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Transcription activation at Class II CRP-dependent promoters: identification of determinants in the C-terminal domain of the RNA polymerase α subunit

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Many transcription factors, including the *Escherichia coli* cyclic AMP receptor protein (CRP), act by making direct contacts with RNA polymerase. At Class II CRP-dependent promoters, CRP activates transcription by making two such contacts: (i) an interaction with the RNA polymerase α subunit C-terminal domain (αCTD) that facilitates initial binding of RNA polymerase to promoter DNA; and (ii) an interaction with the RNA polymerase α subunit N-terminal domain that facilitates subsequent promoter opening. We have used random mutagenesis and alanine scanning to identify determinants within αCTD for transcription activation at a Class II CRP-dependent promoter. Our results indicate that Class II CRP-dependent transcription requires the side chains of residues 265, 271, 285–288 and 317. Residues 285–288 and 317 comprise a discrete 20×10 Å surface on αCTD, and substitutions within this determinant reduce or eliminate cooperative interactions between α subunits and CRP, but do not affect DNA binding by α subunits. We propose that, in the ternary complex of RNA polymerase, CRP and a Class II CRP-dependent promoter, this determinant in αCTD interacts directly with CRP, and is distinct from and on the opposite face to the proposed determinant for αCTD–CRP interaction in Class I CRP-dependent transcription.

Keywords: alpha subunit/catabolite activator protein/cyclic AMP receptor protein/RNA polymerase/transcription activation

Introduction

Transcription initiation requires coordinated protein–protein and protein–DNA interactions, frequently involving one or more transcription factors in addition to the subunits of RNA polymerase. *Escherichia coli* holo RNA polymerase (RNAP) is a complex of four different subunits, with subunit composition $\alpha_2\beta\beta'\sigma$, and is capable of interacting with at least three different promoter elements and with a wide range of protein transcription factors. Transcription factors are known to interact with each of the four RNAP subunits (reviewed in Rhodius and Busby, 1998), but the most studied target is the C-terminal domain of the α subunit (αCTD). Each RNAP α subunit consists of two independent domains joined by a flexible linker. The C-terminal domain (residues 235–329) functions both as a sequence-specific DNA-binding protein (recognizing 'UP elements' within some promoters; Ross *et al*., 1993) and as the target for a number of transcription activator proteins (reviewed by Ebright and Busby, 1995). The surface of αCTD that is involved in sequence-specific interaction with DNA has been identified and characterized (Jeon *et al*., 1995; Gaal *et al*., 1996; Murakami *et al*., 1996). This work is concerned with the identification of residues within αCTD that are required for transcription activation by the cyclic AMP receptor protein (CRP; also known as the catabolite activator protein, CAP), a well-characterized dimeric transcription factor that regulates the expression of >100 genes in response to changes in the intracellular concentration of its allosteric effector, cAMP (reviewed by Kolb *et al*., 1993).

Recent studies have suggested that any specific interaction between RNAP and a DNA-binding protein may be sufficient to recruit RNAP to a promoter and increase the rate of transcription initiation (Dove *et al*., 1997). Activators that function by recruiting RNAP may therefore require nothing more of their 'targets' than proximity to the activator-binding site and a particular array of surface-exposed side chains, and it is possible that an activating region of such a transcription factor might contact different targets at promoters with different architectures. Simple CRP-dependent promoters can be grouped into two classes depending on the location of the 22 bp DNA site for CRP (Ebright, 1993; Busby and Ebright, 1997). At Class I CRP-dependent promoters, a single CRP dimer binds upstream of RNAP, at sites centred close to positions –61, –71, –82 or –92. At Class II CRP-dependent promoters, a single CRP dimer binds close to position –41, overlapping the –35 element, and αCTD binds to the DNA upstream of the CRP dimer (Attey *et al*., 1994; Belyaeva *et al*., 1996; Murakami *et al*., 1997). At both Class I and Class II CRP-dependent promoters, CRP activates transcription by making direct contacts with RNA polymerase. At Class I CRP-dependent promoters, CRP makes a single interaction between a surface-exposed loop (residues 156–164; activating region 1, AR1) in the downstream subunit of the CRP dimer and αCTD (Zhou *et al*., 1993; reviewed by Ebright, 1993). At Class II CRP-dependent promoters, CRP makes two contacts with the RNAP α subunit: AR1 of the upstream subunit of the CRP dimer contacts αCTD (Zhou *et al*., 1994b), and a surface consisting of residues 19, 21, 96 and 101 (activating region 2, AR2) on the downstream subunit of the CRP dimer contacts the α subunit N-terminal domain (Niu *et al*., 1996; reviewed by Busby and Ebright, 1997). At

both classes of promoters, the CRP–αCTD interaction can be disrupted by substitutions within AR1 of CRP (Bell *et al*., 1990; Zhou *et al*., 1994a). However, the quantitative effects of individual alanine substitutions within this loop are different at Class I and Class II CRP-dependent promoters, suggesting that the 'targets' in αCTD contacted by AR1 at the two classes of promoter may differ (Zhou *et al*., 1994a).

Previous studies have identified single amino acid substitutions within αCTD that interfere with activation by CRP at the Class I *lac* P1 promoter. These substitutions fall within the DNA-binding surface, i.e. residues 265–269 and 296–299 (Zou *et al*., 1992; Tang *et al*., 1994; Gaal *et al*., 1996; Murakami *et al*., 1996), and also within an adjacent but distinct set of residues, i.e. residues 258–261 (Tang *et al*., 1994). Niu *et al*. (1996) identified mutant α subunits that were specifically defective in Class II CRP-dependent transcription (i.e. defective in Class II CRP-dependent transcription but not in Class I CRP-dependent transcription or CRP-independent transcription). Their screen defined a determinant in the α subunit N-terminal domain, which was shown to interact with AR2 of CRP, but the determinant which interacts with AR1 was not defined.

In this work, we have used both random mutagenesis and systematic alanine scanning to identify the residues within αCTD that are required for activation by CRP at a Class II CRP-dependent promoter. Since at these promoters CRP makes multiple contacts with RNAP, the importance of the AR1–αCTD interaction varies from one promoter to another (Rhodius *et al*., 1997). To ensure that substitutions which disrupted this interaction would have a measurable effect, we used the semi-synthetic *CC(–41.5)* promoter that contains a consensus CRP-binding site centred at position –41.5 upstream of the *melR* core promoter elements. Wild-type CRP increases expression from this promoter ~40-fold, and >90% of this activation is lost if either AR1 or AR2 of CRP is disrupted (Bell *et al*., 1990; Niu *et al*., 1996; Rhodius *et al*., 1997). Our results indicate that transcription activation at Class II promoters requires residues within a new determinant that is distinct from the determinants known to be required for transcription activation at Class I CRP-dependent promoters.

