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The role of two surface exposed loops in transcription activation by the *Escherichia coli* CRP and FNR proteins

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ABSTRACT

We have investigated a number of mutations that alter the ability of the the *E.coli* transcription factors CRP and FNR to activate transcription. In CRP, some mutations at position 159 (H159L, H159I and Δ159) prevent transcription activation at a number of naturally-occurring and semi-synthetic CRP-dependent promoters. We suggest that some feature of the surface-exposed turn around residue 159 is recognised by RNA polymerase during transcription activation at these promoters. Mutations at position 52 increase CRP activity and reverse the effects of H159L and Δ159, most likely by creating a new contact with RNA polymerase. However this new contact only gives increased expression when the CRP binding site is located 41½ base pairs upstream of the transcription start site and fails to reverse the effects of H159L and Δ159 at promoters where the CRP site is located further upstream. To explain our results we propose that the two surface-exposed turns around residues 52 and 159 contain elements that are potential RNA polymerase docking sites: in the CRP dimer these two active patches are located on adjacent faces of different subunits. FNR, a related transcription activator, contains amino acid sequences homologous to the CRP sequence around position 52. Mutations in this zone (from residues 81 – 88 in FNR) reduce expression from an FNR-dependent promoter without stopping FNR binding to its target. This defines a patch on FNR, which is homologous to the CRP surface-exposed loop around position 52, which is involved in transcription activation, most likely by contacting RNA polymerase.

INTRODUCTION

The *Escherichia coli* cyclic AMP receptor protein (CRP or CAP) controls the transcription of numerous genes involved in carbon source utilisation. In response to increases in the intracellular level of cyclic AMP (cAMP), dimeric CRP undergoes a conformational change that allows the cAMP-CRP complex to bind to specific 22 base-pair sequences at target promoters and activate transcription initiation (1,2). Interestingly, these binding sites for CRP are not all found at the same distance from the transcription start at target promoters. For example, cAMP-CRP binds to a site centred 61½ bp upstream from the *lacP1* transcription start, at the *malT* promoter the site is at −70½, whilst in the *gal* operon, CRP binds 41½ bp upstream of the *galP1* start (2,3). It is not clear whether CRP activates transcription by a common mechanism at these different promoters.

'CRP is a dimer of two identical subunits, each composed of 209 amino acid residues. From crystallographic studies, it is clear that each CRP subunit folds into two domains: the larger N-terminal domain is responsible for cAMP binding and dimer contacts, while the C-terminal domain contains a helix-turn-helix motif involved in DNA-binding (4). Although there are several ways in which CRP might stimulate transcription, most of the available evidence suggests that CRP contacts RNA polymerase (reviewed in 5). However, despite our knowledge of CRP structure, it is not clear which regions are involved in transcription activation. To attempt to locate sites on the surface of CRP that interact with RNA polymerase, a number of groups have isolated positive control mutants: these are mutations that affect transcription activation without altering the ability of CRP to recognise its target site (6–8). Some of these 'positive control' mutations may identify amino acid side chains that directly contact RNA polymerase.

We have focussed our attention on one particular positive control mutant, CRP H159L, that contains a leucine for histidine substitution at position 159. In previous work (7), we showed that the H159L substitution blocks transcription initiation at two synthetic promoters carrying CRP-binding sites located at −41½ and −61½ with respect to the transcription startsite (these distances correspond exactly to the positions of the CRP-binding sites at the *E.coli gal* and *lac* promoters). Since H159 is located in an exposed loop on the surface of CRP, it is possible that the 'patch' recognised by RNA polymerase is a part of this loop. In this work we have further investigated the effects of changing residue 159.

In our previous report (7), we described a second site revertant, K52N, which restores the ability of CRP carrying the H159L mutation to activate transcription. Since this second mutation affects a residue that lies on the opposite face of the CRP subunit to H159, it seems unlikely that changes at position 52 can directly recreate the same contact destroyed by H159L. We found that

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this revertant can only activate transcription at promoters where the CRP site is located 41½ bp upstream of the transcription start. Residue 52 is located on the surface of CRP in a loop between two β-sheets: here we present evidence that the mutation K52N creates a second 'patch' on the surface of CRP that can interact with RNA polymerase.

CRP belongs to a family of bacterial activator proteins which also includes FNR, an *E.coli* transcription factor required for adaptation to growth in the absence of oxygen. Like CRP, the consensus DNA binding site for FNR is also a 22bp inverted repeat: it has been demonstrated that the consensus sites for CRP and FNR differ at just two symmetry-related positions (9−11). Because of their similarities, it is most likely that CRP and FNR activate transcription by a related mechanism. Here, we show that mutations in FNR, in the region homologous to residues 52−59 of CRP, reduce expression from an FNR-dependent promoter without affecting its ability to bind to its target site *in vivo*. Differences in the location of the 'activator patch' in CRP and FNR are discussed.

MATERIALS AND METHODS

We have determined the effects of mutant CRP and FNR derivatives at a number of test promoters illustrated in Figure 1. These promoters were cloned on *Eco*RI-*Hin*dIII fragments into the broad host range, *lac* expression vector, pRW2 (12), such that *lac* expression was under the control of the test promoter. These recombinants were then transformed into a Δ*crp* derivative of Δ*lac* strain M182 or the Δ*fnr* Δ*lac* strain, JRG1728. Mutant alleles of CRP or FNR could then be introduced on compatible plasmids in order to determine their effects on β-galactosidase expression from the test promoters.

