Spacing requirements for interactions between the C-terminal domain of the α subunit of *Escherichia coli* RNA polymerase and the cAMP receptor protein

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During transcription initiation at bacterial promoters, the C-terminal domain of the RNA polymerase α subunit (α CTD) can interact with DNA-sequence elements (known as UP elements) and with activator proteins. We have constructed a series of semi-synthetic promoters carrying both an UP element and a consensus DNA-binding site for the *Escherichia coli* cAMP receptor protein (CRP; a factor that activates transcription by making direct contacts with α CTD). At these promoters, the UP element was located at a variety of distances upstream of the CRP-binding site, which was fixed at position -41.5 bp upstream of the transcript start. At some positions, the UP element caused

enhanced promoter activity whereas, at other positions, it had very little effect. In no case was the CRP-dependence of the promoter relieved. DNase I and hydroxyl-radical footprinting were used to study ternary RNA polymerase–CRP–promoter complexes formed at two of the most active of these promoters, and co-operativity between the binding of CRP and purified α subunits was studied. The footprints show that α CTD binds to the UP element as it is displaced upstream but that this displacement does not prevent α CTD from being contacted by CRP. Models to account for this are discussed.

INTRODUCTION

In *Escherichia coli*, the major RNA polymerase holoenzyme (RNAP) responsible for transcription initiation is a multi-subunit enzyme with the structure $\alpha_2\beta\beta'\sigma^{70}$. The β and β' subunits carry the catalytic site, while the σ subunit is required for specific initiation at promoters. Protein–DNA interactions between σ and two promoter elements located 10 and 35 bp upstream of the transcription start point are required for promoter recognition and transcription initiation [1]. However, at some promoters, transcription initiation also depends on interactions between the C-terminal domain of the α subunit of RNAP (α CTD) and a third promoter sequence element, known as the UP element. In most cases, α CTD-binding elements are 20-bp AT-rich sequences located just upstream of the -35 hexamer, the best-characterized example being the UP element at the ribosomal RNA promoter, *rnnB* P1, which increases promoter activity by over 30-fold [2]. However, α CTD is joined to the rest of α by a flexible linker [3], and thus UP elements are also functional when placed at different locations upstream of promoters, provided that they are located on the same face of the DNA helix [4].

In addition to contacting DNA, α CTD can also contact activator proteins: at many activator-dependent promoters, this contact between α CTD and the activator is essential to recruit RNAP to the promoter and to compensate for defects in one or more of the DNA-sequence elements [3]. One very well-studied transcription factor that interacts directly with α CTD is the *E. coli* cAMP receptor protein (CRP), a cAMP-triggered regulatory factor that can activate transcription initiation at more than 100 different target promoters in *E. coli* (reviewed in [5]). CRP binds

as a dimer to 22-bp sequences that are found located at a variety of positions at promoters. Promoters that carry the DNA site for CRP centred close to position -41 (known as Class II promoters) form a major subset of the CRP regulon. At these promoters, bound CRP activates transcription by making several direct contacts with RNAP. One of these contacts involves interaction between a surface-exposed loop of CRP (activating region 1, AR1) and α CTD [6,7]. The bound CRP dimer prevents α CTD from binding immediately upstream of the -35 hexamer and α CTD is, therefore, displaced upstream of CRP [8].

α CTD–UP-element interactions and α CTD–CRP interactions are both thought to ‘recruit’ α CTD to the DNA, thereby increasing the affinity of RNAP for the promoter [3]. To investigate this, we previously took a Class II CRP-dependent promoter and introduced the *rnnB* P1 α -binding UP element upstream of the CRP binding site [9]. We observed that this UP element increased the activity of the promoter but did not alter its dependence on CRP. This suggested that the contributions of α CTD–UP-element interactions and α CTD–CRP interactions to promoter activity are independent and additive. In the present study we investigated the spacing requirements for interactions between CRP and α CTD by creating a series of promoters in which the distance between the DNA site for CRP and the UP element was altered. We report that UP-element-dependent increases in promoter activity are dependent on the location of the UP element and that, in all cases, activity remains CRP-dependent. We have studied interactions between α subunits and CRP at the promoters where the UP element had the maximum effect, and investigated the dependence of these interactions on AR1 of CRP.