Results

Random mutagenesis screen for substitutions that disrupt activation by CRP at CC(–41.5)

To identify substitutions within αCTD that interfere with transcription activation at a Class II CRP-dependent promoter, we performed random mutagenesis of codons 231–329 of the *rpoA* gene encoding the α subunit of RNAP and screened for mutations that decreased the level of expression from a *CC(–41.5)*::*lac* operon fusion *in vivo*. The region of *rpoA* that encodes residues 231–329 of the α subunit was amplified by error-prone PCR, and the mutagenized fragments were cloned into an *rpoA* expression plasmid (pLAW2). The resulting library of mutagenized plasmids was transformed into M182 Δ*lac* Δ*crp* cells that had been transformed with plasmid pDW300 (encoding CRP) and a plasmid carrying a *CC(–41.5)*::*lac* operon fusion [pRW50/*CC(–41.5)*]. The transformed cells therefore contained wild-type α subunits expressed from the chromosomal *rpoA* gene and mutant α subunits expressed from the multicopy plasmid-borne *rpoA* gene. The transformants were plated onto lactose-MacConkey indicator agar: colonies containing the wild-type pLAW2 plasmid exhibited a Lac^+ phenotype (red colonies). We reasoned that colonies containing pLAW2 derivatives that expressed α subunits that interfered with expression from the *CC(–41.5)* promoter would exhibit a Lac^- phenotype (white or light red colonies; cf. Zou *et al*., 1992; Tang *et al*., 1994; Gaal *et al*., 1996; Niu *et al*., 1996).

Table I. Effect of randomly generated substitutions in αCTD on expression from *CC(–41.5)*

Substitution	No. of isolates	% wild-type activity at *CC(–41.5)*
EG261	1	121 ± 6
RL265	1	86 ± 7
NY268	1	114 ± 7
TA285	1	71 ± 3
TN285	1	59 ± 5
EG286	1	53 ± 10
VA287	3	54 ± 3
VI287	1	61 ± 8
EK288	1	53 ± 3
LF289	1	103 ± 9
ND294	2	129 ± 26
GV296	1	76 ± 7
KE298	1	108 ± 10
SP299	2	86 ± 10
EK302	1	158 ± 17
GD315	1	55 ± 5

The table lists the single amino acid substitutions in αCTD that gave rise to a Lac^- phenotype when the mutant α subunits were expressed in M182 Δ*crp* cells containing plasmids pDW300 (encoding CRP) and pRW50/*CC(–41.5)*. Other alleles that gave rise to a Lac^- phenotype contained the following substitutions: DE259 VA287; ND268 IV275; HY276 EG288 SP313; VA287 RP317; KE298 SP313; single base pair deletion causing a frameshift in codon 292. The promoter activities listed in column 3 were measured by *in vivo* β-galactosidase assays in strain RLG4649 [a crp^+ strain carrying a single copy chromosomal *CC(–41.5)*::*lacZ* fusion] transformed with derivatives of plasmid pLAW2 expressing the mutant α subunits. Data from three independent assays were averaged, and are expressed as a percentage of the activity measured in cells transformed with wild-type pLAW2, with their standard deviation.

We performed four independent mutagenesis reactions, screened ~6000 colonies and isolated 26 independent mutants. The sequence of the mutagenized region of each candidate plasmid was determined, and the amino acid substitutions that interfered with expression from the *CC(–41.5)* promoter were inferred: 20 of the mutants contained single amino acid substitutions (Table I; Figure 1A). Six of the 14 different residues at which substitutions were isolated form a discrete surface-exposed region that has not been implicated previously in DNA binding or transcription activation by CRP (residues 285, 286, 287, 288, 289 and 315). Five of the residues at which substitutions occur are important components of the DNA-binding surface of αCTD (residues 265, 268, 296, 298 and 299; Gaal *et al*., 1996; Murakami *et al*., 1996), and two further residues (294 and 302) are adjacent to the DNA-binding surface. The final substitution, EG261, occurs at a residue that previously has been shown to be important for transcription activation by CRP at the *lac* P1 promoter (Tang *et al*., 1994). Interestingly, in a similar screen using a *CC(–41.5)*::*lacZ* fusion, Niu and Ebright identified four

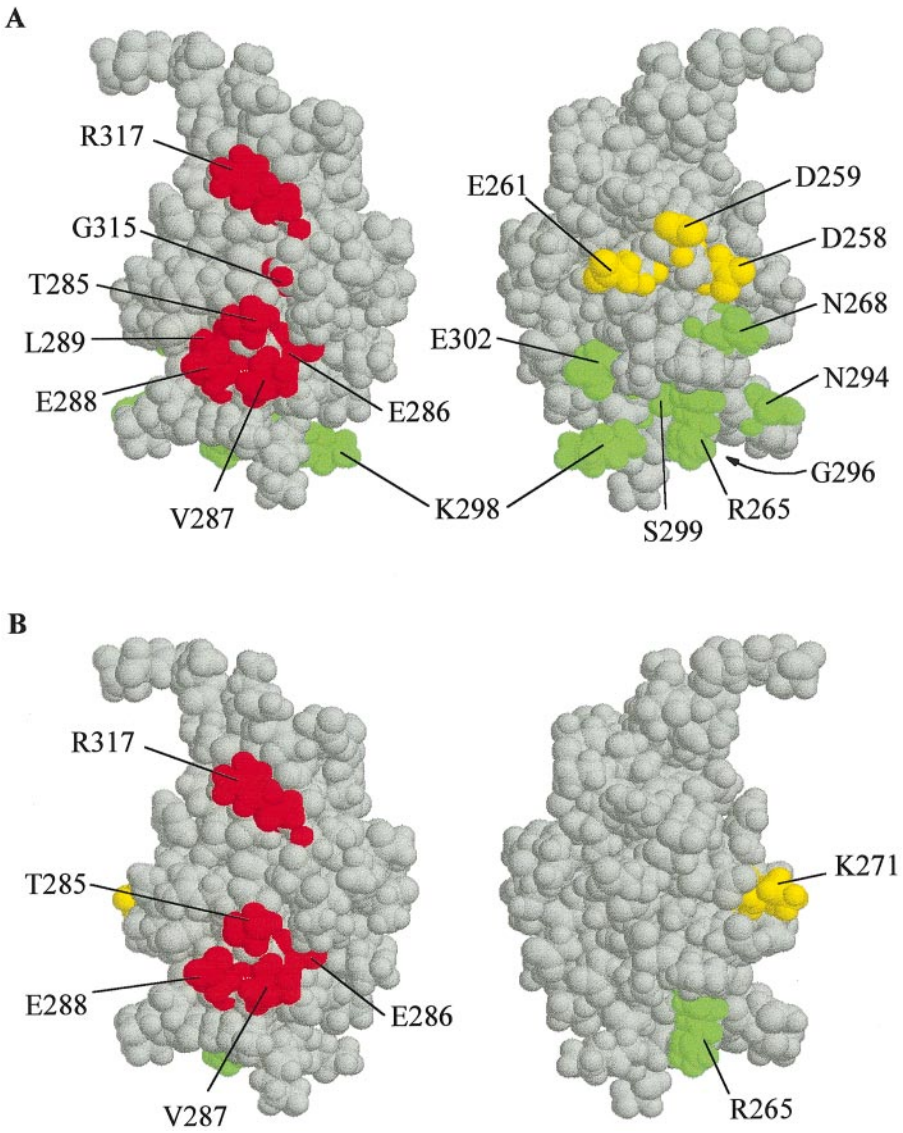

Fig. 1. The solution structure of αCTD (Jeon *et al*., 1995) showing the position of residues implicated in transcription activation at a Class II CRP-dependent promoter. (**A**) Residues at which substitutions generated by random mutagenesis conferred a Lac⁻ colony phenotype on a host carrying a *CC(–41.5)*::*lac* operon fusion (substitutions at positions 258, 259 and 317 were identified in previous work, W.Niu and R.H.Ebright, unpublished data). G296 is obscured by R265. (**B**) Residues at which alanine substitution decreased Class II CRP-dependent transcription *in vivo* and *in vitro*. In both panels, the two views of the structure are related by a 180° rotation. Side chains of residues belonging to the three determinants described in the Discussion are shown in different colours.

