

A simple mechanism for co-dependence on two activators at an *Escherichia coli* promoter

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The *Escherichia coli melAB* promoter is co-dependent upon two transcription activators, MelR and the cyclic AMP receptor protein, CRP. In this study we demonstrate positive co-operativity between the binding of MelR and CRP at the *melAB* promoter, which provides a simple mechanism for its co-dependence. MelR binds to four sites, centred at positions –42.5, –62.5, –100.5 and –120.5 relative to the *melAB* transcription start point. When MelR is pre-bound, CRP is able to bind to a target located between MelR at positions –62.5 and –100.5. This increases the occupation of the two downstream sites for MelR, which is essential for transcription activation. We have identified residues within activating region 1 (AR1) of CRP that are important in transcription activation of the *melAB* promoter. At simple CRP-dependent promoters, the surface of CRP containing these residues is involved in contacting the RNA polymerase α subunit. Our results show that, at the *melAB* promoter, the surface of CRP containing AR1 contacts MelR rather than RNA polymerase. Thus, MelR and CRP activate transcription by a novel mechanism in which they bind co-operatively to adjacent sites and form a bacterial enhanceosome.

Keywords: co-dependence/CRP/*Escherichia coli* protein–protein interactions/transcription activation

Introduction

Regulation of transcription initiation is a key step in the control of gene expression for all organisms, and hundreds of transcription regulators have been studied in prokaryotes and eukaryotes. In prokaryotes, activation of transcription at many promoters is due to the direct interaction of a single activator protein with RNA polymerase (RNAP; Busby and Ebright, 1994). In eukaryotes, transcription activation is more complex, often involving the co-operative binding of multiple transcription activator proteins to an enhancer element within the promoter, forming an enhanceosome (reviewed by Merika and Thanos, 2001).

In bacteria, expression from some promoters is dependent upon two or more activator proteins. Co-dependence of promoter expression provides a simple way of coupling transcription to multiple environmental stimuli. To date,

four mechanisms of co-dependence have been identified at bacterial promoters. (i) The two activators make independent contacts with RNAP, e.g. at the *Escherichia coli ansB* promoter, which is co-dependent on the cyclic AMP receptor protein (CRP) and FNR, CRP and FNR each interact with one of the two RNAP α subunits (Scott *et al.*, 1995). (ii) One activator bends the DNA to facilitate the function of a second activator that can then interact directly with RNAP, e.g. the DNA-bending protein IHF functions in conjunction with a second activator at a number of σ^{54} -dependent promoters (reviewed by Goosen and van de Putte, 1995). (iii) The binding of one activator repositions a second activator from a location where it is unable to activate transcription to a location where it is able to activate transcription, e.g. CRP binding upstream of the *E.coli malK* promoter repositions three molecules of MalT on the DNA such that they are able to activate transcription (Richet *et al.*, 1991). (iv) The function of one activator is inhibited by the presence of a repressor, which is removed by the binding of a second activator, e.g. FNR can only activate transcription at the *E.coli nirB* promoter when the FIS and IHF repressors are removed by the binding of NarL or NarP (Browning *et al.*, 2000).

Interestingly in bacteria, no clear example has been found to date for co-dependence that is due to the co-operative binding of two activators, i.e. the presence of one activator is essential for the binding of the second activator. Here we show that MelR, a member of the AraC/XylS family of transcription activators (reviewed by Gallegos *et al.*, 1997), triggered by melibiose, and CRP, a global transcription regulator, triggered by cyclic AMP (cAMP; reviewed by Kolb *et al.*, 1993), bind co-operatively to adjacent DNA sites at the *E.coli melAB* promoter. This co-operativity results in the recruitment of CRP and increases the occupancy by MelR of the key target site that overlaps the promoter –35 element. Based on this observation, we have constructed artificial promoters where CRP is able to recruit MelR and vice versa. Finally, we have identified a determinant on the surface of CRP that is responsible for interactions with MelR at the *melAB* promoter. This determinant includes residues located within activating region 1 (AR1), the surface of CRP that contacts the C-terminal domain of the RNAP α subunit (α CTD) at simple CRP-dependent promoters (reviewed by Busby and Ebright, 1999). Our results show that mutation of this determinant alters the ability of CRP to be recruited to the *melAB* promoter by MelR, and that CRP functions independently of α CTD at the *melAB* promoter. Thus, MelR and CRP interact directly with one another, and bind co-operatively to the *melAB* promoter in a manner analogous to that of transcription activators in eukaryotic enhanceosomes.