Abbreviations used: RNAP, *Escherichia coli* RNA polymerase holoenzyme; CRP, *E. coli* cAMP receptor protein; α CTD, C-terminal domain of the α subunit of RNAP; AR1, activating region 1 of CRP.

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EXPERIMENTAL

Strains, plasmids and promoter constructions

The bacterial strains, plasmids and DNA fragments used in this study are listed in Table 1. Standard methods for isolation and manipulation of DNA fragments were used throughout. Plasmids carrying different promoter fragments were propagated in the host strain DH5 α . All the promoters used in this work were cloned on *EcoRI*–*HindIII* fragments with the *EcoRI* site upstream of the transcription start site and the *HindIII* site downstream. By convention, promoter sequences are numbered with the transcript start taken as +1, with upstream and downstream sequences prefixed '–' and '+' respectively. The promoters used in this work are denoted CC(–41.5) α (–X) where –41.5 is the position of the centre of the DNA site for CRP, and –X is the position of the non-template strand T (arbitrarily chosen) in the middle of the UP element. The series of promoters created in this work were constructed on *EcoRI*–*HindIII* fragments cloned in the vector pAA121, and are shown in Figure 1. The starting promoter was CC(–41.5) α (–76) (previously referred to as NJS2 in [9]). CC(–41.5) α (–71), α (–67), α (–63) and α (–58) were made by digestion of CC(–41.5) α (–76) with *NcoI* followed by treatment with mung-bean nuclease and religation. CC(–41.5) α (–80) was constructed by digestion of CC(–41.5) α (–76) with *NcoI* followed by treatment with Klenow enzyme and religation. CC(–41.5) α (–70) was made by PCR amplification of CC(–41.5) α (–71) with

primers 5'-TTCAGATCTGAAAATTATTTTAAATTTCCGA-TCTAGGTAAATGTG-3' and 5'-GTAGTCGGTGTGTTT-CAC-3'. This results in the deletion of a G residue at position –62 in CC(–41.5) α (–71). CC(–41.5) α (–61) was made by PCR amplification of CC(–41.5) α (–63) with primers 5'-TTCAGATCTGAAAATTATTTTAAATTTCTAAATGT-GATGTAC-3' and 5'-GTAGTCGGTGTGTTT-CAC-3'. This results in the deletion of two G residues from positions –54 and –55 in CC(–41.5) α (–63). The CC(–41.5) α (–86) promoter was made by annealing the synthetic oligodeoxynucleotides 5'-CATGTCGACC-3' and 5'-CATGGGTCGA-3', and ligating the product into the *NcoI* site of CC(–41.5) α (–76). The CC(–41.5) α (–88) and CC(–41.5) α (–84) promoters were made by resolving multiple inserts of the annealed oligodeoxynucleotides (the resulting sequence depending on the orientation of the inserts). All the derivatives were checked by double-stranded sequencing using the flanking primer 5'-ACCTGACGTCTA-AGAAACC-3'.

All the above constructions resulted in *EcoRI*–*HindIII* fragments with the *HindIII* site located downstream of the transcription start point at position +265. For footprinting and gel-retardation experiments, shorter derivatives of each promoter fragment (denoted D4) were made, with the *HindIII* site located at position +36. These were made by PCR amplification of the pAA121 derivative carrying each promoter using the synthetic oligodeoxynucleotides 5'-AGCAAGCTTTATCTGTATTCA-TGG-3' and 5'-ACCTGACGTCTAAGAAACC-3' as primers.