For experiments with CRP, the *Hin*dIII-*Eco*RI fragment carrying the *crp* gene described by Bell *et al.* (7) was cloned in M13mp18 and mutations were created using synthetic oligonucleotides and the Amersham Site Directed Mutagenesis Kit. In each case the sequence was checked by the dideoxy method using the Pharmacia T7 kit. *Hin*dIII-*Eco*RI fragments carrying mutated derivatives of *crp* were then cloned into plasmid pDU9 (7) to give pDCRP derivatives. These were then transformed into M182Δ*crp* cells carrying pRW2 containing a CRP-sensitive promoter running *lac* expression. Note that pDCRP and pRW2 can be maintained in the same host because they are compatible plasmids encoding resistance to different antibiotics. The promoters *CCpmelR*, *CC+20pmelR* and *galP1*Δ4, illustrated in Fig 1, the plasmids pDU9, pDCRP, pRW2 and strain M182Δ*crp* have been described previously (7).

For experiments with FNR, the *Hin*dIII-*Bam*HI fragment carrying the *fnr* gene described by Spiro *et al.*(13) was cloned in M13mp19 and mutations were introduced as above. *Hin*dIII-*Bam*HI fragments carrying mutated derivatives of *fnr* were then cloned into plasmid pFNR (13). The resulting mutant derivatives were then transformed into the Δ*fnr* strain, JRG1728, carrying pRW2 containing an FNR-sensitive promoter controlling *lac* expression. The promoters *FFpmelR* and *ndh*, illustrated in Figure 1, the plasmid pFNR and strain JRG1728 have been described previously (13).

Cells containing pDCRP or pFNR derivatives were grown in media containing 80 μg/ml ampicillin. Cells carrying pRW2 derivatives were grown in media with 35μg/ml tetracycline. To measure CRP-dependent gene expression, cells were grown aerobically in minimal media containing fructose, whilst for FNR-dependent expression, cells were grown in L-broth plus glucose as previously described (7,13). β-galactosidase activity in cell extracts was measured by the Miller method (14). Arabinose isomerase (the product of the *araA* gene) was assayed exactly as described by Schleif *et al.* (15).

Other strains used in this work were pop2492 which is Δ*crp* and carries a chromosomal *malT-lacZ* fusion (16), and pop1239 which is Δ*crp* *lac*$^+$ *ara*$^+$. Both strains were donated by Olivier Raibaud of Institut Pasteur, Paris. pop1239 is a derivative of HfrG61 with Δ*crp* transduced from CA8439 (17).

Standard methods were used for plasmid isolation, restriction fragment purification and cloning throughout this work (18).

RESULTS AND DISCUSSION

Mutations in CRP at position 159 affect transcription activation

Previously, we reported that CRP carrying the H159L mutation failed to activate transcription from either of two synthetic CRP-dependent promoters *CCpmelR* and *CC+20pmelR* (illustrated in Figure 1) at which synthetic CRP-binding sequences were positioned at −41½ and −61½, respectively, upstream of the

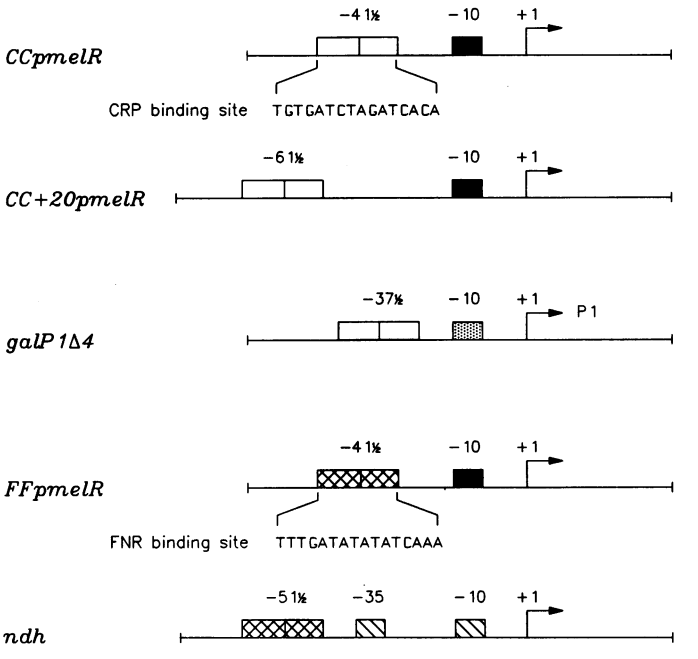

Figure 1. Schematic representation of test promoters used during this work. Line 1 illustrates the synthetic CRP-dependent promoter *CCpmelR* which contains a tight binding 22 bp CRP site (open boxes) cloned upstream from the *melR* −10 sequence (shaded). The CRP site is centred at −41½bp relative to the *melR* transcription startsite (+1). Line 2 illustrates *CC+20pmelR* which carries a CRP binding site positioned 61½bp upstream of the *melR* transcription start. This promoter is derived from *CCpmelR* by the insertion of a 20bp linker between the CRP site (open) and the *melR* −10 sequence (shaded). The *galP1*Δ4 promoter shown on line 3 can be repressed by CRP: it is a derivative of *galP1*, made by removing 4bp between the *galP1* −10 sequence (stippled) and the CRP site (open). *CCpmelR*, *CC+20pmelR* and *galP1*Δ4 have been described previously (10,3,7). *FFpmelR*, line 4, is a synthetic FNR-dependent promoter which carries the 22bp consensus FNR binding site (cross hatched) cloned 41½bp upstream from the *melR* transcription start (10). Line 5 shows the organisation of the *ndh* promoter which is repressed by FNR. The binding site for FNR (cross hatched), −35 and −10 hexamer sequences (hatched) and transcription start (+1) are indicated (13,21).