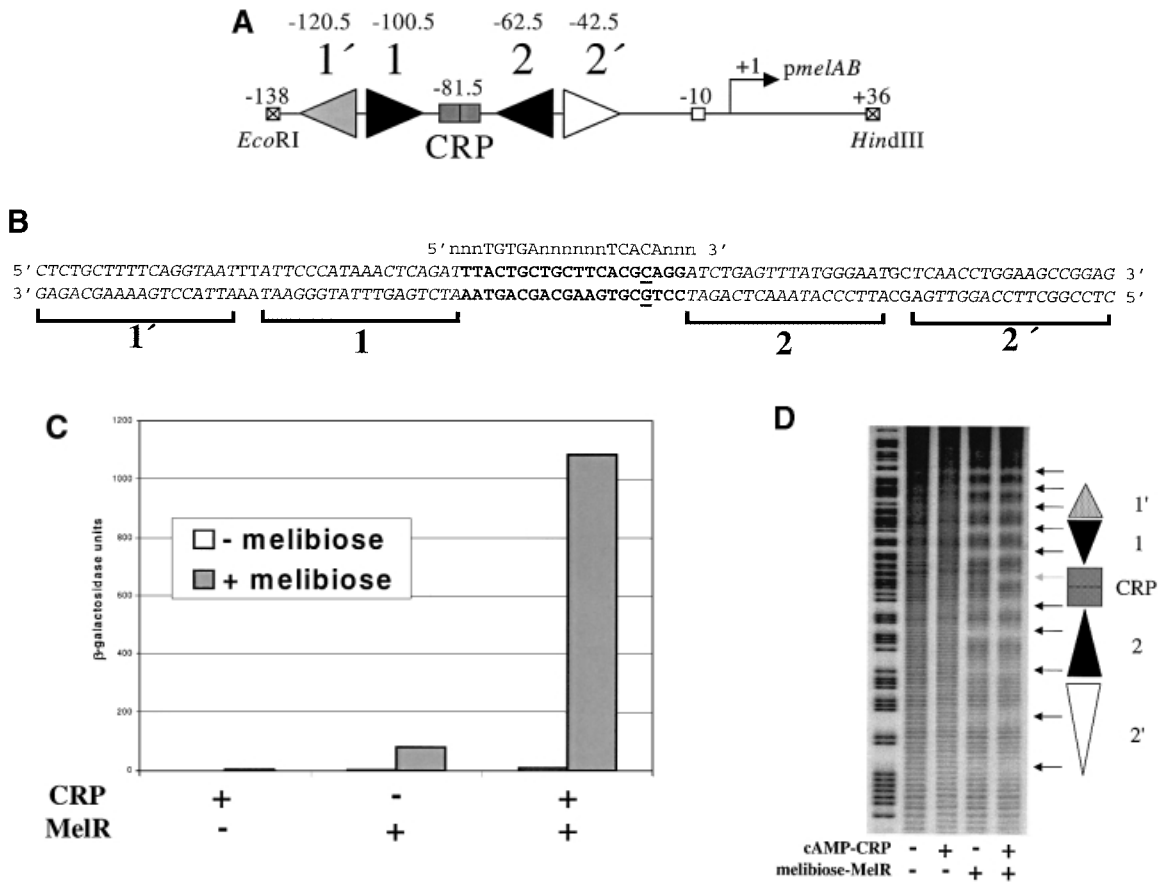


Fig. 1. Role of MeIR and CRP at the *melAB* promoter. (A) Schematic representation of the *melAB* KK43 fragment carrying the *melAB* promoter. DNA sequences are numbered with respect to the transcription start (indicated by the horizontal arrow). The KK43 fragment is bounded by an *EcoRI* site at position -138 and a *HindIII* site at position +36. The different binding sites for MeIR are shown by triangles and the locations of their centres are indicated: filled triangles indicate sites 1 and 2, the lightly shaded triangle indicates site 1' and the open triangle indicates site 2'. The shaded rectangle indicates the target for CRP, centred at position -81.5. The open box indicates the *melAB* promoter -10 hexamer element. (B) Base sequence of positions -129 to -34 of the *melAB* promoter. MeIR binding sites 1', 1, 2 and 2' are shown in italics. The target for CRP is shown in bold. A consensus 22 bp DNA site for CRP is written above the sequence of the *melAB* promoter. Position -75 of the *melAB* promoter is underlined. (C) The activity of the *melAB* promoter was measured in different backgrounds either with or without CRP and MeIR. The figure shows β -galactosidase activities (in Miller units) measured in WAM134 ($\Delta melR \Delta crp \Delta lac$) cells containing the *lac* expression vector, pRW50, carrying the KK43 *melAB* promoter fragment as above. Expression of the *melAB* promoter::*lacZ* fusion was measured either in the absence (open bars) or in the presence (shaded bars) of melibiose in cells carrying plasmid pDCRP, encoding *crp* (+CRP), or the control vector pDU9 (-CRP), and pLG314, encoding *melR* (+MeIR), or the control vector pLGARS (-MeIR). (D) Hydroxyl radical footprint of the KK39 fragment containing the *melAB* promoter. Footprints were performed in the absence and presence of cAMP-CRP and in the absence and presence of melibiose-MeIR as indicated. The position of the different DNA sites for MeIR and the target for CRP are indicated by triangles and a rectangle, respectively. Protections due to MeIR and CRP are indicated by black and grey arrows, respectively.

Results

Co-dependence of the *E. coli melAB* promoter on MeIR and CRP

Transcription initiation at the *E. coli melAB* promoter is co-dependent upon MeIR and CRP. In our previous work (Belyaeva *et al.*, 2000) we showed that MeIR binds to four sites at the *melAB* promoter (sites 1', 1, 2 and 2') centred at positions -120.5, -100.5, -62.5 and -42.5, respectively, relative to the transcription start point (Figure 1A and B). In order to activate transcription, MeIR must occupy its most downstream site, site 2'. CRP alone does not bind to the *melAB* promoter. However, in the presence of MeIR, CRP is able to bind to the *melAB* promoter, and increases the occupancy of site 2' by MeIR. Hence, in the absence of MeIR, CRP does not activate transcription at the *melAB*

promoter whereas, in the presence of MeIR, CRP activates transcription ~10-fold (Figure 1C).

We have now used hydroxyl radical footprinting to study the binding of MeIR and CRP at the *melAB* promoter (Figure 1D). MeIR alone protects two sets of five consecutive minor grooves, corresponding to two dimers of MeIR binding to sites 1' and 1, and sites 2 and 2', respectively. CRP alone does not bind to the *melAB* promoter. However, in the presence of MeIR, addition of CRP gives an extra protection at positions -82, -83 and -84 relative to the transcription start point, and protection of MeIR binding sites 2 and 2' is increased. These results, which accord with previous DNase I footprints (Belyaeva *et al.*, 2000), suggest that, in the presence of MeIR, CRP binds to a target centred between MeIR binding sites 1 and 2, and that this increases the occupancy of sites 2 and 2' by MeIR. Thus, CRP and MeIR, present together at the *melAB*