Table 1 Bacterial strains, plasmids and promoter fragments

	Description	Source
Bacterial strains		
M182	Δlac <i>E. coli</i> K12 strain	Casadaban and Cohen [10]
M182 Δcrp	Δcrp derivative of M182	Busby et al. [11]
BL21(DE3)	<i>E. coli</i> strain with inducible T7 RNA polymerase	Groberg and Dunn [12]
DH5 α	Δlac <i>recA</i> strain	Hanahan [13]
Bacterial plasmids		
pAA121	pBR322-derived vector for cloning <i>EcoRI</i> – <i>HindIII</i> fragments	Kelsall et al. [14]
pRW50	Broad host range <i>lac</i> expression vector for cloning <i>EcoRI</i> – <i>HindIII</i> fragments	Lodge et al. [15]
pDCRP	pBR322 derivative carrying wild-type <i>crp</i>	Bell et al. [16]
pDCRP HL159	Derivative of pDCRP encoding CRP HL159	Bell et al. [16]
pDU9	Derivative of pDCRP with <i>crp</i> gene deleted	Bell et al. [16]
pDR540	Vector carrying <i>lac</i> promoter	Pharmacia
pHTT7f1-NH α	Plasmid encoding His-tagged α under control of the bacteriophage T7 gene 10 promoter	Tang et al. [17]
pHT <i>lac</i> 1-NH α	Plasmid encoding His-tagged α under control of the <i>lac</i> promoter	This work
Promoter fragments (all <i>EcoRI</i> – <i>HindIII</i> fragments)		
CC(–41.5)	Fragment carrying CRP-dependent promoter with consensus DNA site for CRP centred at position –41.5 upstream of the <i>melR</i> transcript start	Gaston et al. [28]
NJS1	Derivative of CC(–41.5) with <i>EcoRI</i> – <i>Bgl</i> II linker inserted upstream of DNA site for CRP	Savery et al. [9]
CC(–41.5) α (–76)	Derivative of NJS1 with UP-element cloned between <i>EcoRI</i> and <i>Bgl</i> II sites upstream of DNA site for CRP (previously called NJS2)	Savery et al. [9]
CC(–41.5) α (–88)	Derivative of CC(–41.5) α (–76) with 12 bp inserted*	This work
CC(–41.5) α (–86)	Derivative of CC(–41.5) α (–76) with 10 bp inserted*	This work
CC(–41.5) α (–84)	Derivative of CC(–41.5) α (–76) with 8 bp inserted*	This work
CC(–41.5) α (–80)	Derivative of CC(–41.5) α (–76) with 4 bp inserted*	This work
CC(–41.5) α (–71)	Derivative of CC(–41.5) α (–76) with 5 bp deleted†	This work
CC(–41.5) α (–70)	Derivative of CC(–41.5) α (–76) with 6 bp deleted†	This work
CC(–41.5) α (–67)	Derivative of CC(–41.5) α (–76) with 9 bp deleted†	This work
CC(–41.5) α (–63)	Derivative of CC(–41.5) α (–76) with 13 bp deleted†	This work
CC(–41.5) α (–61)	Derivative of CC(–41.5) α (–76) with 15 bp deleted†	This work
CC(–41.5) α (–58)	Derivative of CC(–41.5) α (–76) with 18 bp deleted†	This work

* Fragment inserted between UP element and CRP site.

† Fragment deleted between UP element and CRP site.

Figure 1 Base sequences of promoters constructed in this study

Shows the sequences of the non-template strand of the promoters used. The sequences from the upstream *Eco*RI site (GAATTC) to the transcript start (+1) are shown in each case. The locations of the UP element (bold and double-underlined) and the DNA sites for CRP (bold) are shown. CC(−41.5) refers to the position of centre of the DNA site for CRP, while the α(−X) refers to the position of the UP element where X is taken as a T in the centre of the sequence.

PCR products were digested with *Eco*RI and *Hind*III, and the resulting fragments were cloned between the *Eco*RI and *Hind*III sites of pAA121.

Assays of promoter activity *in vivo*

To assay the activity of the different promoters, *Eco*RI–*Hind*III fragments were cloned into the broad-host-range low-copy-number *lac* expression vector, pRW50, placing the *lac* genes under the control of the promoter. The resulting plasmids were transformed into the Δ *lac* strains M182 or M182 Δ *crp*, and transformants were grown in Luria broth supplemented with appropriate antibiotics. β -Galactosidase expression was measured exactly as described previously [15,16] using the Miller [18] method, and β -galactosidase activities were taken to be proportional to the activity of the cloned promoter.

