Interactions between the *Escherichia coli* cAMP receptor protein and the C-terminal domain of the α subunit of RNA polymerase at Class I promoters

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The *Escherichia coli* cAMP receptor protein (CRP) is a factor that activates transcription at over 100 target promoters. At Class I CRP-dependent promoters, CRP binds immediately upstream of RNA polymerase and activates transcription by making direct contacts with the C-terminal domain of the RNA polymerase α subunit (α CTD). Since α CTD is also known to interact with DNA sequence elements (known as UP elements), we have constructed a series of semi-synthetic Class I CRP-dependent promoters, carrying both a consensus DNA-binding site for CRP and a UP element at different positions. We

previously showed that, at these promoters, the CRP– α CTD interaction and the CRP–UP element interaction contribute independently and additively to transcription initiation. In this study, we show that the two halves of the UP element can function independently, and that, in the presence of the UP element, the best location for the DNA site for CRP is position –69.5. This suggests that, at Class I CRP-dependent promoters where the DNA site for CRP is located at position –61.5, the two α CTDs of RNA polymerase are not optimally positioned. Two experiments to test this hypothesis are presented.

INTRODUCTION

Transcription in *Escherichia coli* is effected by a single multi-subunit DNA-dependent RNA polymerase (RNAP) of subunit structure, $\alpha_2\beta\beta'\sigma$. Transcription initiation requires the σ subunit: direct interactions between σ and promoter –10 and –35 elements are essential for promoter recognition and the initiation of RNA synthesis [1]. However, at some promoters, transcription initiation also depends on interactions between the C-terminal domains of the two α subunits (α CTD) and a third promoter element, the UP element [2]. α CTD-binding elements cover two helical turns of promoter DNA (≈ 20 bp) and, in many cases, are found just upstream of the promoter –35 hexamer. Recent results suggest that, at UP elements, the two α CTDs of RNAP bind adjacent to each other on the same side of the promoter DNA [3–5]. Although it is not clear whether α CTD recognizes motifs in the major or minor grooves of target promoter DNA, it is apparent that each α CTD covers approximately half of the UP element sequence. Moreover, each α CTD can be independently positioned and there is considerable flexibility in its possible location [5,6]. This flexibility appears to be due to the linker that joins α CTD to the rest of α and hence to RNAP [7,8].

At many activator-dependent promoters, α CTD contacts the activator in addition to contacting DNA. This protein–protein contact is essential to compensate for defects in one or more of the promoter DNA sequence elements [9]. The best studied activator 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 [10]). CRP binds as a dimer to 22 bp sequences that are located at a variety of positions within target promoters. At one major subset of CRP-dependent promoters (known as Class I promoters), a single DNA site for CRP is located upstream of the promoter –35 element, centred near position –61.5, –71.5, –81.5 or –91.5 (reviewed in [11]). Bound CRP activates transcription initiation at these promoters by making a direct contact with RNAP: this contact involves

interaction between a surface-exposed loop of CRP (activating region 1) and α CTD [11,12], which increases the affinity of RNAP for the promoter DNA, thereby recruiting RNAP to the target promoter. Interestingly, the flexible linker that joins α CTD to the rest of RNAP permits the same CRP–RNAP contact to be made, irrespective of the location of the bound CRP [13,14].

Recent studies suggest that activating region 1 of CRP and UP element sequences are contacted by distinct surfaces of α CTD [15]. Thus, α CTD can make simultaneous, separate contacts with CRP and promoter DNA, and the contributions of α CTD–promoter DNA and α CTD–CRP interactions to promoter activity may be independent and additive. In support of this, we previously took a Class I CRP-dependent promoter and introduced the *rrnB* P1 α -binding UP element immediately downstream of the CRP-binding site [16]. We observed that the *rrnB* P1 UP element increased the activity of this promoter, but did not alter its dependence on CRP, confirming that, at least in this system, the contributions of α CTD–UP element interactions and α CTD–CRP interactions to promoter activity are independent and additive. In this work, we have extended our study to show that CRP-dependent transcription can be stimulated by half of the UP element. We then investigated the effects of altering the location of the UP element and the DNA site for CRP, in order to determine the optimal juxtaposition of CRP and α CTD. Finally, the implications of our findings for other Class I CRP-dependent promoters (such as the *lac* promoter) are considered.