substitutions in αCTD that gave weak phenotypic changes on Lac-tetrazolium indicator plates: RC317 (part of the surface defined by residues 285–289 and 315; Figure 1A), DA258, DG259 and DN259 (part of the surface defined by residue 261; Figure 1A) (W.Niu and R.H.Ebright, unpublished data).

To quantify the effects of the substitutions listed in Table I, the pLAW2 derivatives were transformed into a reporter strain carrying a single copy chromosomal *CC(–41.5)*::*lacZ* fusion, and β-galactosidase activity was measured. Substitutions at positions 285, 286, 287, 288 and 315 had the greatest effect, decreasing expression to between 53 and 71% of the wild-type level. Some substitutions in the DNA-binding surface (at residues 265, 296 and 299) also decreased expression (Table I). Curiously, a number of the substitutions that gave rise to a Lac⁻ phenotype on indicator plates did not decrease the level of β-galactosidase expression measured in this direct assay. The plate phenotypes may have been misleading, or the effect of these substitutions may be greater in the growth conditions prevailing during growth on plates than in the fast growing, well-aerated cultures used for the assay.

Alanine scan to identify residues within αCTD that are important for activation by CRP at CC(–41.5)

A complication of the random mutagenesis procedure is that the substitutions obtained may confer their phenotype by introducing a clash, rather than by removing an essential side chain. Furthermore, the randomly generated library of mutants screened was not saturated, i.e. we did not carry out the screen in a manner that would have resulted in our obtaining every potential substitution that would cause a reduction in CRP-dependent activation at *CC(–41.5)*. To determine the importance of each side chain

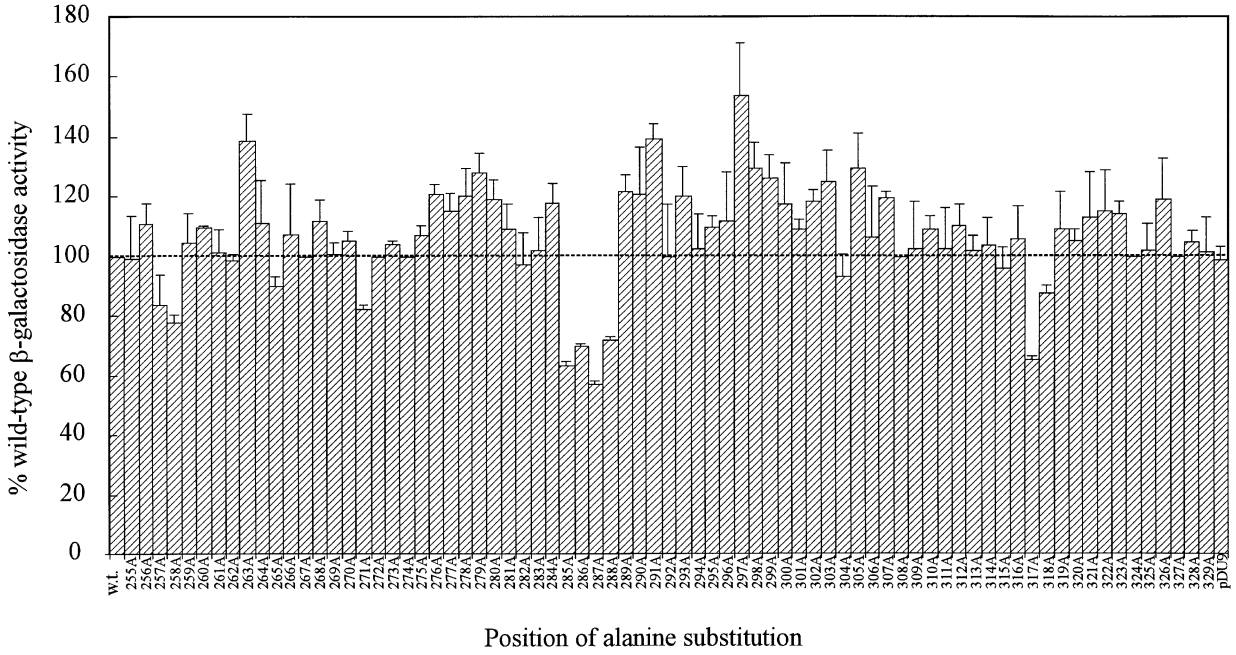

Fig. 2. Effect of single alanine substitutions within αCTD on expression from *CC(–41.5)*. RLG4649 (*crp*$^+$) cells carrying a chromosomal *CC(–41.5)*::*lacZ* fusion were transformed with derivatives of plasmids pHTf1α (substitutions at 255–271) or pREIIα (substitutions at positions 273–329) expressing α subunits with single alanine substitutions at the positions indicated on the *x*-axis, or with a control plasmid that did not express *rpoA* (pDU9). Cultures were grown to mid-log in L-broth + 100 μg/ml ampicillin. The activities shown (with SD) are the average of three independent experiments, and are expressed as a percentage of the average activity obtained from cells transformed with plasmids expressing wild-type α subunits. Residues 267, 272, 274, 308, 324 and 327 are alanines in the wild-type protein and were not changed.

within αCTD for Class II CRP-dependent transcription, we therefore performed alanine scanning (Cunningham and Wells, 1989). Lysogens carrying a chromosomal *CC(–41.5)*::*lacZ* fusion were transformed with a set of plasmids encoding the RNAP α subunit in which residues 255–329 were each changed individually to alanine, and the level of *lacZ* expression was determined *in vivo* from β-galactosidase assays.

Alanine substitution of residues 257, 258, 265, 271, 285–288, 317 and 318 resulted in defects in Class II CRP-dependent transcription, with substitution of residues 285–288 and 317 causing the largest defects (Figure 2). Consistent with the location of the randomly generated substitutions isolated in the earlier screen, these results suggest that there may be three discrete determinants for Class II CRP-dependent transcription on the surface of αCTD: residues 285–289, 315, 317 and 318 form a contiguous patch on the surface of αCTD, residues 265, 268, 294, 296, 298, 299 and 302 correspond closely to the DNA-binding surface (Gaal *et al*., 1996; Murakami *et al*., 1996), and residues 257–259, 261 and 271 form another contiguous patch on the opposite face of the domain to residues 285–289, 315, 317 and 318 (Table I; Figures 1 and 2).