Purification of α subunits

Plasmid pHT*taef*I-NH α was constructed by replacing the T7 promoter of pHTT7f1-NH α with the *tac* promoter from plasmid pDR540 (Pharmacia, Uppsala, Sweden). The *tac* promoter region of pDR540 was amplified by PCR using the primers 5'-GCTC-TAGATTGTTATCCGCTCAC-3' and 5'-ACCTGACGTCTA-AGAAACC-3'. The resulting PCR product was cut with *Hind*III, treated with Klenow enzyme to produce a blunt end and then cut with *Xba*I. This fragment was ligated into vector DNA prepared from pHTT7f1-NH α by cutting with *Bgl*II, treating with Klenow enzyme to produce a blunt end and then cutting with *Xba*I. His-tagged RNAP α subunits were purified in non-denaturing conditions from M182 Δ *crp* cells transformed with pHT*taef*I-NH α . The procedure was essentially as described in Tang et al. [17],

except that the His-tagged alpha was eluted from a Ni²⁺-NTA agarose column (Quiagen, Hilden, Germany) by a gradient of 5–75 mM imidazole in 20 mM Tris/HCl (pH 7.9)/500 mM NaCl/10% glycerol.

Footprinting and gel-retardation experiments

DNase I and hydroxyl-radical footprinting studies *in vitro* were performed using purified holo-RNAP and CRP or CRP HL159, purified as described in [16] using the method of Ghosaini et al. [19]. Footprinting of proteins bound to labelled *Pst*I–*Hind*III fragments containing the CC(−41.5) α (−63) and CC(−41.5) α (−76) promoters was performed exactly as in our previous work [8,20–22]. The non-template strands of the shortened D4 derivative fragments were end-labelled using [γ -³²P]ATP at the *Hind*III end. Footprinting gels were calibrated with Maxam–Gilbert 'G' sequencing reactions of the labelled fragments.

Gel-retardation assays were performed with *Eco*RI–*Hind*III fragments end-labelled using [γ -³²P]ATP, essentially as described by Kolb et al. [23], except that 2 mM spermidine and 20 μ g/ml sonicated herring-sperm DNA were included in incubation mixes, the gel running buffer was 0.5 $\times$ Tris/borate/EDTA containing 2% glycerol, and samples were run on 6% acrylamide gels containing 7.5% glycerol and 0.5 $\times$ Tris/borate/EDTA buffer.

RESULTS AND DISCUSSION

Spacing requirements for UP-element function

The starting point for this work was the NJS1 promoter, a semi-synthetic Class II CRP-dependent promoter, carrying a DNA site for CRP centred at position −41.5. We constructed a series

Figure 2 Activity of different promoters

Shows the β -galactosidase activities measured in M182 crp^+ Δlac and M182 Δcrp Δlac cells carrying different Class II CRP-dependent promoters (with the UP element located at different positions) cloned into the *lac* expression vector pRW50. The control NJS1 promoter is a Class II CRP-dependent promoter lacking an UP element. Cells were grown exponentially in Luria broth containing 35 μ g/ml tetracycline and β -galactosidase expression was measured by the Miller protocol [18]: activities are expressed in Miller units of β -galactosidase activity. Each value is the mean $\pm$ standard deviation of at least three independent assays. Cells carrying the vector pRW50 with no promoter insert contain less than 25 units of β -galactosidase activity.

of derivatives in which the UP element from the *E. coli rrnB* P1 promoter was cloned upstream of the DNA site for CRP, at different locations from position -58 to position -88 (shown in Figure 1). To determine the effect on promoter activity of changing the spacing between the CRP site and the UP element, *EcoRI*–*HindIII* fragments carrying each of the promoters were cloned into the *lac* expression vector, pRW50, and β -galactosidase levels were measured in M182 crp^+ and M182 Δcrp host cells. The results, shown in Figure 2, confirm that CRP-dependent promoter activity can be increased 3–4-fold by the UP element, and that this increase is dependent on the precise location of the UP element (compare the activities of the different promoters with the activity of the NJS1 promoter lacking an UP element). UP-element-dependent increases in activity are ‘face-of-the-helix’-dependent, with peaks of activity when the UP element is located at positions -63 , -76 and -86 . The results with the M182 Δcrp host cells show that, in each case, promoter activity is dependent on CRP. Thus, although the presence of the UP element increases promoter activity, it cannot relieve the dependence on CRP.