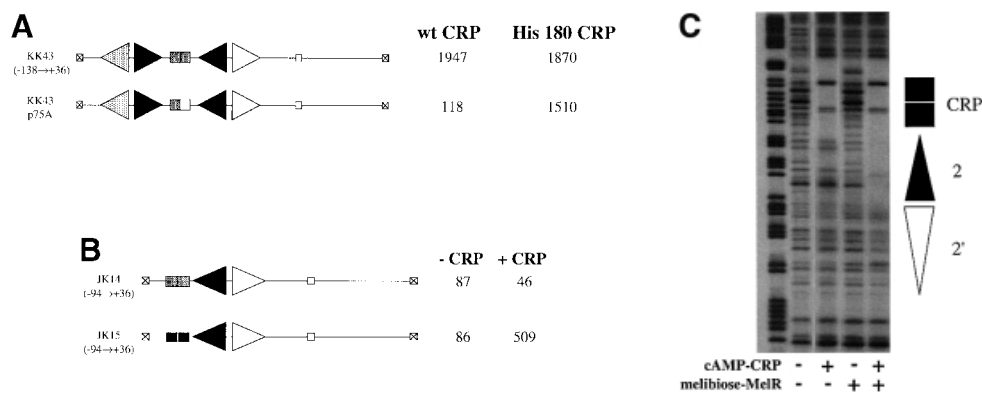


Fig. 2. Interactions of CRP at the *melAB* promoter. **(A)** Activity of the wild-type and p75A mutant *melAB* promoters carried on the KK43 fragment. The fragments with the two promoters are illustrated schematically using the same conventions as in Figure 1A: triangles represent MelR binding sites and the rectangle represents the target for CRP, with the open rectangle indicating the p75A mutation. The promoters were cloned into the *lac* expression vector, pRW50, and the figure shows β -galactosidase activities (in Miller units) measured in WAM134 ($\Delta melR \Delta crp \Delta lac$) cells containing the pRW50 derivatives, pLG314 encoding MelR, and pDCRP encoding either wild-type (wt) CRP or His180 CRP. **(B)** Activity of the JK14 and JK15 *melAB* promoters. The fragments with the two promoters are illustrated schematically using the same conventions as in Figure 1A: triangles represent MelR binding sites and the rectangle represents the target for CRP, with the dark shading indicating a consensus DNA site for CRP. The promoters were cloned into the *lac* expression vector, pRW50, and the figure shows β -galactosidase activities (in Miller units) measured in WAM134 ($\Delta melR \Delta crp \Delta lac$) cells containing the pRW50 derivatives, pLG314 encoding MelR, and either pDCRP encoding wild-type CRP (+CRP) or the vector pDU9 (-CRP). Note that, in the absence of pLG314, the JK15 promoter is inactive. **(C)** DNase I footprinting of complexes at the JK15 fragment. Footprints were performed in the absence and presence of cAMP-CRP and in the absence and presence of melibiose and 150 nM MelR as indicated. The position of MelR binding sites and the target for CRP are indicated by triangles and a rectangle, respectively.

promoter, protect 11 consecutive minor grooves of the DNA, corresponding to a region of >100 bp, suggesting the formation of an ordered nucleoprotein complex. The fact that the locations of the hydroxyl radical footprints created by MelR are the same in both the presence and absence of CRP argues that CRP does not provoke any gross change in the position of MelR binding at the different sites.

The DNA sequence at the *melAB* promoter between MelR-binding sites 1 and 2 contains a poor match with the consensus DNA site for CRP (Figure 1B), and this accounts for the lack of binding found with CRP alone. We believe that the CRP-dependent protection of this region seen in the presence of MelR is due to the binding of CRP. To confirm this, we mutated position -75 relative to the transcription start point, a position that matches the sequence of a consensus DNA site for CRP. This substitution resulted in a large decrease in transcription activation at the *melAB* promoter (Figure 2A). It has been shown previously that the effects of substitutions at this position of a DNA site for CRP can be suppressed by the RH180 substitution in the DNA binding domain of CRP (R.H.Ebright, personal communication). Therefore, we compared transcription activation by wild-type CRP and RH180 CRP at the wild-type *melAB* promoter and at the promoter containing the substitution at position -75. As expected, the RH180 substitution had little effect on CRP-dependent activation of the wild-type *melAB* promoter. However, the decrease in transcription activation due to the substitution at position -75 was suppressed by the RH180 substitution in CRP (Figure 2A). This confirms that CRP binds directly to the region between sites 1 and 2 for MelR, and permits the exact positioning of the target for CRP, equidistant from sites 1 and 2.

A consensus CRP site short circuits the need for sites 1' and 1 at the *melAB* promoter

Previous studies have shown that sites 1' and 1 for MelR at the *melAB* promoter are essential for transcription activation by CRP (Belyaeva *et al.*, 2000). One explanation for this may be that CRP is only able to bind to the *melAB* promoter in the presence of MelR bound both upstream and downstream. To investigate this, we worked with the JK14 promoter, a derivative that lacks sites 1' and 1 for MelR. First, we confirmed that transcription at the JK14 promoter is not activated by CRP (Figure 2B). We then created the JK15 promoter derivative that contains a correctly positioned consensus DNA site for CRP upstream of site 2. In contrast to the situation with the JK14 promoter, the JK15 promoter can be activated by MelR and CRP (Figure 2B). This activation is dependent on the precise location of the DNA site for CRP, since the insertion of 2 bp just upstream of MelR binding site 2 in the JK15 promoter results in the complete loss of CRP-dependent transcription activation (the JK15+2 promoter; data not shown). These results show that the requirement for MelR-binding sites 1 and 1' can be short-circuited by a consensus DNA site for CRP and, thus, at the wild-type *melAB* promoter, MelR binding at sites 1' and 1 must function to recruit CRP to its poor DNA site.

Co-operative binding of MelR and CRP

We have used DNase I footprinting at the JK15 promoter to investigate the co-operative interactions between MelR and CRP. At this promoter, both MelR and CRP are able to bind independently to their respective DNA sites. However, at a sufficiently low concentration, MelR is only able to bind to sites 2 and 2' in the presence of CRP (Figure 2C). Thus, whilst MelR recruits CRP at the wild-

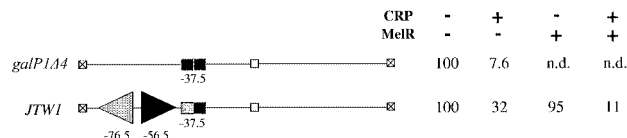


Fig. 3. Activity of the *galP1Δ4* and *JTW1* promoters. Fragments with the two promoters are illustrated schematically: *galP1Δ4* contains a DNA site for CRP (shown as a rectangle) at a location where CRP represses promoter activity, whilst *JTW1* is a derivative carrying a weakened DNA site for CRP (lighter shaded rectangle) and an upstream element carrying MeIR binding sites 1' and 1. The promoters were cloned into the *lac* expression vector, pRW50, and the figure shows β -galactosidase activities measured in different backgrounds either with or without CRP and MeIR. The activities (in arbitrary units) were measured in WAM134 ($\Delta melR \Delta crp \Delta lac$) cells containing the pRW50 derivatives, and either plasmid pDCRP, encoding *crp* (+CRP), or the control vector pDU9 (-CRP), together with either pLG314, encoding *melR* (+MeIR), or the control vector pLGARS (-MeIR).

type *melAB* promoter, the reciprocal interaction is seen at the *JK15* promoter, where CRP can recruit MeIR.