In vitro transcription at a Class II CRP-dependent promoter

The reporter strains used in the experiments described above contained wild-type α subunits that may have diminished the observed effects of the mutant α subunits. Because of this, and the other complications inherent in interpreting results obtained from a multicomponent system *in vivo*, we complemented our *in vivo* analyses with *in vitro* transcription experiments, using purified RNAP reconstituted with α subunits carrying selected alanine substitutions. His-tagged α subunits carrying alanine substitutions at positions 258, 261, 265, 271, 285, 286, 287, 288 or 317 were overexpressed, purified and reconstituted into RNAP with wild-type β, β′ and σ^{70} subunits [although substitution of residue 261 had no effect in the alanine scan performed at *CC(–41.5) in vivo*, αEA261 was included because of the reported importance of residue 261 at the Class I CRP-dependent *lac* P1 promoter; Tang *et al*. (1994)]. The activity of each RNAP preparation was normalized to transcription from a template carrying the constitutive *lac*UV5 promoter. The ability of each RNAP to transcribe from a template carrying the *CC(–41.5)* promoter, in the absence or presence of wild-type CRP, was then measured.

None of the RNAPs produced a detectable level of transcript from the *CC(–41.5)* promoter in the absence of CRP. Transcription by each RNAP was stimulated by the presence of wild-type CRP, but the level of CRP-dependent transcription differed greatly between the mutant RNAPs (Figures 3 and 4). The greatest defects in CRP-dependent transcription were found with RNAPs carrying αTA285 or αVA287 subunits, which produced ~20% of the amount of CRP-dependent transcript produced by wild-type RNAP. Alanine substitutions at positions 265, 271, 286, 288 and 317, but not 258 or 261, also resulted in defects in Class II CRP-dependent transcription in this assay. The *in vitro* transcription experiments demonstrate that alanine substitution of residues 265, 271, 285–288 and 317 of the α subunit directly affects the level of transcription from the *CC(–41.5)* promoter, and exclude the possibility that the phenotypes observed *in vivo* could have resulted from indirect effects, such as altered levels of CRP expression. We conclude that, for residues 265, 271, 285–288 and

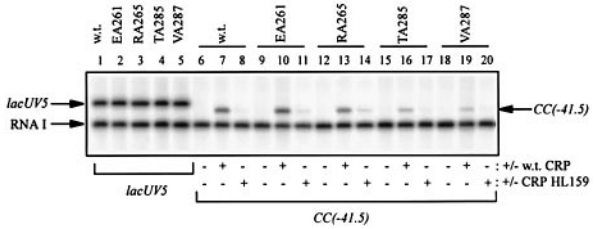

Fig. 3. *In vitro* transcription from *CC(–41.5)* in the presence of wild-type or HL159 CRP. The figure shows the transcripts produced in a typical multiround transcription experiment using purified RNAP containing α subunits with alanine substitutions at the positions indicated. In lanes 1–5 the template DNA was pSR/*lac*UV5, and in lanes 6–20 the template DNA was pSR/*CC(–41.5)*. Where indicated, 20 nM wild-type CRP or CRP carrying the HL159 substitution was added to the reaction mixtures prior to the addition of RNAP. The position of transcripts initiated at the *lac*UV5 or *CC(–41.5)* promoters, and the position of the plasmid-encoded transcript, RNA I, are indicated.

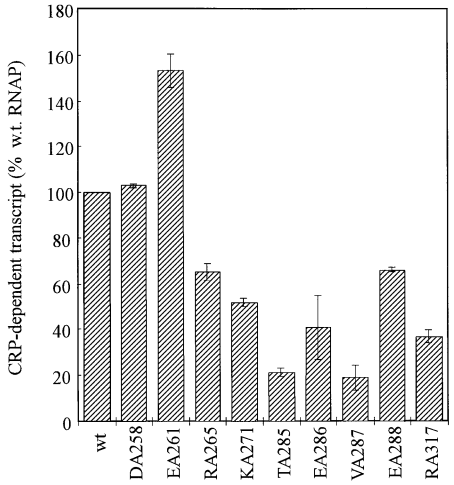

Fig. 4. Effect of alanine substitutions in αCTD on transcription from *CC(–41.5) in vitro*. Purified RNAP was reconstituted using α subunits containing alanine substitutions at the positions indicated. Multiround transcription experiments were performed using supercoiled pSR/*CC(–41.5)* template, and the amount of transcript produced by each RNAP in the presence of 20 nM wild-type CRP was measured. Values (±1 SD) are expressed as a percentage of the amount of transcript produced by RNAP containing wild-type α subunits, and are the averages of at least three independent determinations.

317, side chain atoms beyond C^{β} make favourable interactions in Class II CRP-dependent transcription.

Transcription from *CC(–41.5)* was also measured in the presence of CRP carrying a Leu for His substitution at position 159, which completely inactivates AR1 (Rhodius *et al*., 1997). Each RNAP produced a low, but detectable, level of transcript in the presence of this mutant activator protein (~10% of the level produced by wild-type RNAP in the presence of wild-type CRP; Figure 3; data not shown). As the HL159 substitution in CRP causes a greater defect in transcription activation than any of the single alanine substitutions in αCTD, it appears that more than one side chain must be removed from αCTD in order to destroy the AR1–αCTD interaction completely.

Interactions between the α subunit of RNAP and DNA

To determine if any of the alanine substitutions within αCTD that interfered with transcription activation by

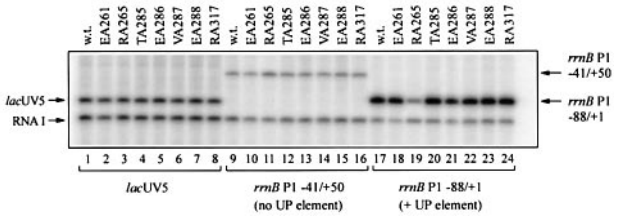

Fig. 5. *In vitro* transcription from *rrnB* P1 in the absence and presence of the UP element. The figure shows the transcripts produced in a multiround transcription experiment using purified RNAP containing α subunits with alanine substitutions at the positions indicated. The results presented are representative examples of an experiment which was performed in triplicate. In lanes 1–8 the template DNA was pSR/*lac*UV5, in lanes 9–16 the template DNA was pRLG2230 (*rrnB* P1 –41 to +50: no UP element), and in lanes 17–24 the template DNA was plasmid pRLG862 (*rrnB* P1 –88 to +1: contains a UP element). The position of transcripts initiated at the *lac*UV5 or *rrnB* P1 promoters, and the position of the plasmid-encoded transcript, RNA I, are indicated.