The requirement for AR1 is not removed by an UP element

Transcription activation at many CRP-dependent promoters is dependent on direct interactions between AR1 of CRP and a target site in the α subunit of RNAP [7,21,24,25]: this interaction is disrupted by a Leu-for-His substitution at position 159 in CRP (this CRP derivative is denoted HL159) [16,24]. To assess the role of AR1– α CTD interactions at the new series of promoters, plasmid pDCRP, encoding either wild-type CRP or the HL159 derivative, was introduced into the M182 Δcrp host strain carrying the different promoters in pRW50, and β -galactosidase activities were measured. The results, shown in Table 2, indicate

that activity of all the promoters can be increased by the presence of plasmid-encoded wild-type CRP, and that the HL159 substitution reduces activation substantially in each case. In most

Table 2 CRP-dependence of promoters

This Table lists the β -galactosidase levels made in M182 and M182 Δcrp cells that were grown aerobically in Luria broth containing 35 μ g/ml tetracycline and 80 μ g/ml ampicillin, and were harvested during exponential growth. The M182 Δcrp cells contained pRW50 carrying different promoters plus pDU9, pDCRP or pDCRP HL159. β -Galactosidase levels were measured by the Miller protocol [18] and are expressed as Miller units. Each assay was performed independently at least three times and the values shown in the table are the averages of these determinations. The % value shown in brackets in the HL159 column is a measure of expression with CRP carrying the HL159 substitution compared to expression with wild-type CRP. Cells carrying the vector pRW50 with no promoter insert contained less than 25 units of β -galactosidase activity.

Promoter	β -Galactosidase expression (in Miller units) by pRW50 carrying promoters in			
	M182 Δcrp cells containing			
	M182 cells	pDU9	pDCRP	
NJS1	1501	71	1983	160 (8%)
CC(–41.5) α (–88)	2579	98	3501	161 (5%)
CC(–41.5) α (–86)	3245	51	4618	266 (6%)
CC(–41.5) α (–84)	2702	101	3192	238 (7%)
CC(–41.5) α (–80)	1675	80	2616	125 (5%)
CC(–41.5) α (–76)	4384	143	4858	648 (8%)
CC(–41.5) α (–71)	2081	69	2586	191 (5%)
CC(–41.5) α (–70)	1685	56	2354	187 (8%)
CC(–41.5) α (–67)	4567	222	7832	1704 (21%)
CC(–41.5) α (–63)	7175	266	12310	1485 (12%)
CC(–41.5) α (–61)	4039	70	5148	1066 (20%)
CC(–41.5) α (–58)	734	68	873	95 (11%)

Figure 3 DNase I and hydroxyl-radical footprint analyses of complexes formed at the CC(−41.5)α(−76) promoter

Shows autoradiograms of sequence gels run to analyze products after (a) DNase I and (b) hydroxyl-radical cleavage of complexes formed between a DNA fragment carrying the CC(−41.5)α(−76) promoter (end-labelled on the lower strand), purified CRP or CRP HL159 (100 nM) and RNA polymerase (100 nM) as indicated. In each case, lane 1 of each autoradiogram contains a Maxam–Gilbert sequencing reaction specific for G residues, and the shaded bars indicate protection by α , CRP and the rest of RNA polymerase, as indicated.

cases, the HL159 substitution reduces promoter activity by over 90 %, although this reduction is less at promoters where the UP element is positioned closer to the DNA site for CRP. Thus, AR1 of CRP is required for full activation of each promoter, even when the presence of the UP element causes a several-fold increase in activity, irrespective of the location of the UP element.

Footprinting studies of ternary RNAP–CRP–promoter complexes

The largest effect of the UP element is seen at the CC(−41.5)α(−63) promoter (Figure 1 and Table 2). At this promoter it is likely that the UP element and the DNA site for CRP are ideally juxtaposed so that α CTD can make simultaneous interactions with CRP and the UP element. We have used DNase I and hydroxyl-radical footprinting to investigate the location of CRP and α CTD in complexes at this promoter. In parallel, experiments were performed with the CC(−41.5)α(−76) promoter, where the UP element is located more than one turn of the DNA helix further upstream.