EXPERIMENTAL

Strains, plasmids and promoters

The starting bacterial strains, plasmids and DNA fragments used in this study are described in Table 1 of our previous paper [16]. The CRP-dependent promoters used in this study are derivatives of the CC(–72.5) promoter. All the promoters described here were carried on *EcoRI*–*HindIII* fragments, with the *EcoRI* site and *HindIII* site respectively upstream and downstream of the promoter sequences. For cloning and for propagation, these

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.

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Figure 1 Base sequences of Class I CRP-dependent promoters

The sequences of the non-template strand of the CC(-72.5), NJS4, NJS5 and NJS6 promoters are shown, from the upstream *EcoRI* site (GAATTC) to the transcript start (+1) in each case. The -10 hexamer is singly underlined and the UP element (and segments thereof) is boxed. The locations of the DNA sites for CRP are shown (double underlined).

EcoRI–*HindIII* fragments were cloned into the general cloning vector, pAA121. For measurement of promoter activities, the *EcoRI*–*HindIII* fragments were cloned into the broad host-range, low copy-number *lac* expression vector, pRW50, to give promoter::*lac* fusions. For the *in vitro* transcription assays, promoter fragments were transferred to the plasmid pSR as described by Savery et al. [15]. Recombinant plasmids were propagated in the *E. coli* K12 Δlac strain, M182, or in the Δcrp derivative, M182 Δcrp . Standard methods for isolation and manipulation of DNA fragments were used throughout. By convention, promoter sequences are numbered with the transcript start taken as +1, with upstream and downstream sequences prefixed '–' and '+' respectively. Many of the promoters used in this work are denoted CC(–X) α (–Y) where –X is the position of the centre of the DNA site for CRP, and –Y is the position of the last of four thymines on the non-template strand in the middle of the UP element (arbitrarily chosen, as in our previous work [17]).

Construction of NJS4, NJS5 and NJS6

The CC(–72.5) promoter was constructed from the *E. coli melR* promoter: CC(–72.5) carries a single consensus DNA site for CRP centred at position –72.5 (i.e. between base pairs –72 and –73) upstream of the *melR* –10 region and transcript start point [18]. Savery et al. [16] described the construction of NJS4 in which the 22 bp UP element from the *E. coli rrnB P1* promoter was inserted immediately downstream of the CRP site in CC(–72.5) (Figure 1). The derivatives NJS5 and NJS6 were constructed by exploiting the *DraI* site in the centre of the UP element in NJS4 and the *NruI* site at the corresponding position in CC(–72.5). NJS5 (Figure 1), which carries just the upstream part of the UP element, was constructed by ligating the *EcoRI*–*DraI* fragment from NJS4 to the *NruI*–*HindIII* fragment from CC(–72.5). NJS6 (Figure 1), which carries just the downstream part of the UP element, was made by ligating the *EcoRI*–*NruI* fragment from CC(–72.5) to the *DraI*–*HindIII* fragment from NJS4.