CRP also caused defects in sequence-specific interactions between the α subunit of RNAP and DNA, we measured transcription at the UP element-dependent *rrnB* P1 promoter *in vitro*. Templates carrying *rrnB* P1 promoter derivatives with or without a UP element were transcribed by RNAPs reconstituted with α subunits carrying single alanine substitutions at positions 261, 265, 285–288, 317 (Figure 5) or 271 (data not shown). Consistent with previous reports (Gaal *et al*., 1996; Murakami *et al*., 1996), of the substitutions tested here, only RA265 severely reduced transcription from the *rrnB* P1 promoter containing a UP element. We conclude that the defects in CRP-dependent transcription caused by the other substitutions cannot be ascribed to altered interactions with DNA.

Interactions between the α subunit of RNAP and CRP

We previously described a series of semi-synthetic promoters that carry the *rrnB* P1 UP element at different locations adjacent to a consensus CRP site, and showed that, at some of these promoters, purified α subunits and CRP bind cooperatively. The observed cooperativity is dependent on AR1 of CRP, and is destroyed if CRP carries the HL159 substitution, suggesting that the cooperativity directly reflects α subunit–CRP interactions (Savery *et al*., 1995; Lloyd *et al*., 1998). Figure 6 shows the results of electrophoretic mobility shift experiments with the *CC(–41.5)* α*(–63)* promoter, a derivative of *CC(–41.5)* in which the *rrnB* P1 UP element is located immediately upstream of the CRP-binding site (Lloyd *et al*., 1998). Purified His-tagged wild-type α subunits and αEA261 subunits exhibit ~4-fold cooperativity with wild-type CRP. In contrast, purified His-tagged αVA287 and αGD315 subunits exhibited no cooperativity with CRP in interacting with *CC(–41.5)* α*(–63)*. Thus, the VA287 and GD315 substitutions reduce α subunit–CRP interactions.

Discussion

We have identified seven residues within αCTD whose side chains are important for transcription activation by CRP at *CC(–41.5)* both *in vivo* and *in vitro* (R265, K271, T285, E286, V287, E288, R317). The location of these residues, together with the location of non-alanine substitu-

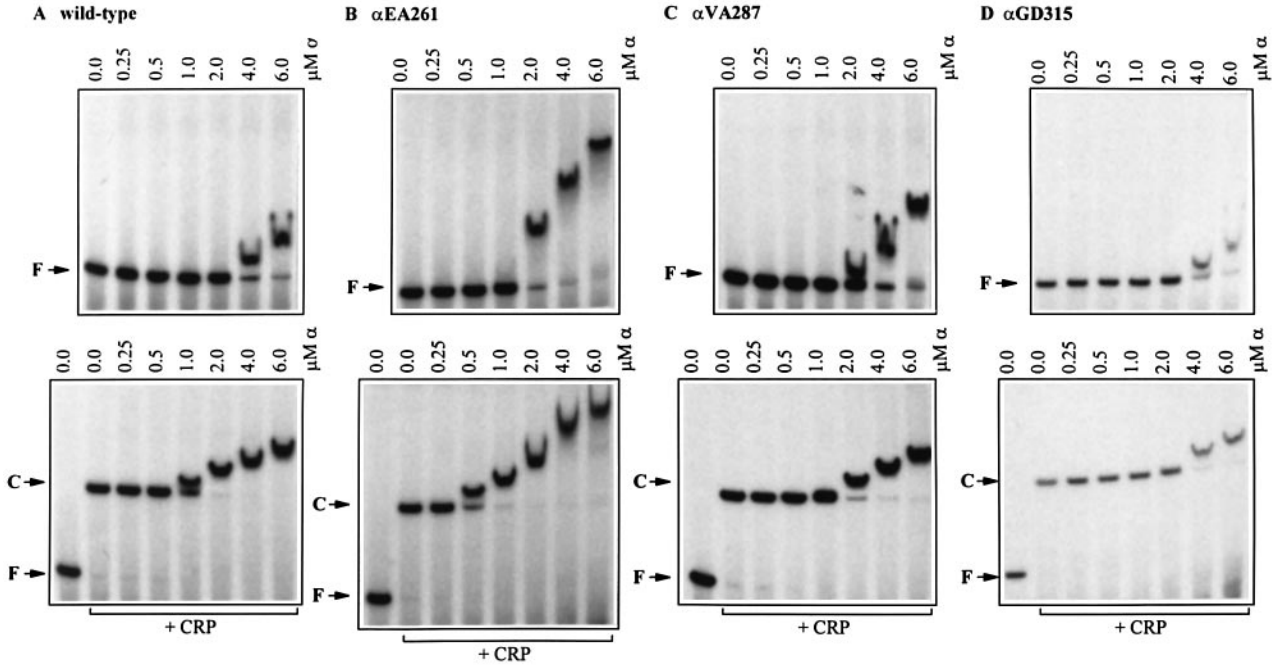

Fig. 6. Interactions between purified CRP and RNAP α subunits at the *CC(–41.5)* α*(–63)* promoter. The figure shows autoradiograms of electrophoretic mobility shift assays analysing the binding of purified wild-type (**A**) and mutant (**B**, **C** and **D**) α subunits to end-labelled fragments carrying the *CC(–41.5)* α*(–63)* promoter. The results presented are representative examples of an experiment which was performed in triplicate. The labelled DNA (~0.4 nM) was incubated with the concentrations of each α subunit indicated, in the presence or absence of 100 nM wild-type CRP. The positions of the unbound DNA ('F') and the binary CRP–DNA complex ('C') are indicated by arrows.

tions that affect expression from *CC(–41.5) in vivo*, suggests that αCTD contains at least two, and possibly three, separate determinants for transcription activation at Class II CRP-dependent promoters (Figure 1).

The determinant that has the greatest effect on transcription activation by CRP at *CC(–41.5)*, both *in vivo* and *in vitro*, includes residues 285–289, 315, 317 and 318. This determinant is not required for α subunit–DNA interaction but is required for cooperative α subunit–CRP interactions (Figures 5 and 6). We propose that this surface of αCTD makes direct protein–protein interactions with AR1 of CRP in the ternary complex of RNAP, CRP and a Class II CRP-dependent promoter. Consistent with this proposal, the dimensions of this determinant (~20×10 Å; Figure 1; Jeon *et al.*, 1995) are comparable with the dimensions of AR1 (~14×11 Å; Niu *et al*., 1994), and the side chain identities of the two most critical residues within the determinant, T285 and V287, suggest possible hydrogen-bonded and hydrophobic interactions with the side chains of the two most critical residues of AR1, T158 and P160 (Zhou *et al*., 1994a). Interestingly, there are precedents for the involvement of this region of αCTD in transcription activation: substitutions at residues 289 and 317 decreased transcription activation by the CRP homologue FNR *in vivo* (Lombardo *et al*., 1991), and substitutions at 286, 287, 289, 290 and 300 decreased transcription activation by bacteriophage P2 Ogr *in vivo* (Wood *et al*., 1997).