We reasoned that the co-operative interactions between MeIR and CRP may be sufficient to repress transcription initiation at a promoter where MeIR and CRP bind to sites overlapping those for RNAP. In order to investigate this, we performed an experiment with the *galP1Δ4* promoter, which is a derivative of the *E. coli galP1* promoter. In previous work, we demonstrated factor-independent transcription initiation at *galP1* but found that this initiation could be stimulated by the binding of CRP to a site centred at position -41.5 (Bingham *et al.*, 1986). However, when the DNA site for CRP was moved to position -37.5 in the *galP1Δ4* promoter, we found that CRP repressed the factor-independent transcription initiation (Bell *et al.*, 1990). In this study we constructed a derivative of the *galP1Δ4* promoter that contains a weakened DNA site for CRP such that CRP is less able to repress the promoter (Figure 3). A DNA element carrying MeIR-binding sites 1' and 1 was introduced immediately upstream of the DNA site for CRP, with identical spacing to that found at the *melAB* promoter. Expression from this promoter, *JTW1*, was then measured in different backgrounds either with or without CRP and MeIR (Figure 3). Our results show that MeIR alone hardly affects expression from the *JTW1* promoter, whilst in the presence of CRP, MeIR increases repression 3-fold, presumably because of co-operative interactions between MeIR and CRP.

Substitutions within CRP that affect transcription activation at the *melAB* promoter

To identify the residues of CRP that are important in transcription activation at the *melAB* promoter, a library of randomly mutated *crp* genes was created using error-prone PCR. Plasmids expressing mutated *crp* genes were transformed into a reporter strain carrying a *melAB* promoter::*lacZ* fusion, and colonies were screened on MacConkey lactose agar for CRP mutants that gave altered levels of expression from the *melAB* promoter whilst retaining the ability to bind DNA, as judged by repression of the *galP1Δ4* promoter. Six independent mutants were isolated, three with increased levels of expression, and three with decreased levels of expression from the *melAB* promoter. Sequencing of the mutated *crp* genes identified

Table I. Activation by mutant CRP at different promoters

Amino acid substitution	β -galactosidase activity (%) ^a <i>pmelAB::lac</i> fusion	β -galactosidase activity (%) ^b <i>CC(-61.5)::lac</i> fusion
Wild-type CRP	100	100
His159→Leu	95	5
Random mutants		
Thr158→Ile	225	10
Pro160→Ser	52	18
Gln164→Arg	56	18
Alanine scan mutants*		
Lys152→Ala	95	100
Gln153→Ala	95	100
Pro154→Ala	97	100
Asp155→Ala	92	95
Met157→Ala	105	95
Thr158→Ala	120	10
His159→Ala	80	90
Pro160→Ala	63	87
Asp161→Ala	105	95
Gly162→Ala	90	25
Met163→Ala	105	90
Gln164→Ala	105	100
Ile165→Ala	110	100
Lys166→Ala	61	100

^a β -galactosidase activities were measured in WAM134 ($\Delta melR \Delta crp \Delta lac$) cells containing pRW50 carrying the KK43 *melAB* promoter fragment, pLG314 encoding *melR*, and pDCRP or pYZCRP (*) encoding different mutant *crp* derivatives as indicated.

^b β -galactosidase activities were measured in WAM134 ($\Delta melR \Delta crp \Delta lac$) cells containing pRW50 carrying the *CC(-61.5)* promoter fragment and pDCRP or pYZCRP (*) encoding different mutant *crp* derivatives as indicated.

Activities are expressed relative to the activity measured in cells carrying pDCRP or pYZCRP (*) encoding wild-type CRP (taken as 100%). The results listed are the average of at least three independent determinations.

three substitutions within CRP. Two of these, PS160 and QR164, resulted in decreased levels of transcription activation at the *melAB* promoter, and the third, TI158, resulted in an increase in transcription activation (Table I). None of the three substitutions affected the ability of CRP to bind DNA as judged by band shift assays *in vitro* and repression of the *galP1Δ4* promoter *in vivo* (data not shown). Remarkably, all three substitutions identify residues that are located within AR1 of CRP, a surface that contacts α CTD during transcription activation at simple CRP-dependent promoters (i.e. promoters that are activated by a single CRP dimer alone; reviewed by Busby and Ebright, 1999). All three substitutions have previously been shown to reduce CRP-dependent transcription activation at simple CRP-dependent promoters (Zhou *et al.*, 1993, 1994; Niu *et al.*, 1994, 1996), and this was confirmed by measuring activation at the *CC(-61.5)* promoter, a simple Class I CRP-dependent promoter that is often used as a paradigm (Table I).

Previous studies by Ebright and colleagues exploited alanine scanning to find the key residues of AR1, and identified Thr158 as providing the most important side-chain for activation at simple CRP-dependent promoters (Niu *et al.*, 1994; Zhou *et al.*, 1994). Thus, expression from the *melAB* promoter was also measured in the presence of mutant CRP derivatives containing single alanine substitutions at amino acids 152–166 (Table I; note that residue

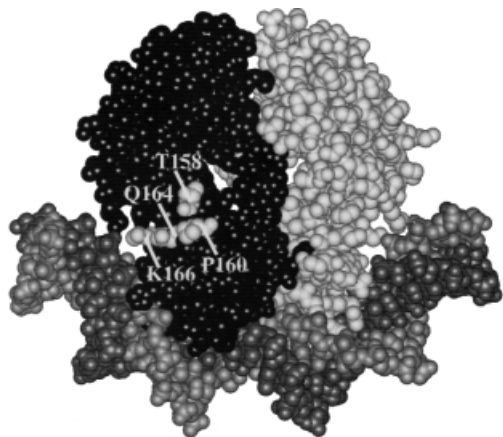


Fig. 4. Model of CRP. The figure shows a space-filling model of CRP bound to a 22 bp consensus DNA site (Parkinson *et al.*, 1996). Residues important in transcription activation at the *melAB* promoter are indicated.