melR transcription startsite (7). Experiments both *in vivo* and *in vitro* showed that, whilst CRP bound to target sites at these promoters, it failed to interact productively with RNA polymerase to activate transcription. Since H159 lies in a surface-exposed turn of CRP, we reasoned that H159 might make an essential contact with RNA polymerase. To investigate this, we made a number of different changes in the *crp* gene, cloned in plasmid pDCRP, that altered codon 159. To determine the effects of these changes on CRP activity, the pDCRP derivatives were introduced into M182Δ*crp* cells carrying pRW2 containing *CCpmelR* or *CC+20pmelR*. The data in Figure 2 show that expression from both *CCpmelR* and *CC+20pmelR* is dependent on CRP and is suppressed by the mutations H159L or H159I as well as by deletion of H159. In contrast, substantial activity from both promoters was found with CRP H159A and H159S.

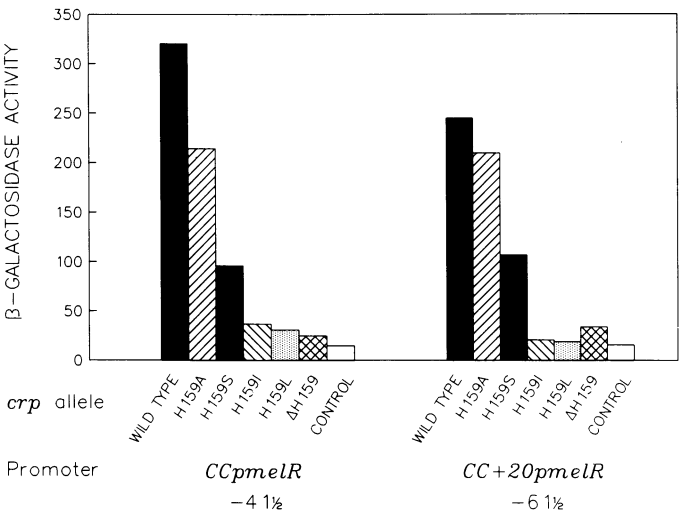

Figure 2. Transcription activation by CRP and derivatives carrying different changes at position 159. The height of the differently shaded bars in the figure represent β-galactosidase expression, determined in M182Δ*crp* cells carrying derivatives of the *lac* expression vector, pRW2, containing either the *CCpmelR* or *CC+20pmelR* promoter sequences. pDCRP derivatives carrying wild type CRP or CRP containing different changes at position 159 were introduced as indicated on the abcissa. M182Δ*crp* cells transformed with pDU9, in which the *Eco*RI-*Hin*dIII fragment carrying *crp* has been replaced by a polylinker, were used as a control for these experiments. Cells were grown aerobically in minimal medium containing fructose as a carbon source and β-galactosidase assays were performed as described in Materials and Methods.

We checked that none of these mutations prevented binding of CRP to its target site by exploiting *galP1*Δ4 (Figure 1, line 3): this promoter is active in the absence of CRP, but expression is strongly inhibited by CRP. We previously showed that the *galP1*Δ4 promoter can be used to measure CRP binding to its target site *in vivo* (7). pDCRP derivatives carrying H159L, H159I, ΔH159, H159A, H159S or wild type CRP were transformed into M182Δ*crp* cells carrying the *galP1*Δ4 promoter controlling *lac* expression in pRW2. In each case, expression from *galP1*Δ4 was totally suppressed, indicating that the different mutations at position 159 have little or no effect on DNA binding (data not shown).

These results show that H159 cannot be essential for the activation function of CRP since changes to alanine or serine produce only small effects. It is possible that the total blockage of CRP-dependent expression by H159L, H159I and ΔH159 is due to conformational effects on neighbouring residues. Interestingly, the different substitutions at position 159 have similar effects at both *CCpmelR* and *CC+20pmelR*, suggesting that the region around position 159 plays the same role at these two promoters despite the differing location of the CRP site. To check that altering H159 affects expression from natural CRP-dependent promoters as well as semi-synthetic promoters, we measured expression of the wild-type *malT*, *lac* and *araBAD* promoters in the presence of CRP H159L.

The effect of the H159L substitution on the *malT* promoter, where the CRP site is centred at −70½ (16), was determined by introducing derivatives of pDU9 carrying either *crp* or *crp* H159L into the Δ*crp* strain pop2492, that carries a *malT-lacZ* fusion (16). The levels of β-galactosidase expression in cells containing either CRP or CRP H159L are shown in Table I.