The second determinant includes residues 265, 268, 294, 296, 298, 299 and 302, which correspond closely to the DNA-binding surface of αCTD (Jeon *et al*., 1995; Gaal *et al*., 1996; Murakami *et al*., 1996). We propose that this determinant makes non-specific protein–DNA interactions with the DNA segment adjacent to the CRP site in the ternary complex of RNAP, CRP and a Class II CRP-dependent promoter. This is analogous to the proposed role of this determinant in Class I CRP-dependent transcription (Blatter *et al*., 1994; Busby and Ebright, 1994; Gaal *et al*., 1996), Mor-dependent transcription (Artsimovitch *et al*., 1996) and Ogr-dependent transcription (Wood *et al*., 1997). Consistent with this proposal, results from DNase I footprinting (Attey *et al*., 1994), hydroxyl radical footprinting (Belyaeva *et al*., 1996) and experiments with a chemical DNA-cleaving reagent (Murakami *et al.*, 1997) indicate that αCTD is close to, or in contact with, the DNA segment immediately upstream of the CRP site in the ternary complex of RNAP, CRP and a Class II CRP-dependent promoter. In addition, substitution of this region of the *CC(–41.5)* promoter with the UP element from *rrnB* P1 increases promoter strength (Savery *et al*., 1995; Lloyd *et al*., 1998).

Random mutagenesis and alanine scanning implicate residues 257, 258, 259, 261 and 271 as a potential third determinant for Class II CRP-dependent transcription (Table I; Figure 2; W.Niu and R.H.Ebright, unpublished data). These residues form a discrete surface-exposed patch that is on the opposite face of αCTD to the 285–289, 315, 317, 318 determinant (Figure 1). Residues within this determinant have been proposed to make direct interactions with AR1 of CRP at the Class I CRP-dependent *lac* promoter (Tang *et al*., 1994). However, the role and relative importance of these residues at the Class II CRP-dependent promoter studied here is unclear; the effects of substitutions in this determinant are small *in vivo* and, except for substitution of residue 271, not detectable *in vitro* (Table I; Figures 2–4). It is possible that these substitutions affect interactions other than the αCTD–CRP and αCTD–DNA contacts.

Table II. Bacterial strains and plasmids used in this work

	Brief description	Source/reference
Bacterial strains		
M182	*E.coli* K12 Δ*lac crp*$^+$	Casadaban and Cohen (1980)
M182 Δ*crp*	Δ*crp* derivative of M182	Busby *et al*. (1983)
RLG 4649	λ lysogen of M182 carrying chromosomal *CC(–41.5)::lacZ* fusion	this work
BL21 DE3	*E.coli* λDE3. Encodes T7 RNAP under control of *lac*UV5 promoter	Novagen Inc.
XL1-Blue	*E.coli recAI* [F′ *lacI*^{q}]	Stratagene
Plasmids		
pLAW2 (and derivatives)	Plasmid carrying *rpoA* encoding RNAP α subunit (and derivatives carrying random mutations in codons 231–329)	Zou *et al*. (1992); this work
pREIIα (and derivatives)	Plasmid carrying r*poA* encoding RNAP α subunit (and derivatives carrying alanine substitutions at positions 273–329)	Blatter *et al*. (1994); Gaal *et al*. (1996); Wood *et al*. (1997); M.Kainz and R.L.Gourse (in preparation)
pHTf1α (and derivatives)	Plasmid carrying *rpoA* encoding RNAP α subunit (and derivatives carrying alanine substitutions at positions 255–271)	Tang *et al*. (1994); Gaal *et al*. (1996)
pHTT7f1NHα (and derivatives)	Plasmid carrying *rpoA* encoding RNAP α subunit (and derivatives) with hexa-His coding region between codons 1 and 2, under control of a T7 promoter	Tang *et al*. (1995); this work
pMKSe2	Plasmid carrying *rpoB*, encoding RNAP β subunit, under control of *lac* promoter	Severinov *et al*. (1993)
pT7β′	Plasmid carrying *rpoC*, encoding RNAP β′ subunit, under control of T7 promoter	Zalenskaya *et al*. (1990)
pLHN12σ	Plasmid carrying *rpoD*, encoding RNAP σ^{70} subunit, under control of T7 promoter	R.Burgess (unpublished)
pDW300	pLG339 derivative carrying *crp* gene	West *et al*. (1993)
pDCRP (and derivatives)	pBR322 derivative carrying *crp* gene (and derivatives)	West *et al*. (1993)
pDU9	Derivative of pDCRP with *crp* deleted	Bell *et al*. (1990)
pSR/*CC(–41.5)*	pBR322 derivative carrying *CC(–41.5)* promoter cloned upstream of transcription terminator	this work
pSR/*lac*UV5	pBR322 derivative carrying *lac*UV5 promoter cloned upstream of transcription terminator	this work
pRLG2230	pRLG770 derivative (Ross *et al*., 1990) carrying *rrnB* P1 promoter (–41 to +50) cloned upstream of transcription terminator	W.Ross and R.L.Gourse (unpublished)
pRLG862	pRLG770 derivative carrying *rrnB* P1 promoter (–88 to +1) cloned upstream of transcription terminator	Ross *et al*. (1990)
pRW50/*CC(–41.5)*	Broad host range, low copy transcriptional fusion vector containing *CC(–41.5)* promoter upstream of *lac* genes	West *et al*. (1993)
pAA121/*CC(–41.5)* α*(–63)*	pBR322 derivative carrying *CC(–41.5)* α*(–63)* promoter	Lloyd *et al*. (1998)

One interpretation of our results is that the face of αCTD that interacts with AR1 of CRP at Class II CRP-dependent promoters differs from the face that interacts at Class I CRP-dependent promoters. An alternative interesting possibility is that, at any CRP-dependent promoter, αCTD is able to interact with DNA in either of two orientations; one that presents the 285–289, 315, 317, 318 determinant to CRP, and one that presents the 257–259, 261, 271 determinant to CRP, with the preferred orientation depending on the architecture of the promoter and the sequence of the DNA segment contacted by αCTD. Consistent with this, we have observed that substitutions in the 285–289, 315, 317, 318 determinant, in addition to substitutions in the 257–259, 261, 271 determinant, reduce transcription at Class I CRP-dependent promoters (N.J. Savery, S.J.W.Busby and R.L.Gourse, unpublished data; W.Niu and R.H.Ebright, unpublished data). Further work is now essential to elucidate the variables affecting the relative utilization of the two patches at Class I and Class II CRP-dependent promoters.

Materials and methods

Strains and plasmids

Table II lists the strains and plasmids used in this work. Standard methods for isolation and manipulation of DNA fragments were used throughout. Strain RLG4649 [a λ lysogen encoding a chromosomal *CC(–41.5)*::*lacZ* fusion] was constructed from strain M182 by the method of Simons *et al*. (1987), as described in Rao *et al*. (1994). Plasmid pSR/*CC(–41.5)* was constructed by cloning an *Eco*RI–*Hin*dIII fragment carrying the *CC(–41.5)* promoter (Gaston *et al*., 1990) into plasmid pSR (Kolb *et al*., 1995). The promoter fragment used for the construction of both strain RLG4649 and plasmid pSR/*CC(–41.5)* carried the *CC(–41.5)* DNA sequence from position –80 upstream of the transcription start point to position +35 downstream. Plasmid pSR/*lac*UV5 was constructed by cloning an *Eco*RI–*Hin*dIII fragment, carrying *lac*UV5 promoter sequence from –60 to +40, from RLG593 (Ross *et al*., 1990) into plasmid pSR.