156 of CRP is Ala). Substantial decreases in transcription activation were seen with alanine substitutions of Pro160 and Lys166 (Table I). Taken together, our results argue that residues Thr158, Pro160, Gln164 and Lys166 of CRP participate in transcription activation at the *melAB* promoter: these residues identify a surface of CRP that overlaps with AR1 (Figure 4).

Table I compares the effects of different substitutions in CRP on expression from the *melAB* promoter with expression from *CC(-61.5)*. The results show that substitutions at certain positions in CRP (e.g. Thr158 and His159) have different effects at the two promoters. This suggests that the role of the AR1-containing surface of CRP at the *melAB* promoter is different from its role at simple CRP-dependent promoters such as *CC(-61.5)*, where it interacts directly with α CTD. Thus, we have investigated the involvement of α CTD in CRP-dependent activation at the *melAB* promoter by using a preparation of *E.coli* RNAP, reconstituted with truncated α subunits that lack the C-terminal 73 residues. To do this, we used *in vitro* transcription assays with pSR plasmid carrying the KK43 fragment, such that transcripts initiating at the *melAB* promoter run to an efficient terminator in the pSR vector. In our previous work with purified wild-type RNAP, we showed that synthesis of the transcript initiating at the *melAB* promoter was dependent on the presence of purified MelR and melibiose (Belyaeva *et al.*, 2000). The transcript could be quantified by reference to the factor-independent synthesis of the RNA-1 transcript, and we showed that initiation of the transcript at the *melAB* promoter was stimulated nearly 2-fold by the presence of cAMP-CRP in the incubation (the reasons why CRP-dependent activation *in vitro* is much less than *in vivo* are not clear). Figure 5 shows the results of an experiment in which the transcript initiating at the *melAB* promoter was quantified after *in vitro* assays using preparations of *E.coli* RNAP reconstituted with wild-type full-length α subunits or truncated α -256 subunits that lack the C-terminal 73 residues, purified MelR and purified CRP. The results show that the truncation of the RNAP α subunit causes a reduction in transcription initiation from the *melAB* promoter. However, CRP is able to activate transcription from the *melAB* promoter with RNAP containing both full-

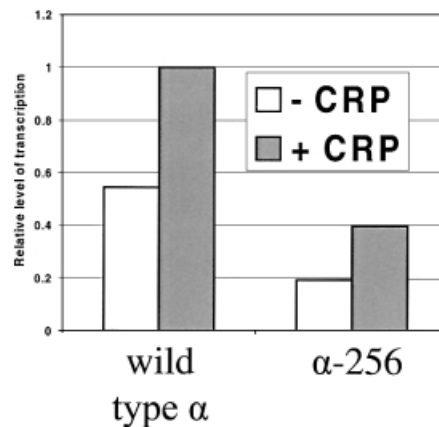


Fig. 5. *In vitro* transcription from the *melAB* promoter. The bar chart illustrates amounts of transcripts initiating at the *melAB* promoter on the KK43 fragment cloned in plasmid pSR. Transcripts were quantified relative to the factor-independent RNA-1 transcript. Assays were performed in the presence of melibiose-MelR and in the absence (open bars) or presence (shaded bars) of cAMP-CRP. Assays were performed with purified RNAP reconstituted with either full-length α (wild-type α) or truncated α lacking the C-terminal 73 residues (α -256).

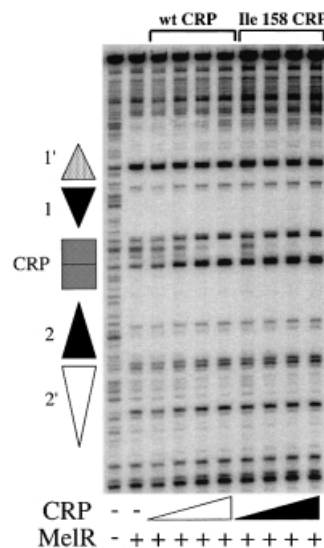


Fig. 6. DNase I footprint of complexes at the *melAB* promoter. The figure shows footprints obtained in the absence and presence of melibiose-MelR as indicated. Footprints were performed with no CRP, wild-type (wt) CRP (2, 5, 10 or 20 nM) or Ile158 CRP (2, 5, 10 or 20 nM) as shown. The position of MelR binding sites and the target for CRP are indicated by triangles and a rectangle, respectively.

length and truncated α subunits. The fact that the same level of CRP-dependent transcription activation is seen *in vitro* with both RNAP preparations argues that CRP activates transcription at the *melAB* promoter independently of α CTD. We conclude that the residues within AR1 that are important in transcription activation at the *melAB* promoter are not involved in a contact with α CTD.

In order to investigate further the role of residues in and around AR1 in transcription activation at the *melAB* promoter, we purified CRP carrying the T158 substitution that improves CRP-dependent activation of the *melAB* promoter *in vivo*. We then used DNase I footprinting to study the binding of the wild-type and mutant CRP to the *melAB* promoter. The results in Figure 6 show that, in the

presence of MeIR, both wild-type and mutant CRP bind to the CRP target centred at position –81.5 between MeIR binding sites 1 and 2. However, mutant Ile158 CRP binds to the promoter with a greater affinity than wild-type CRP and, consequently, the binding of MeIR to site 2' is increased. The simplest explanation for this is that residues in and around AR1 of CRP interact directly with a cognate surface of MeIR at the *melAB* promoter and that substitution of these residues results in altered binding of CRP and MeIR to the *melAB* promoter.