Table I. Effects of CRP and CRP H159L on expression from different promoters

Strain[1]	*Promoter and enzyme assayed*[2]	*Supplement to medium*[3]	*β-galactosidase activity*[4]
pop2492 [pDU9]	*pmalT-lacZ*	–	15
pop2492 [pDCRP]	*pmalT-lacZ*	–	900
pop2492 [pDCRP H159L]	*pmalT-lacZ*	–	50
pop1239 [pDU9]	*plac-lacZ*	1mM IPTG	42
pop1239 [pDCRP]	*plac-lacZ*	–	30
pop1239 [pDCRP]	*plac-lacZ*	1mM IPTG	1815
pop1239 [pDCRP H159L]	*plac-lacZ*	1mM IPTG	98
Strain[1]	*Promoter and enzyme assayed*[2]	*Supplement to medium*[3]	*AraA activity*[5]
pop1239 [pDU9]	*para-araA*	–	0.01
pop1239 [pDU9]	*para-araA*	10mM Arabinose	0.25
pop1239 [pDCRP]	*para-araA*	–	0.04
pop1239 [pDCRP]	*para-araA*	10mM Arabinose	1.80
pop1239 [pDCRP H159L]	*para-araA*	–	0.04
pop1239 [pDCRP H159L]	*para-araA*	10mM Arabinose	2.40

Notes:
1. pop2492 and pop1239 are *crp*-delete strains. pDCRP is a pBR322-based plasmid carrying a *crp* insert and pDU9 is the control plasmid with no insert.
2. pop2492 carries a fusion between *malT* and *lacZ*. In pop1239 expression of *lacZ* and *araA* products are driven by their respective promoters.
3. Cells were grown in M9 minimal medium with fructose as a carbon source, 80 μg/ml ampicillin and additions of IPTG or arabinose as shown for induction of the *lac* or *ara* promoters respectively.
4. β-galactosidase activities were measured by the standard method and are expressed in Miller units. Each assay was performed independently at least three times.
5. *AraA* product activities (arabinose isomerase) were measured as described in reference 15. The units are μmoles of product/minute/mg of total protein.

From the data, it is clear that the *malT* promoter is strongly activated by CRP but this activity is sharply reduced in the presence of CRP H159L. Similarly, the effect of H159L on expression from the wild type *lac* promoter (where CRP binds to a site centred at −61½) was determined by introducing CRP or CRP H159L into the Δ*crp lac*$^+$ strain, pop1239. The data in Table I show that CRP is essential for transcription of the *lac* operon and that CRP-dependent expression is suppressed by H159L. Finally, we determined the effect of CRP H159L on *araBAD* expression in the strain pop1239. Cells were grown in media supplemented with arabinose and the level of arabinose isomerase (*araA*) activity was assayed. The data in Table I show that expression of *araA* is dependent on CRP but, unlike *lac* and *malT*, this activity is not reduced in the presence of H159L. At the *araBAD* promoter CRP binds to a site at −93½ but this is separated from the *araBAD* transcription site by AraC binding sites I_1 and I_2 (24).

Mutations at position 52 affect a surface-exposed loop that can play a role in transcription activation

In our previous study we described a second site revertant, carrying an asparagine for lysine substitution at position 52, that restores activity to CRP H159L (7). Surprisingly, this doubly-mutated CRP, H159L K52N, is only active at the *CCpmelR* promoter, where the CRP site is positioned at −41½. To determine whether K52N could 'reeducate' CRP carrying other changes at position 159, K52N was introduced into the ΔH159 derivative of CRP. Effects on CRP activity at *CCpmelR* and *CC+20pmelR* were then measured as above. The data in Figure 3, show that CRP-dependent expression of *CCpmelR* is blocked by both H159L and ΔH159, but is restored by K52N. We also introduced K52N into CRP carrying the G162C substitution: according to Eschenlauer and Reznikoff (8), changes at G162, which is in the same surface-exposed loop as H159, affect the positive control function of CRP. Data in Figure 3 show that G162C stops expression from *CCpmelR* and that expression is restored by the K52N substitution. Note that the doubly-mutated CRP derivatives activate transcription better than wild-type CRP at this promoter. In contrast, whilst H159L, ΔH159 and G162C also block CRP-dependent transcription from *CC+20pmelR*, their effects are not reversed by K52N.

These results argue that the effect of K52N is independent of the nature of the change at position 159 (or 162). Inspection of the CRP stucture (references 4 & 19 and Figure 8) shows that position 52 is located in a second surface-exposed turn between β sheets 4 and 5 on the opposite face of the CRP monomer to position 159. Thus, it is unlikely that K52N can recreate the same contact destroyed by H159L. Rather, we suggest that K52N allows a new contact between CRP and RNA polymerase that is only productive at promoters such as *CCpmelR* where the CRP site is located 41½ base pairs upstream of the transcription startpoint. Some evidence for this comes from the data in Figure 4 that show the effects of a number of different amino acid substitutions at residue 52 on CRP-dependent expression from *CCpmelR* and *CC+20pmelR*. The results show that the mutations K52D, K52N or K52L cause 3−5 fold increases in expression from *CCpmelR* whilst not increasing the activity of *CC+20pmelR*.