Random mutagenesis of the αCTD coding region

Error-prone PCR (Zhou *et al*., 1991) was used to prepare a library of random *rpoA* mutations. The *rpoA* αCTD coding region from plasmid pLAW2 was amplified by four independent PCRs using *Taq* DNA polymerase and oligonucleotide primers that flanked the *Hin*dIII site adjacent to codon 231 and the *Bam*HI site downstream of the translation stop codon. After restriction with *Hin*dIII and *Bam*HI, the products were cloned into pLAW2 to generate a library of pLAW2 derivatives carrying random mutations in the segment encoding αCTD. M182 Δ*crp* cells carrying pDW300, encoding CRP, and pRW50/*CC(–41.5)*, encoding a *CC(–41.5)*::*lac* fusion, were transformed by electroporation with samples from the pLAW2 library, and the transformed cells were plated onto MacConkey indicator agar containing 10 g/l lactose, 100 μg/ml ampicillin, 35 μg/ml tetracycline and 25 μg/ml kanamycin (this reporter strain was used because it gives a stronger Lac$^+$ phenotype on the indicator plates than a reporter strain containing a chromosomal *crp* gene).

Measurement of β-galactosidase activity in vivo

Cultures were inoculated to an A_{600} of ~0.007 and grown to mid-log phase (A_{600} ~0.35–0.40) in Lennox Broth containing 100 μg/ml ampicillin

at 37°C with vigorous aeration. Cultures of strains transformed with derivatives of plasmid pLAW2 also contained 1 mM isopropyl-β-D-galactopyranoside (IPTG). Specific β-galactosidase activity was determined by the method of Miller (1972).

Protein purification and reconstitution of RNA polymerase

RNAP α subunits carrying a hexa-His tag between the first and second codons were prepared using plasmid pHTT7f1NHα. Derivatives of pHTT7f1NHα carrying mutant *rpoA* alleles were constructed by replacing the *Hin*dIII–*Bam*HI fragment, which encodes αCTD, with fragments from plasmids encoding the different amino acid substitutions (Tang *et al*., 1994; Gaal *et al*., 1996; Wood *et al*., 1997; M.Kainz and R.L.Gourse, in preparation). Overexpression of the α subunits in strain BL21 DE3 and purification of α by Ni^{2+}-affinity chromatography were performed essentially as described in Tang *et al*. (1995) and Gaal *et al.* (1996). Preparation of inclusion bodies containing β, β′ or σ^{70} subunits from strains XL1-Blue [pMKSe2], BL21 DE3 [pT7β′] and BL21 DE3 [pLHN12σ], respectively, and reconstitution of RNA polymerase were also performed as described in Tang *et al*. (1995). Wild-type CRP and CRP HL159 were purified by the method of Ghosaini *et al*. (1988) from M182 Δ*crp* cells transformed with plasmids pDCRP or pDCRP HL159.

In vitro transcription assays

In vitro transcription reactions (25 µl) were performed in a buffer containing 100 mM KCl, 40 mM Tris–acetate pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 100 µg/ml bovine serum albumin (BSA), 200 µM ATP, 200 µM CTP, 200 µM GTP, 10 µM UTP and 5 µCi of [α-^{32}P]UTP (DuPont). CRP, if added, was present at 20 nM. Reactions that contained CRP also contained 0.2 mM cAMP. Supercoiled template DNA was prepared using a Qiagen midiprep kit, and was added at a final concentration of 0.2 nM. Reactions were started by the addition of RNAP and incubated at 22°C for 15 min. Samples were analysed by denaturing gel electrophoresis and quantified using a PhosphorImager (Molecular Dynamics) and ImageQuant software. Concentrations of RNAP, chosen to give the same amount of transcription from the constitutive *lac*UV5 promoter in the absence of CRP, were 2.4 nM wild-type RNAP, 1.8 nM RNAP αDA258, 2.2 nM RNAP αEA261, 12.5 nM RNAP αRA265, 2.3 nM RNAP αKA271, 6.7 nM RNAP αTA285, 4.7 nM RNAP αEA286, 2.6 nM RNAP αVA287, 5.9 nM RNAP αEA288 and 5.9 nM RNAP αRA317.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed with *Eco*RI–*Hin*dIII fragments prepared from plasmid pAA121/*CC(–41.5)* α*(–63)* and end-labelled with [γ-^{32}P]ATP and polynucleotide kinase. Reaction mixtures contained ~0.4 nM labelled DNA, 20 mM HEPES pH 8.0, 5 mM $MgCl_2$, 50 mM potassium glutamate, 1 mM DTT, 2 mM spermidine, 20 µg/ml sonicated herring sperm DNA, 10% glycerol and proteins as indicated in the figure legends. Reaction mixtures that contained CRP also contained 0.2 mM cAMP. Reactions were incubated for 20 min at 37°C and were then run on 6% acrylamide gels containing 7.5% glycerol and 0.5× TBE buffer. Gel running buffer was 0.5× TBE containing 2% glycerol.

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References

Artsimovitch,I., Murakami,K., Ishihama,A. and Howe,M.M. (1996) Transcription activation by the bacteriophage Mu Mor protein requires the C-terminal regions of both α and σ^{70} subunits of *Escherichia coli* RNA polymerase. *J. Biol. Chem.*, **271**, 32343–32348.

Attey,A., Belyaeva,T., Savery,N., Hoggett,J., Fujita,N., Ishihama,A. and Busby,S. (1994) Interactions between the cyclic AMP receptor protein and the alpha subunit of RNA polymerase at the *E.coli gal* P1 promoter. *Nucleic Acids Res*., **22**, 4375–4380.

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., Bown,J., Fujita,N., Ishihama,A. and Busby,S. (1996) Location of the C-terminal domain of the RNA polymerase alpha subunit in different open complexes at the *E.coli* galactose operon regulatory region. *Nucleic Acids Res*., **24**, 2243–2251.

Blatter,E.E., Ross,W., Tang,H., Gourse,R.L. and Ebright,R.H. (1994) Domain organization of RNA polymerase α subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. *Cell*, **78**, 889–896.

Busby,S. and Ebright,R.H. (1994) Promoter structure, promoter recognition, and transcription activation in prokaryotes. *Cell*, **79**, 743–746.

Busby,S. and Ebright,R.H. (1997) Transcription activation at class II CAP-dependent promoters. *Mol. Microbiol*., **23**, 853–859.

Busby,S., Kotlarz,D. and Buc,H. (1983) Deletion mutagenesis of the *Escherichia coli* galactose operon promoter region. *J. Mol. Biol.*, **167**, 259–274.