Discussion

MeIR and CRP bind together co-operatively at the *E.coli melAB* promoter to form a large, ordered nucleoprotein complex that covers >100 bp of DNA. The presence of CRP results in an increase in occupancy by MeIR of site 2' that overlaps the promoter –35 element. Our studies have shown that the removal of sites 1' and 1 upstream of the target for CRP abolishes CRP-dependent transcription activation because upstream-bound MeIR is essential for the recruitment of CRP. However, at the promoter (JK14) lacking MeIR-binding site 1' and 1, the introduction of a consensus DNA site for CRP (in the JK15 promoter) restores CRP-dependent transcription activation. This argues that the role of MeIR bound at sites 1' and 1 is to recruit CRP to its target and that upstream-bound MeIR is not essential for transcription activation. Working with the JK15 promoter, we were then able to show that pre-bound CRP can recruit MeIR to an adjacent binding site. Thus, MeIR and CRP bind co-operatively and this provides the mechanism of co-dependence at the *melAB* promoter. We were able to exploit this co-operative binding to create a promoter where MeIR and CRP can function co-operatively together to repress an otherwise factor-independent promoter.

MeIR and CRP interact at the *melAB* promoter to activate transcription, but this interaction can be exploited to repress transcription (Figure 3). In contrast, the anti-activator CytR interacts with CRP to repress transcription at some *E.coli* promoters (reviewed by Valentin-Hansen *et al.*, 1996). Interestingly, the CytR–CRP interaction can be exploited to activate transcription at an artificial derivative of the *lac* promoter (Rasmussen *et al.*, 1996). Repression of transcription by CytR is dependent upon a direct protein–protein interaction with CRP involving specific residues within both CytR and CRP (Kallipolitis *et al.*, 1997; Meibom *et al.*, 1999). Similarly, at the *melAB* promoter, activation by CRP appears to be dependent on direct protein–protein interactions with MeIR.

In the second part of this study, we identified substitutions within CRP that alter MeIR-dependent transcription activation at the *melAB* promoter. These substitutions identify the surface of CRP that interacts with MeIR (Figure 4). Surprisingly, these substitutions fall within or adjacent to AR1, the surface of CRP that interacts with α CTD at simple CRP-dependent promoters. Since CRP-dependent activation of the *melAB* promoter is independent of α CTD, we suggest that the role of the CRP surface containing AR1 at the *melAB* promoter is to contact MeIR rather than α CTD. Thus, at this complex promoter, AR1 of CRP has been diverted from its 'usual' task of interacting directly with RNAP.

In conclusion, MeIR and CRP bind to the *melAB* promoter co-operatively to adjacent sites in order to activate transcription in a co-dependent manner. This represents a novel mechanism of co-dependence upon two transcription activators at a bacterial promoter. Since this is such a simple mechanism, it is surprising that more examples have not been found at bacterial promoters, especially as this mechanism appears to be widely used in eukaryotes. One of the problems with this mechanism may be that it requires the two activators to be 'committed' to each other by carrying complementary contact surfaces, and such 'commitment' carries the price of evolutionary inflexibility. With the great increase in our knowledge of bacterial genome sequences, and with many parallel studies on different regulatory regions, it will be especially interesting to see whether the model we have developed for co-dependence at the *E.coli melAB* promoter turns out to be a 'one-off' or a paradigm for many other cases. It will also be interesting to see whether CRP can interact directly with any other member of the large AraC/XylS family of transcription activators.

Materials and methods

Strains, plasmids and promoters

The *E.coli* K-12 strain used in this work was WAM134, a $\Delta melR$ $\Delta crp::cm$ derivative of WAM131 (Belyaeva *et al.*, 2000).

Different *EcoRI*–*HindIII* fragments carrying promoters were cloned into the plasmid vectors pRW50 and pSR. pRW50 is a low copy number *lac* expression vector encoding resistance to 35 μ g/ml tetracycline (Lodge *et al.*, 1992), whilst pSR is a pBR322 derivative for cloning promoter fragments upstream of the λ *oop* transcription terminator (Kolb *et al.*, 1995). By convention, *melAB* promoter sequences are numbered with respect to the transcription start point with upstream and downstream locations denoted by '–' and '+' prefixes, respectively. The *melAB* promoter fragments all carry the *HindIII* site at position +36 and the *EcoRI* site at position –312 (KK81), –138 (KK43), –188 (KK39) or –94 (JK14), as described by Belyaeva *et al.* (2000). The Class I CRP-dependent *CC(-61.5)* promoter was also cloned on an *EcoRI*–*HindIII* fragment and used as described by Bell *et al.* (1990) and Zhou *et al.* (1994).

Plasmid pLG314, encoding *melR* expressed from the CRP-independent *galP2* promoter, was constructed by subcloning the *EcoRI*–*BamHI* fragment from plasmid pCM118-314 (Michán *et al.*, 1995) into the *tet^R* gene of the vector plasmid pLG339, which encodes resistance to 25 μ g/ml kanamycin (Stoker *et al.*, 1982). pLG339 Δ ARS is a derivative of pLG339 carrying a deletion between the *EcoRI* and *SalI* sites that results in the loss of *tet^R* (Belyaeva *et al.*, 2000). Wild-type and mutant *crp* derivatives were cloned in plasmids pDCRP (Bell *et al.*, 1990) and pYZCRP (Zhou *et al.*, 1991), which are pBR322 derivatives encoding resistance to 80 μ g/ml ampicillin. As a control plasmid, we used pDU9, a derivative of pDCRP with the *crp* gene replaced by the *EcoRI*–*HindIII* pUC9 polylinker (Bell *et al.*, 1990).

Construction of KK43 p75A

The starting fragment was KK43 (Figure 1A). To construct KK43 p75A, the *EcoRI*–*BglII* fragment was replaced with a PCR-amplified fragment generated from KK43 using primer D26440 (5'-AACTCAGATCTTTCGTGAAGCAGC-3') and a primer upstream of the *EcoRI* site.

Construction of JK15 and JK15+2

The starting material was the JK14 fragment (Figure 2B). To construct JK15, the JK14 fragment was extended using PCR with primers D27153 (5'-GCAGAATTCAAATGTGATGTACATCACAGGATCTGAGTTTATGG-3') and D14314 (5'-GCAAAGCTTGGATGTCAGGTCGACGGATCTC-3'). To construct JK15+2, the JK14 fragment was extended using PCR with primers D19007 (5'-GCAGAATTCAAATGTGATGTACATCACAGGATCTGAGTTTATGG-3') and D14314.