Figure 3 and Figure 4 show the results of footprinting with the CC(−41.5)α(−76) promoter and the CC(−41.5)α(−63) promoter, respectively. As expected, in the DNase I footprints with the CC(−41.5)α(−76) promoter, purified CRP binds to and protects the DNA site for CRP, while the ternary CRP–RNAP–promoter complexes give an extended footprint from the upstream limit of the UP-element sequence to beyond the transcription start site (Figure 3a). In the absence of CRP, holo-

RNAP does not form an open complex but gives a footprint in the region of the UP element, likely to be due to protection by the α subunits. The protection seen with CRP carrying the HL159 substitution (which disrupts AR1) is different to that with wild-type CRP: with the mutant protein, additional DNase I-sensitive bands are seen between the CRP site and the UP element, as previously described for the *galP1* promoter [20].

The hydroxyl-radical footprints allow precise identification of the bands protected by protein. Figure 3(b) indicates that bound CRP protects three sets of bases from −29 to −33, −40 to −43 and −50 to −52 on the non-template strand. Three further areas of protection, each separated by approximately 10 bp, are apparent upstream of the CRP site, corresponding to the bases protected by α CTD and extending to the upstream limit of the cloned UP element. As reported by Belyaeva et al. [8], this phasing of protected bands shows that CRP and α CTD bind to the same face of the DNA, and that α CTD is binding to, and protecting bases within, the UP element.

With the CC(−41.5)α(−63) promoter, the result of DNase I footprinting is very similar (Figure 4a): purified CRP binds to and protects the DNA site for CRP, whereas the ternary CRP–RNAP–promoter complex gives an extended footprint from the upstream limit of the UP-element sequence to beyond the transcription start site. Interestingly, however, no additional DNase I-sensitive bands are seen in the footprint with CRP carrying the HL159 substitution. Hydroxyl-radical footprinting (Figure 4b) reveals the same three areas of protection due to CRP, but, in contrast to the CC(−41.5)α(−76) promoter, only

Figure 4 DNase I and hydroxyl-radical footprint analyses of complexes formed at the CC(-41.5) α (-63) promoter

Shows autoradiograms of sequence gels run to analyze products after (a) DNase I and (b) hydroxyl-radical cleavage of complexes formed between a DNA fragment carrying the CC(-41.5) α (-63) promoter (end-labelled on the lower strand), purified CRP or CRP HL159 (100 nM) and RNA polymerase (100 nM) as indicated. In each case, lane 1 contains a Maxam-Gilbert sequencing reaction specific for G residues, and the shaded bars indicate protection by α , CRP and the rest of RNA polymerase, as indicated.

two further upstream protected sets of bases are observed: these are due to α CTD binding to the UP element, which, at the CC(-41.5) α (-63) promoter, is closer to the DNA site for CRP. Thus, CRP and the α subunits bind immediately adjacent to each other. These results indicate that the ternary complexes formed at the CC(-41.5) α (-63) and the CC(-41.5) α (-76) promoters differ, and that the position of the UP element determines the location of at least one α CTD.

Interactions between CRP and purified α subunits bound to adjacent sites

We have used band-shift assays to investigate the binding of purified α subunits, in either the presence or absence of CRP, to DNA fragments carrying the CC(-41.5) α (-63) promoter. Figure 5(a) shows the titration of fragments with the purified α subunits alone, while Figure 5(b) shows the result of the titration performed in the presence of wild-type CRP. In the absence of CRP, 4 μ M α subunit is required to shift the fragment, whereas in the presence of CRP, only 1 μ M is required. This indicates cooperativity, with the presence of CRP increasing the binding of α subunit 4-fold. Figure 5(c) shows a similar experiment performed with CRP carrying the HL159 substitution that interrupts AR1: in the conditions of the experiment 4 μ M α subunit is required to shift the promoter fragment. Since this is the concentration required to shift the fragment in the absence of CRP, we conclude that co-operativity between CRP and α subunits in binding to the CC(-41.5) α (-63) promoter is dependent on AR1 of CRP. Similar experiments were also per-

formed with promoter derivatives in which the spacing between the DNA site for CRP and the UP element was altered: in each case, the co-operative interactions were reduced (G. S. Lloyd, unpublished work).

