revealed that CRP 2, 3 and 5 bound the cAMP–CRP complex with highest affinity, that CRP 4 bound activated CRP with slightly lower affinity, and that CRP 1 exhibited much lower affinity for activated CRP. These results were in accord with prediction based on approximation to the consensus sequence shown in Fig. 2 for the ten important bases in the two 11 bp half-sites. All ten agreed with the consensus sequence in CRP 2, CRP 3 and CRP 5; all but one of the ten agreed with the consensus sequence in CRP 4, and only seven of the ten agreed with the consensus sequence in CRP 1.

The spacing of the five CBSs in the control region of the *mtl* operon proved to be noteworthy (see Fig. 1). Thus, with the exception of the centre-to-centre distance between CRP 3 and CRP 4 (73 bp), all CBSs are 43 or 44 bp apart. This distance corresponds to four helical turns in A-DNA (11 bp per turn) and to just slightly more than four turns in B-DNA (10–10.5 bp per turn). These results suggest that CRP 1, CRP 2 and CRP 3 must be localized to one side of the DNA helix, each separated from the preceding or following site by close to four turns. Further, CRP 4 and CRP 5 must similarly be localized to one side of the DNA helix, being separated from each other by four turns. Finally, CRP 3 and CRP 4 are separated from each other by 73 bp, or about seven helical turns. Thus, all five CBSs are probably localized to the same side of the DNA. Based on these findings, we propose the conformational model for CRP binding to the five CBSs in the control region of the *mtl* operon of *E. coli* shown in Fig. 7. If CRP binding bends the DNA 90° (Botsford & Harman, 1992; Kolb *et al.*, 1993a), and all five binding sites are on the same side of the helix, then the DNA should form a 450° loop as indicated in the figure. RNA polymerase should then interact with the DNA (–35 and –10 sites) at a position within this looped region. As interaction of more than a single CRP dimer with RNA

Fig. 7. Model for *mtl* operon transcriptional initiation and repression complexes. Proteins of the transcriptional initiation complex depicted include RNA polymerase and five cAMP receptor protein dimers (CRP 1–5) bound to their respective binding sites in the operator–promoter region of the *mtl* operon (see also Fig. 1). CRP is presumed to bend the DNA in the direction of the major groove by 90°. The black arrow indicates the transcription direction, 5'→3'. The DNA may assume a helical configuration due to CRP binding as suggested by Schultz *et al.* (1991), thereby enabling one or more CRP dimer(s) to contact RNA polymerase. The fructose repressor, FruR, bound to its operator site (O; indicated by an open square) may displace this initiation complex. The proteins are not drawn to scale.

polymerase has been demonstrated (Joung *et al.*, 1993; Busby *et al.*, 1994), it is possible that one or more of the bound CRP dimer(s) interact directly with RNA polymerase.

The FruR binding site identified previously (Ramseier *et al.*, 1995) lies between the –10 and –35 regions directly in the centre of the 'CRP loop'. The FruR protein has been shown to bind to the *mtl* operator–promoter region and inhibit transcriptional initiation two- to fourfold (Chin *et al.*, 1989; Ramseyer *et al.*, 1995). Whether this inhibition is due to competition for binding of RNA polymerase or has an alternative explanation has yet to be determined. It is important to note that the mechanism by which the mannitol repressor (MtlR) which may possibly function in conjunction with FruR (Figge *et al.*, 1994) exerts its effect on *mtl* operon transcriptional initiation is unknown. It is interesting, however, that loss of CRP function apparently abolishes mannitol inducibility (M. H. Saier, Jr, unpublished results). These observations collectively suggest that multiple protein–protein–DNA interactions govern transcriptional initiation in the *mtl* operon.

The results summarized above reveal that the structure of the *mtl* operon control region is among the most complex so far found in bacteria, with at least eight distinct binding sites for known transcription factors, i.e. for CRP (five binding sites), FruR (one binding site), RNA polymerase (two binding sites) and possibly the mannitol repressor. Some of these proteins presumably interact to form

activated or inhibited transcriptional initiation complexes, activated or inhibited, respectively, for open complex formation. This degree of complexity resembles that for eukaryotic transcriptional initiation. We anticipate that as initiation complexes in bacteria are characterized further, the currently recognized differences in complexity between eukaryotic and prokaryotic transcriptional initiation complexes will vanish.

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