Construction of JTW1

The starting fragments were *galP1Δ4* (Bell *et al.*, 1990) and KK43 (Figure 1A). To construct JTW1, an *NcoI*–*HindIII* fragment was generated by PCR amplification from *galP1Δ4* using primer D29613 (5'-AATAATCCATGGTGCACACTGCATCTTTTTATGCTATGG-3') and a primer downstream of the *HindIII* site. An *EcoRI*–*NcoI* fragment was generated by PCR amplification from KK43 using primer D29614 (5'-AATAATCCATGGGCAGTAAATCTGAGTTTATGGG-3') and a primer upstream of the *EcoRI* site. The *EcoRI*–*NcoI* and *NcoI*–*HindIII* fragments were ligated together to form the JTW1 promoter.

Screen of library of mutant *crp* derivatives

Error-prone PCR (Zhou *et al.*, 1991) was used to construct a library of mutant *crp* derivatives. pDCRP was used as a template with primers D3407 (5'-AGGCGTATCACGAGGCCCT-3') and D4600 (5'-GTAGTCGGTGTGTTTAC-3'), which anneal upstream of the *EcoRI* and downstream of the *HindIII* sites, respectively. *EcoRI*–*HindIII* fragments containing mutagenized *crp* derivatives were subcloned into pDCRP. After eight libraries of pDCRP derivatives had been screened by monitoring expression of the KK43::*lacZ* fusion, the base sequence of the entire *EcoRI*–*HindIII* insert was determined for all the selected mutants.

Proteins

MelR was overexpressed using the T7 expression plasmid, pCM117-303, as described by Michán *et al.* (1995). The overexpressed MelR was purified using the method of Caswell *et al.* (1992), as described by Belyaeva *et al.* (2000). CRP was purified using the method of Ghosaini *et al.* (1988), as described by Savery *et al.* (1998). Holo DNA-dependent RNAP containing either a full-length or a truncated α subunit was a gift from M.Thomas (see Meng *et al.*, 2000).

In vitro transcription assays

These were performed using the system described by Kolb *et al.* (1995), as described by Savery *et al.* (1998). Briefly, the KK81 fragment carrying the *melAB* promoter was cloned into plasmid pSR and the resulting recombinant was purified by caesium chloride gradient centrifugation and used as template for RNA synthesis. After addition of different factors and purified RNAP, a cocktail of labelled nucleoside triphosphate and heparin was added. Transcription initiation at the *melAB* promoter generates an easily detectable discrete transcript that terminates at the *oop* terminator, with the control RNA-1 from the ColE1 origin acting as an internal control. The incubation mixture contained 5% glycerol, 20 mM Tris pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 50 μ g/ml BSA, 200 μ M cAMP, 10 nM melibiose and 10 nM supercoiled plasmid DNA template. MelR (1500 nM) and CRP (50 nM) were added as appropriate, and incubated with 20 nM RNAP for a further 5 min prior to the addition of 200 μ M ATP, CTP and GTP, 10 μ M α -³²P-labelled UTP and 100 μ g/ml of heparin. ³²P-labelled RNA was analysed on calibrated polyacrylamide sequencing gels precisely as before (Savery *et al.*, 1998). Band intensities were quantified using ImageQuant software (Molecular Dynamics).

Footprinting

DNase I and hydroxyl radical footprinting experiments were performed using protocols described by Savery *et al.* (1996). In these experiments, incubations contained 4–10 nM of the purified KK81 or KK39 *EcoRI*–*HindIII* fragment that had been end-labelled at the *HindIII* site with [γ -³²P]ATP by polynucleotide kinase. Incubations contained 10 nM melibiose, 1000 nM MelR unless otherwise stated, and 200 μ M cAMP, 75 nM CRP unless otherwise stated. After DNase I or hydroxyl radical treatment, footprint patterns were analysed on polyacrylamide sequencing gels that were calibrated with Maxam–Gilbert sequence ladders.

Measurement of promoter activities in vivo

DNA fragments containing the promoters were cloned into pRW50, a low copy number *lac* expression vector, to generate promoter::*lac* fusions. β -galactosidase levels in cells were measured by the Miller (1972) method: cells were grown in media either with or without melibiose exactly as in our previous work (Webster *et al.*, 1987), and all assays were performed at least three times on independent transformants. Assays were performed in a Δ *melR* Δ *crp* (WAM134) background carrying different plasmids encoding *crp* and *melR*.