Casadaban,M.J. and Cohen,S.N. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli. J. Mol. Biol*., **138**, 179–207.

Cunningham,B. and Wells,J. (1989) High-resolution epitope mapping of hGH–receptor interactions by alanine scanning mutagenesis. *Science*, **244**, 1081–1085.

Dove,S.L., Joung,J.K. and Hochschild,A. (1997) Activation of prokaryotic transcription through arbitrary protein–protein contacts. *Nature*, **386**, 627–630.

Ebright,R.H. (1993) Transcription activation at class I CAP-dependent promoters. *Mol. Microbiol*., **8**, 797–802.

Ebright,R.H. and Busby,S. (1995) The *Escherichia coli* RNA polymerase α subunit: structure and function. *Curr. Opin. Genet. Dev*., **5**, 197–203.

Gaal,T., Ross,W., Blatter,E.E., Tang,H., Jia,X., Krishnan,V.V., Assa-Munt,N., Ebright,R.H. and Gourse,R.L. (1996) DNA-binding determinants of the α subunit of RNA polymerase: novel DNA-binding domain architecture. *Genes Dev.*, **10**, 16–26.

Gaston,K., Bell,A., Kolb,A., Buc,H. and Busby,S. (1990) Stringent spacing requirements for transcription activation by CRP. *Cell*, **62**, 733–743.

Ghosaini,L.R., Brown,A.M. and Sturtevant,J.M. (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.

Jeon,Y.H., Negishi,T., Shirakawa,M., Yamazaki,T., Fujita,N., Ishihama,A. and Kyogoku,Y. (1995) Solution structure of the activator contact domain of the RNA polymerase α subunit. *Science*, **270**, 1495–1497.

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 Eσ^{38} for overlapping promoters and ability to support CRP activation. *Nucleic Acids Res*., **23**, 819–826.

Lloyd,G.S., Busby,S.J.W. and Savery,N.J. (1998) Spacing requirements for interactions between the C-terminal domain of the α subunit of *Escherichia coli* RNA polymerase and the cyclic AMP receptor protein. *Biochem. J*., **330**, 413–420.

Lombardo,M., Bagga,D. and Miller,C.G. (1991) Mutations in *rpoA* affect expression of anaerobically regulated genes in *Salmonella typhimurium*. *J. Bacteriol.*, **173**, 7511–7518.

Miller,J. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Murakami,K., Fujita,N. and Ishihama,A. (1996) Transcription factor recognition surface on the RNA polymerase α subunit is involved in contact with the DNA enhancer element. *EMBO J*., **15**, 4358–4367.

Murakami,K., Owens,J.T., Belyaeva,T.A., Meares,C.F., Busby,S.J.W. and Ishihama,A. (1997) Positioning of two alpha subunit carboxy-terminal domains of RNA polymerase at promoters by two transcription factors. *Proc. Natl Acad. Sci. USA*, **94**, 11274–11278.

Niu,W., Zhou,Y., Dong,Q., Ebright,Y.W. and Ebright,R.H. (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.H. (1996) Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. *Cell*, **87**, 1123–1134.

Rao,L., Ross,W., Appleman,J.A., Gaal,T., Leirmo,S., Schlax,P.J., Record,M.T.,Jr and Gourse,R.L. (1994) Factor-independent activation of *rrnB* P1; an 'extended' promoter with an upstream element that dramatically increases promoter strength. *J. Mol. Biol.*, **235**, 1421–1435.

Rhodius,V.A. and Busby,S.J.W. (1998) Positive activation of gene expression. *Curr. Opin. Microbiol.*, **1**, 152–159.

Rhodius,V.A., West,D.M., Webster,C.L., Busby,S.J.W. and Savery,N.J. (1997) Transcription activation at class II CRP-dependent promoters: the role of different activating regions. *Nucleic Acids Res.*, **25**, 326–332.

Ross,W., Thompson,J.F., Newlands,J.T. and Gourse,R.L. (1990) *E.coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. *EMBO J.*, **9**, 3733–3742.

Ross,W., Gosink,K.K., Salomon,J., Igarishi,K., Zou,C., Ishihama,A., Severinov,K. and Gourse,R.L. (1993) A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. *Science*, **262**, 1407–1413.

Savery,N.J., Rhodius,V.A., Wing,H.J. and Busby,S.J.W. (1995) Transcription activation at *Escherichia coli* promoters dependent on the cyclic AMP receptor protein: effects of binding sequences for the RNA polymerase α subunit. *Biochem. J.*, **309**, 77–83.

Severinov,K., Soushko,M., Goldfarb,A. and Nikiforov,V. (1993) Rifampicin region revisited—new rifampicin-resistant and streptolydigin-resistant mutants in the beta-subunit of *Escherichia coli* RNA-polymerase. *J. Biol. Chem.*, **268**, 14820–14825.

Simons,R.W., Houman,F. and Kleckner,N. (1987) Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene*, **53**, 85–96.

Tang,H., Severinov,K., Goldfarb,A., Fenyo,D., Chait,B. and Ebright,R.H. (1994) Location, structure, and function of the target of a transcriptional activator protein. *Genes Dev.*, **8**, 3058–3067.

Tang,H., Severinov,K., Goldfarb,A. and Ebright,R.H. (1995) Rapid RNA polymerase genetics: one-day, no-column preparation of reconstituted recombinant *Escherichia coli* RNA polymerase. *Proc. Natl Acad. Sci. USA*, **92**, 4902–4906.

West,D., Williams,R., Rhodius,V., Bell,A., Sharma,N., Zou,C., Fujita,N., Ishihama,A. and Busby,S. (1993) Interactions between *Escherichia coli* cyclic AMP receptor protein and RNA polymerase at class II promoters. *Mol. Microbiol.*, **10**, 789–797.

Wood,L.F., Tszine,N.Y. and Christie,G.E. (1997) Activation of P2 late transcription by P2 Ogr protein requires a discrete contact site on the C-terminus of the α subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.*, **274**, 1–7.

Zalenskaya,K., Lee,J.Y., Gujuluva,C.N., Shin,Y.K., Slutsky,M. and Goldfarb,A. (1990) Recombinant RNA-polymerase—inducible overexpression, purification and assembly of *Escherichia coli rpo* gene-products. *Gene*, **89**, 7–12.

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., Busby,S. and Ebright,R.H. (1993) Identification of the functional subunit of a dimeric transcription factor protein by use of oriented heterodimers. *Cell*, **73**, 375–379.

Zhou,Y., Merkel,T.J. and Ebright,R.H. (1994a) 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.

Zhou,Y., Pendergrast,P., Bell,A., Williams,R., Busby,S. and Ebright,R. (1994b) The functional subunit of a dimeric transcription activator protein depends on promoter architecture. *EMBO J.*, **13**, 4549–4557.

Zou,C., Fujita,N., Igarishi,K. and Ishihama,A. (1992) Mapping the cAMP receptor protein contact site on the α subunit of *Escherichia coli* RNA polymerase. *Mol. Microbiol.*, **6**, 2599–2605.

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