A simple explanation for the results in Figure 4 is that removal of K52 unmasks a second activating 'patch' on the surface of CRP that is productive at promoters where CRP binds at −41½, but not other distances. We reasoned that it may be possible to identify new mutations that destroyed this second activating 'patch'. Thus, starting from CRP K52N H159L, we altered residues E54, G56 or E58, all of which lie in the same surface-exposed loop as residue K52, and measured the effects of these changes on expression from *CCpmelR*. The results in Figure 5, show that replacing E54 has very little effect on expression of *CCpmelR*, whilst the changes G56A and E58N reduce expression between 5−6 fold. To check that these new mutations did not affect CRP binding to target sites, we measured their effects on expression from the *galP1*Δ4 promoter (Figure 1, line 3). The

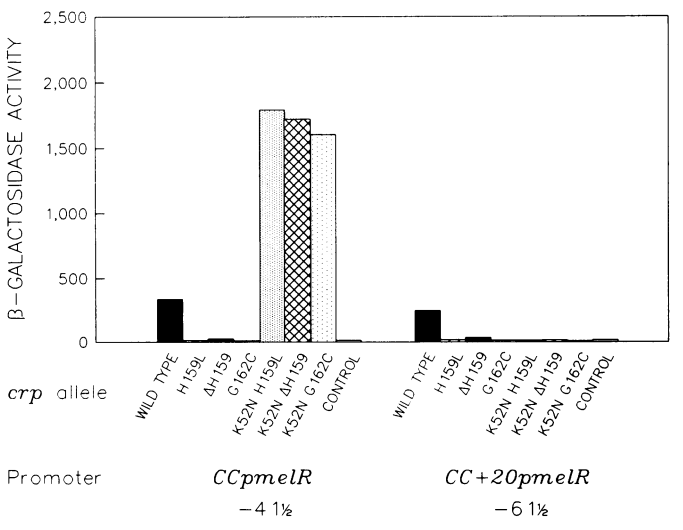

Figure 3. Activation of *CCpmelR* and *CC+20pmelR* by CRP and derivatives. The height of the bars in the histogram represent the activities of the *CCpmelR* and *CC+20pmelR* promoters, cloned in pRW2, measured in M182Δ*crp* cells. pDCRP, or derivatives carrying changes in CRP at positions 52, 159 and 162, were introduced as indicated. pDU9, in which the *crp* gene has been removed, was used as a control.

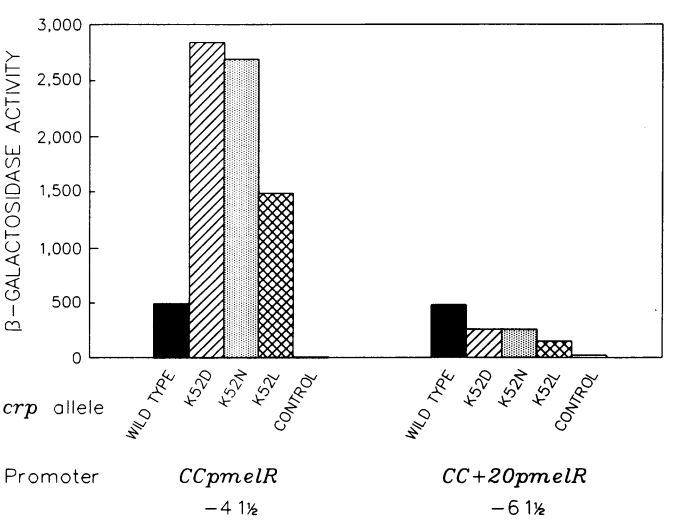

Figure 4. Effect of different substitutions at position 52 of CRP on activation of *CCpmelR* and *CC+20pmelR*. The bars represent β-galactosidase expression in M182Δ*crp* cells carrying *CCpmelR* or *CC+20pmelR* cloned in the *lac* expression vector, pRW2. pDCRP encoding either wild type CRP or CRP carrying different substitutions at position 52 were introduced as indicated.

data in Figure 5 show that each mutant CRP repressed expression from *galP1*Δ4 to same extent as wild-type CRP, showing that each CRP derivative was fully able to bind to target sites *in vivo*. Thus, changes at G56 and E58 interfere with activation of expression from *CCpmelR* by CRP K52N H159L. Interestingly, the mutations G56A or E58N have very little effect on the ability of wild type CRP to activate *CCpmelR* (not shown). The simplest explanation for our data is that changes at position 52 alter the conformation of the loop between β-sheets 4 and 5 to facilitate interaction with RNA polymerase, and that this improvement can be reversed by the changes at G56 or E58.

Mutations in FNR that affect transcription activation

The results with CRP suggested that two surface exposed loops, containing residues 52−58 and 156−163, can play a role in transcription activation. Since CRP and FNR are related in both structure and function, it is likely that they activate transcription by similar mechanisms. Therefore, we asked if the corresponding regions of FNR are also important for positive control function. Alignment of the amino acid sequences of CRP and FNR (9) shows that residues around H159 of CRP are not present in the corresponding region of FNR: in particular, residues H159 and G162, which are reported sites of CRP positive control mutations (7,8), are not conserved. However, there are significant homologies between residues 52−58 of CRP and the corresponding region of FNR containing residues 81−87 (Figure 6: note that the numbers are different because of the additional 29 amino acids at the N-terminal of FNR).

To investigate if this part of FNR is important for transcription activation, we altered the amino acids from residue 80 to 88 and tested for effects on FNR function. The entire *fnr* gene was cloned into M13, codons 80−88 were subjected to mutagenesis using a randomly-doped synthetic oligonucleotide and mutant *fnr* genes were then cloned into pFNR. Derivatives of pFNR were then transferred into the Δ*fnr* strain, JRG1728. To determine the effects of the changes on FNR activity we exploited the FNR-dependent promoter, *FFpmelR*, illustrated in line 4 of Figure 1: this promoter is similar to *CCpmelR* save that it carries a consensus sequence for FNR-binding rather than CRP-binding, 41½ bp upstream of the *melR* transcription start (10,13). The data in the left half of Figure 7 show the effects of mutations at different positions in FNR on expression from *FFpmelR*. The results show that the changes I81T, T82P, G85A, D86A, E87K and Q88E result in substantial decreases in FNR-dependent expression from *FFpmelR*, whilst the mutations T80N, E83A and Q84H have but small effects.