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References

- Bell,A., Gaston,K., Williams,R., Chapman,K., Kolb,A., Buc,H., Minchin,S., Williams,J. and Busby,S. (1990) Mutations that alter the ability of the *Escherichia coli* cyclic AMP receptor protein to activate transcription. *Nucleic Acids Res.*, **18**, 7243–7250.
- Belyaeva,T., Wade,J., Webster,C., Howard,V., Thomas,M., Hyde,E. and Busby,S. (2000) Transcription activation at the *Escherichia coli melAB* promoter: the role of MelR and the cyclic AMP receptor protein. *Mol. Microbiol.*, **36**, 211–222.
- Bingham,A., Ponnambalam,S., Chan,B. and Busby,S. (1986) Mutations that reduce expression from the P2 promoter of the *Escherichia coli* galactose operon. *Gene*, **41**, 67–74.
- Browning,D., Cole,J. and Busby,S. (2000) Suppression of FNR-dependent transcription activation at the *Escherichia coli nir* promoter by Fis, IHF and H-NS: modulation of transcription initiation by a complex nucleo-protein assembly. *Mol. Microbiol.*, **37**, 1258–1269.
- Busby,S. and Ebricht,R. (1994) Promoter structure, promoter recognition, and transcription activation in prokaryotes. *Cell*, **79**, 743–746.
- Busby,S. and Ebricht,R. (1999) Transcription activation by catabolite activator protein (CAP). *J. Mol. Biol.*, **293**, 199–213.
- Caswell,R., Williams,J., Lyddiatt,A. and Busby,S. (1992) Overexpression, purification and characterisation of the *E. coli* MelR transcription activator protein. *Biochem. J.*, **287**, 493–499.
- Gallegos,M.-T., Schleif,R., Bairoch,A., Hofmann,K. and Ramos,J.-L. (1997) AraC/XylS family of transcriptional regulators. *Microbiol. Mol. Biol. Rev.*, **61**, 393–410.
- Ghosaini,L., Brown,A. and Sturtevant,J. (1988) Scanning calorimetric study of the thermal unfolding of catabolite activator protein from *Escherichia coli* in the absence and presence of cyclic mononucleotides. *Biochemistry*, **27**, 5257–5261.
- Goosen,N. and van de Putte,P. (1995) The regulation of transcription initiation by integration host factor. *Mol. Microbiol.*, **16**, 1–7.
- Kallipolitis,B., Norregaard-Madsen,M. and Valentin-Hansen,P. (1997) Protein–protein communication: structural model of the repression complex formed by CytR and the global regulator CRP. *Cell*, **89**, 1101–1109.
- Kolb,A., Busby,S., Buc,H., Garges,S. and Adhya,S. (1993) Transcriptional regulation by cAMP and its receptor protein. *Annu. Rev. Biochem.*, **62**, 749–795.
- Kolb,A., Kotlarz,D., Kusano,S. and Ishihama,A. (1995) Selectivity of the *Escherichia coli* RNA polymerase σ^{38} for overlapping promoters and ability to support CRP activation. *Nucleic Acids Res.*, **23**, 819–826.
- Lodge,J., Fear,J., Busby,S., Gunasekaran,P. and Kamini,N. (1992) Broad host-range plasmids carrying the *E. coli* lactose and galactose operons. *FEMS Microbiol. Lett.*, **74**, 271–276.
- Meibom,K., Sogaard-Andersen,L., Mironov,A. and Valentin-Hansen,P. (1999) Dissection of a surface-exposed portion of the cAMP–CRP complex that mediates transcription activation and repression. *Mol. Microbiol.*, **32**, 497–504.
- Meng,W., Savery,N., Busby,S. and Thomas,M. (2000) The *Escherichia coli* RNA polymerase α subunit linker: length requirements for transcription activation at CRP-dependent promoters. *EMBO J.*, **19**, 1555–1566.
- Merika,M. and Thanos,D. (2001) Enhanceosomes. *Curr. Opin. Genet. Dev.*, **11**, 205–208.
- Michán,C., Busby,S. and Hyde,E. (1995) The *Escherichia coli* MelR transcription activator: production of a stable fragment containing the DNA-binding domain. *Nucleic Acids Res.*, **23**, 1518–1523.
- Miller,J. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Niu,W., Zhou,Y., Dong,Q., Ebricht,Y. and Ebricht,R. (1994) Characterization of the activating region of *Escherichia coli* catabolite gene activator protein (CAP). I. Saturation and alanine-scanning mutagenesis. *J. Mol. Biol.*, **243**, 595–602.

- Niu,W., Kim,Y., Tau,G., Heyduk,T. and Ebright,R. (1996) Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. *Cell*, **87**, 1123–1134.
- Parkinson,G., Wilson,C., Gunasekara,A., Ebright,Y., Ebright,R. and Berman,H. (1996) Structure of the CAP–DNA complex at 2.5 Å resolution: a complete picture of the protein–DNA interface. *J. Mol. Biol.*, **260**, 395–408.
- Rasmussen,P., Holst,B. and Valentin-Hansen,P. (1996) Dual-function regulators: the cyclic AMP receptor protein and CytR can act either to repress or to activate transcription depending on the context. *Proc. Natl Acad. Sci. USA*, **93**, 10151–10155.
- Richet,E., Vidal-Ingigliardi,V. and Raibaud,O. (1991) A new mechanism for coactivation of transcription: repositioning of an activator triggered by the binding of a second activator. *Cell*, **66**, 1185–1195.
- Savery,N., Belyaeva,T. and Busby,S. (1996) Protein:DNA interactions. In Docherty,K. (ed.), *Gene Transcription: DNA Binding Proteins, Essential Techniques*. Wiley/BIOS Press, Chichester, UK, pp. 1–5 and 21–33.
- Savery,N., Lloyd,G., Kainz,M., Gaal,T., Ross,W., Ebright,R., Gourse,R. and Busby,S. (1998) Transcription activation at class II CRP-dependent promoters: identification of determinants in the C-terminal domain of the RNA polymerase α subunit. *EMBO J.*, **17**, 3439–3447.
- Scott,S., Busby,S. and Beacham,I. (1995) Transcriptional co-activation at the *ansB* promoters: involvement of the activating regions of CRP and FNR when bound in tandem. *Mol. Microbiol.*, **18**, 521–531.
- Stoker,N., Fairweather,N. and Spratt,B. (1982) Versatile low-copy number plasmid vectors for cloning in *Escherichia coli*. *Gene*, **18**, 335–341.
- Valentin-Hansen,P., Sogaard-Andersen,L. and Pedersen,H. (1996) A flexible partnership: the CytR anti-activator and the cAMP-CRP activator protein, comrades in transcriptional control. *Mol. Microbiol.*, **20**, 461–466.
- Webster,C., Kempell,K., Booth,I. and Busby,S. (1987) Organisation of the regulatory region of the *Escherichia coli* melibiose operon. *Gene*, **59**, 253–263.
- Zhou,Y., Zhang,X. and Ebright,R. (1991) Random mutagenesis of gene-sized DNA molecules by use of PCR with *Taq* DNA polymerase. *Nucleic Acids Res.*, **19**, 6052.
- Zhou,Y., Zhang,X. and Ebright,R. (1993) Identification of the activating region of catabolite gene activator protein (CAP): isolation and characterization of mutants of CAP specifically defective in transcription activation. *Proc. Natl Acad. Sci. USA*, **90**, 6081–6085.
- Zhou,Y., Merkel,T. and Ebright,R. (1994) Characterization of the activating region of *Escherichia coli* catabolite gene activator protein (CAP). II. Role at class I and class II CAP-dependent promoters. *J. Mol. Biol.*, **243**, 603–610.

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