Conclusions

In the present study, we have shown that an UP element cloned upstream of the CRP binding site at a Class II CRP-dependent promoter can increase promoter activity from a number of different locations. At every promoter tested, AR1 of CRP makes a contribution to transcription activation, and optimal activity is observed when the UP element is located at position -63. The hydroxyl-radical-footprint data suggest that, at the CC(-41.5) α (-63) promoter, the two α CTDs of RNAP fit into the two adjacent major grooves immediately upstream of bound CRP and on the same face of the DNA (see the top line of Figure 6). We propose that, at the CC(-41.5) α (-63) promoter, α CTD bound to the UP element and CRP are perfectly juxtaposed, allowing optimal α CTD-AR1 interactions to be made: the band-shift assays show that CRP and purified α subunits bind co-operatively. As the UP element is moved further upstream, it becomes less effective, presumably because α CTD is unable to contact AR1 of CRP and the UP element simultaneously. However, peaks of promoter activity are found when the UP element is moved to positions -76 and -86 (Figure 2): again, this activity is dependent on AR1 of CRP (Table 2). At these positions, we suppose that the UP element is again on the same face of the DNA helix as AR1 of CRP, and hence, α CTD is able

Figure 5 Interactions between purified CRP and RNAP α subunits at the CC(–41.5) α (–63) promoter

Shows autoradiograms of gel-retardation assays analyzing the binding of increasing concentrations of purified wild-type α subunits to end-labelled fragments carrying the CC(–41.5) α (–63) promoter. Initial incubation mixtures contained approx. 0.4 nM labelled fragment and different concentrations of purified α as indicated: (a) reaction mixtures contained no CRP; (b) reaction mixtures contained 100 nM wild-type CRP (except the first lane with no CRP); and (c) reaction mixtures contained 100 nM CRP HL159 (except the first lane with no CRP).

to contact AR1 of CRP and the UP element simultaneously. However, this poses a serious problem: since, according to the footprinting, α CTD 'tracks' upstream as the UP element is moved upstream, and since the CRP dimer remains fixed to its site, how is the same α CTD–AR1 contact made as α CTD is moved upstream? Figure 6 suggests three models to explain the observation that α CTD displaced upstream can still contact AR1 on CRP. In model 1, the C-terminal domains of both α subunits remain anchored to the UP element when it is moved further upstream: the distance between AR1 of CRP and its target in α CTD is therefore increased, and a direct contact is only possible after distortion of the DNA. In model 2, the gap between CRP and the UP element is bridged by the two α CTDs shuttling between two positions, one of which involves optimal interactions with the UP element while the other allows α CTD to contact CRP. Finally, model 3 supposes that only one α CTD follows the UP element to its upstream position, while the other remains in contact with the upstream monomer of the CRP dimer. Although the experimental approaches outlined here do not permit us to distinguish between these three possibilities, recently developed

At CC(–41.5) α (–63):

At CC(–41.5) α (–76):

Model 1

Model 2

Model 3

Figure 6 The organization of the RNAP subunits and CRP at class II CRP-dependent promoters

The top line of the Figure shows the likely organization of transcriptionally competent complexes at the CC(–41.5) α (–63) promoter: the two α CTDs bind immediately upstream of the CRP dimer and make independent contacts with both the UP element and AR1 of CRP. Below are shown three models (1, 2 and 3) to account for the organization of the RNAP and CRP subunits at the CC(–41.5) α (–76) promoter, where the UP element is located more than one turn of the DNA helix further upstream. The models provide an explanation for the observation that α CTD binds to the displaced UP element and yet is still able to make contact with AR1 of CRP. Model 1 supposes that the α CTD–AR1 contact is facilitated by a distortion in the DNA. Model 2 supposes that α CTD 'dances' on the DNA between the UP element and a position adjacent to the CRP dimer [as at the CC(–41.5) α (–63) promoter]. Model 3 supposes that the two α CTDs are separated with one α CTD contacting AR1 of CRP and that the other α CTD contacting the UP element (note that a similar model has been proposed by Eichenberger et al. [27] to account for flexibility in the positioning of α CTD at Class I CRP-dependent promoters).

techniques which allow the two α CTDs of RNAP to be individually labelled with a chemical 'tag' may solve the problem [26].

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