To check whether the mutations altered FNR such that it was unable to bind to its target site, we exploited the *E.coli ndh* promoter to monitor DNA binding function. Expression from this promoter (illustrated in line 5 of Figure 1) is sharply repressed by FNR during conditions of anaerobic growth, due to FNR binding close to the *ndh* transcription start (20,21). The *ndh* promoter was cloned in pRW2 (13), transferred to the Δ*fnr* strain, JRG1728, and the effects of FNR carrying different mutations were determined. The data in the right hand half of Figure 7 show that FNR blocks expression from the *ndh* promoter

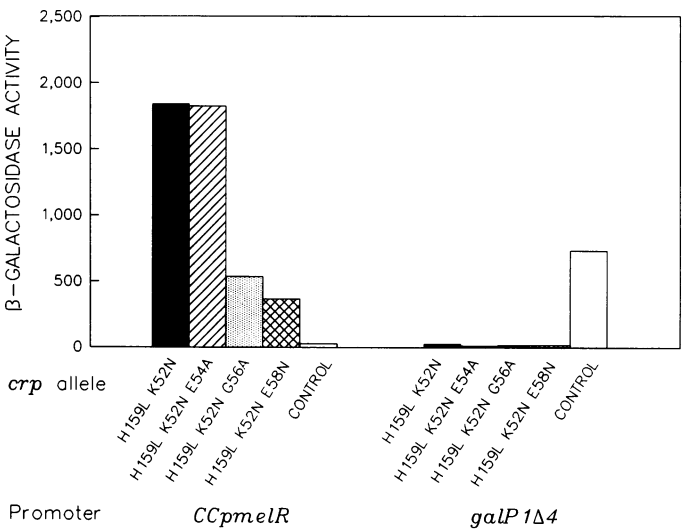

Figure 5. Effect of substitutions around position 52 on the activity of CRP H159L K52N. The height of the shaded bars represent β-galactosidase expression from the *CCpmelR* (left hand panel) and *galP1*Δ4 (right hand panel) promoters, cloned in pRW2, measured in M182Δ*crp* cells.

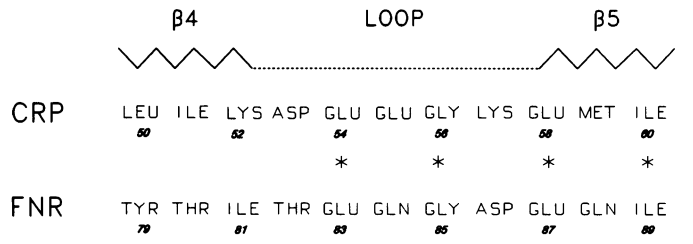

Figure 6. Partial sequence alignment of CRP and FNR. The amino acid sequence of CRP from residues 50−60 has been aligned with the corresponding region of FNR, which comprises residues 79−89 (9). Note that the numbering for the two proteins is different because FNR contains an additional amino terminal 29 residues that are absent in CRP. Amino acid residues that are identical in CRP and FNR are highlighted by an asterisk.

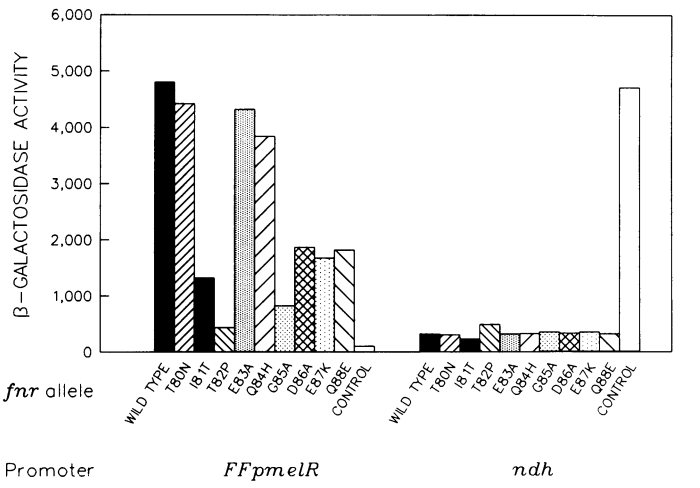

Figure 7. Effect of different mutations on FNR activity. The bars indicate β-galactosidase expression from *FFpmelR* (left hand panel) and from the *ndh* promoter (right hand panel), cloned in pRW2, measured in JRG1728 cells. pFNR, which carries wild type *fnr*, and derivatives carrying different changes in FNR from residues 80−88, were introduced as indicated. Cells were grown anaerobically in L-broth plus glucose as described in Materials and Methods. A derivative of pFNR, in which the *fnr* gene has been replaced by a linker, was used as a control.

and that mutations at positions 80–88 have minimal effects on repression. This suggests that the changes have little or no effect on FNR binding to DNA and that the reductions in expression from *FFpmelR* are due to alterations in the transcription activation function of FNR.

CONCLUSIONS

The location of positive control 'patches' in CRP and FNR

In this work we have described a number of mutations in CRP and FNR that primarily cause defects in transcription activation. We can be sure that the measured effects are not due to changes in DNA binding, since, in each case, we could show that the mutated form of the activator could act as a repressor. Further, in most of this work, we used semi-synthetic promoters carrying tight-binding consensus CRP- or FNR-binding sequences. Thus, we can discount effects due to small changes in CRP or FNR concentrations due to interference with autoregulatory mechanisms at the *crp* (22) or *fnr* promoters (9,23). Since, at many promoters, CRP, and by inference, FNR, appear to act by contacting RNA polymerase (reviewed in 5), the simplest explanation for the effects we describe here is that the mutations alter this contact with RNA polymerase, either directly or indirectly.

Several lines of evidence point to the importance of the surface-exposed loop around position 159 in CRP. After mutagenesis of the entire *crp* gene, Ebright and collaborators (personal communication) isolated numerous positive control mutations just in this region. Eschenlauer and Reznikoff (8) reported that *crp* carrying the mutation G162C conferred a positive control phenotype. We have shown that certain changes at position 159 stop the transcription activation function of CRP *in vivo* and *in vitro*. Taken together, the simplest explanation is that some feature of the surface of CRP around position 159 makes a direct contact with RNA polymerase.

In this work we show that the H159L mutation stops CRP activation at two natural promoters, *plac* and *pmalT* and two semi-synthetic promoters *CCpmelR* and *CC+20pmelR*. Since the location of the CRP site varies from −70½ at *pmalT* (16) through −61½ at *plac* and CC+20*pmelR* to −41½ at CC*pmelR*, the implication is that the same 'patch' on CRP is used to contact RNA polymerase at each of these promoters, despite their different architectures. Interestingly, the H159L mutation did not stop transcription activation at the *araBAD* promoter. In this case, CRP binds further upstream, and is separated from RNA polymerase by the binding of a second activator, AraC. Lobell and Schleif (24) recently proposed that the primary role of CRP at the *araBAD* promoter is to break a repression loop formed by AraC binding to operators on either side of the CRP site: CRP acts as a 'loop breaker' and contact with RNA polymerase in the open complex is not possible. Our present observations are consistent with this proposal: although CRP H159L has lost its ability to contact polymerase, it can still bind normally to target sites, and this, apparently, is sufficient for it to activate *paraBAD*, whilst failing at *plac*, where direct contacts are essential.

The suggestion that CRP exploits some feature of the loop around position 159 to contact RNA polymerase and hence activate transcription, immediately poses a number of problems. How could the same contact be used at promoters with widely different organisations? Secondly, since FNR is homologous to

Figure 8. Structure of the CRP dimer. The protein is represented as an α-carbon backbone trace with the DNA binding recognition helices of the two subunits positioned in two adjacent major grooves on the DNA duplex. The surface exposed loop containing residues 52–58 of the right hand subunit is indicated by the filled arrow, whilst the loop carrying residues 156–163 on the left hand subunit is indicated by the open arrow. From the figure, it is clear that the loop containing H159 from one subunit lies on the same face of the CRP dimer as the loop containing residue K52 from the adjacent subunit. The atomic coordinates for the CRP structure, as described by Weber and Steitz (4), were obtained from the Brookhaven Protein Data Bank file 2GAP. The figure was generated using the Desk Top Molecular Modeller Program from Oxford University Press.

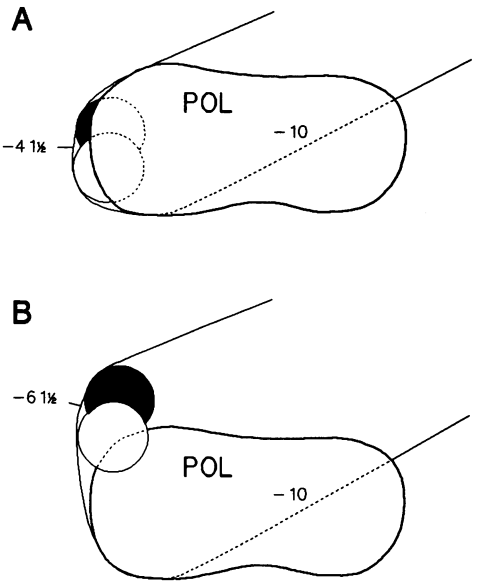

Figure 9. Interaction of CRP and RNA polymerase at *CCpmelR* and *CC+20pmelR*. **Panel A** shows the relative positions of RNA polymerase and CRP, as determined by footprinting studies, at *CCpmelR*, where the CRP site is centred at −41½. The adjacent faces in the CRP dimer are shaded differently to illustrate that one contains residues around position 159 (shaded) and the other contains residues around position 52 (open), as shown in Figure 8. In the open complex, it is clear that RNA polymerase can bind alongside the CRP dimer and protect DNA sequences both upstream and downstream of CRP (3,27,28). Thus, in the open complex at *CCpmelR*, RNA polymerase is positioned such that it can interact with either the 159 loop on one subunit or the 52 loop on the other. **Panel B** shows the organisation of CRP and RNA polymerase at *CC+20pmelR*, where the CRP binding site is centred at −61½. In this case, footprinting studies suggest that RNA polymerase only extends as far as the promoter-proximal CRP subunit. Therefore, we propose that the activating contacts between CRP and RNA polymerase must involve residues around the 159 loop on the promoter-proximal CRP subunit. This may explain why the effects of H159L at *CC+20pmelR* are not reversed by K52N: RNA polymerase cannot 'stretch' far enough upstream to make a productive interaction with the 52 loop on the adjacent subunit. Note, however, that some limited contacts between polymerase and the 52 loop in the upstream CRP subunit at *CC+20pmelR* may be possible since the changes K52N, D or L cause significant reductions in expression from *CC+20pmelR* (right hand panel of Fig. 4).

CRP, is there a similar patch in FNR? Finally, since CRP and FNR both bind as a dimer (25,26) does polymerase contact just one or both subunits? We believe that results with CRP carrying the mutation K52N throw some light on these issues.

The observation that mutations at position 52 reverse the effects of H159L at the *CCpmelR* promoter was surprising, since K52 is on the opposite face of CRP to the 159 region. However, several lines of evidence suggest that the K52N mutation creates (or improves) a second patch on CRP that can interact with RNA polymerase. Firstly, position 52 is in a surface-exposed loop and mutations here clearly could alter the conformation of the loop. Secondly, all the different substitutions we made at position 52 (52D, N and L in Figure 4 and 52Q and V, unpublished work) caused an increase in expression from *CCpmelR*, as if substitution of K52 was 'unmasking' a hidden motif in CRP, resulting in CRP containing two 'patches' that can contact polymerase. Further, the increased expression from *CCpmelR* due to K52N was suppressed by changes at neighbouring positions in the loop (Figure 5). Finally, K52N reactivates CRP carrying different changes at position 159 or 162 (Figure 3): an active patch unmasked by K52N would not be affected by the nature of the group at these positions. However, if K52N was reactivating CRP H159L by triggering a conformation change that altered the 159 loop, it would be unlikely to also directly compensate for Δ159 and G162C.

We propose that, at *CCpmelR*, RNA polymerase normally contacts CRP via the surface-exposed loop around position 159, but in the double mutant, H159L K52N, this contact is not possible and a different contact is made with the alternative loop around position 52. Although, at first sight, this appears to be sterically impossible because positions 52 and 159 are on opposite faces of the CRP molecule, it is striking that, in the dimer, the 159 loop on one subunit is adjacent to the 52 loop in the neighbouring subunit (Figure 8). Footprint studies of open complexes at promoters where the CRP site is centred at −41½ clearly show that RNA polymerase makes contacts with promoter DNA both upstream and downstream of the CRP dimer (3,27,28). Taking this and the well-characterised CRP-induced bending of DNA (29) into consideration, we and others have proposed that RNA polymerase binds alongside the CRP dimer in an arrangement such as that sketched in Figure 9 (3,19,30,31). Because of the two-fold symmetry axis in CRP, adjacent faces of the CRP dimer are different, one carrying the loop around position 159 and the other displaying the 52 loop. Thus, when RNA polymerase is positioned alongside the CRP dimer bound at −41½, it can make contact with the 52 loop on one subunit or the 159 loop on the other (Figure 9). In the absence of the normal 159 loop contact, it is easy to see how a simple mutation could alter the 52 loop to allow a new interaction. On this model, it is clear that only one of the two 159 loops in the CRP dimer can be directly involved in a contact with polymerase at a particular promoter: clearly the two 159 loops in the dimer are on different faces and could not both be contacted by the same RNA polymerase molecule at the same time. This implies that, although CRP is a dimer, only one subunit is actually needed to make the contact necessary to activate transcription.

A puzzling aspect of the effects of mutations at position 52 in CRP is that they result in increased expression at *CCpmelR* but not at *CC+20pmelR* (Figures 3 and 4) or other promoters at which the CRP site is located further upstream than −41½ (unpublished results). Presumably the contact unmasked by the changes at position 52 is only productive when CRP is bound at −41½. To explain this, we suggest that whilst RNA polymerase can easily contact both CRP subunits at *CCpmelR*, it can only interact cooperatively with one subunit at *CC+20pmelR*. This is illustrated in the sketches in Figure 9, which are based on the observation that whilst RNA polymerase contacts bases both upstream and downstream of CRP at *CCpmelR*, it only contacts bases downstream of CRP at *CC+20pmelR* (3). A simple explanation for our results would be that RNA polymerase contacts the 159 loop in the downstream subunit of the CRP dimer at *CC+20pmelR* but, in the absence of this favorable contact, it cannot 'reach' the compensating contact in the 52 loop of the neighbouring subunit.

In summary, we are suggesting that the CRP monomer contains two potential activating patches which are located on opposite faces. However, when CRP binds as a dimer to an inverted repeat, the two potential activating patches are displayed on adjacent faces of different subunits. Thus, when RNA polymerase lies alongside the CRP dimer, it can make two distinct contacts. In cases where the CRP binds further upstream it can only make one contact. An important test of this is to examine other members of the CRP family of activators. Interestingly, the primary sequence of FNR exhibits little homology to CRP in the region of the 159 loop but significant homology in the zone corresponding to the 52 loop region in CRP. Our data clearly show that mutations in this region of FNR (from positions 81−88) block the transcription activation function of FNR whilst not stopping target site recognition. Thus we propose that the 'activating patch' in FNR is located at the position corresponding to the surface exposed CRP loop including position 52 rather than that including position 159. It will be interesting to see whether other dimeric activator proteins like CRP and FNR use one or other or both patches during gene activation.

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