Alteration of the location of the UP element and CRP site of NJS4

The unique *BamHI* site of the NJS4 promoter was exploited to create a series of short deletions ($\Delta 1$, $\Delta 2$, $\Delta 4$ and $\Delta 6$) and insertions (+2, +3 and +4) immediately downstream of the UP element. To facilitate presentation, the NJS4 promoter has been renamed CC(–72.5) α (–46), and subsequent promoters are named by the same convention (see above). Figure 2A shows the base sequence of the resulting promoters. To construct CC(–71.5) α (–45), CC(–70.5) α (–44), CC(–68.5) α (–42) and CC(–66.5) α (–40), PCR was used to amplify the

CC(–72.5) α (–46) promoter cloned in pAA121. The primers used were 5'-ACCTGACGTCTAAGAAACC-3' (which hybridizes upstream of the *EcoRI* site of the pAA121 vector) and 5'-AGGGATCCATGGAGAAATTTAAATAA-3' [for CC(–71.5) α (–45)], 5'-AGGGATCCATGGGAAATTTAAATAA-3' [for CC(–70.5) α (–44)], 5'-AGGGATCCATGAAA-TTTAAATAA-3' [for CC(–68.5) α (–42)] or 5'-AGGGATCCGAAATTTAAATAA-3' [for CC(–66.5) α (–40)]. *EcoRI*–*BamHI* fragments (carrying the CRP site and UP element) were purified from each of the resulting PCR products and ligated to the *BamHI*–*HindIII* fragment from the starting CC(–72.5) α (–46) promoter to give each of the new promoters. To construct the CC(–74.5) α (–48), CC(–75.5) α (–49) and CC(–76.5) α (–50) promoters, the *NcoI* sites of the CC(–70.5) α (–44), CC(–71.5) α (–45) and CC(–72.5) α (–46) promoters respectively were filled in using the Klenow fragment of DNA polymerase.

Construction of the CC(–n) α (–44) series of promoters

Starting with the CC(–70.5) α (–44) promoter, a series of derivatives was generated in which the DNA site for CRP was placed at different positions (Figure 2B). To facilitate subsequent *in vitro* studies, extra spacer DNA was placed upstream of the CRP site (compare Figures 2A and 2B). To do this, an *EcoRI*–*BamHI* fragment carrying DNA for the CRP site was purified after PCR using the CC(–70.5) α (–44) promoter with primers: 5'-ATCAGGAATTCAGAGATCTGCGTATCGATGCAATTCGAGCTCGGTACCCG-3' and 5'-AAGGATCCA-TGTGATGTACATCA-3'. Starting with the CC(–70.5) α (–44) promoter, PCR was then used to generate a set of fragments carrying a *BglII* site at different positions upstream of the UP element. To do this, PCR was performed with the primer 5'-AGCAAGCTTTATCTGTATTTCATGG-3' (which covers the downstream *HindIII* site) and different primers encoding a *BglII* site immediately upstream of the UP element sequence (sequences available on request). Products from each PCR reaction were restricted with *BglII* and *HindIII*, purified and then ligated to the *EcoRI*–*BamHI* fragment carrying the DNA site for CRP and the upstream extension. This generated the CC(–61.5) α (–44), CC(–65.5) α (–44), CC(–69.5) α (–44), CC(–70.5) α (–44), CC(–71.5) α (–44), CC(–72.5) α (–44), CC(–74.5) α (–44) and CC(–76.5) α (–44) promoter sequences shown in Figure 2(B). To construct CC(–78.5) α (–44), the *XbaI* site of CC(–74.5) α (–44) was filled in with the Klenow fragment of DNA polymerase. Similarly, CC(–80.5) α (–44) was constructed by filling in the *SaI* site of CC(–76.5) α (–44). Starting with the CC(–80.5) α (–44) promoter, PCR was again used to generate fragments carrying a *BglII* site further upstream of the UP element. Products from each PCR reaction were again restricted with *BglII* and *HindIII*, purified and ligated to the *EcoRI*–*BamHI*

Figure 2 Base sequences of Class I CRP-dependent promoters

The sequences of the non-template strands of different promoters constructed in this study are shown. In each case the base sequence from the upstream *EcoRI* site (GAATTC) to the transcript start (+1) is shown. The -10 hexamer is singly underlined and the UP element is boxed. The locations of the DNA sites for CRP are shown (double underlined). The different promoters are named according to the location of the UP element and the DNA site for CRP. (A) A set of promoters where both the location of the UP element and the DNA site for CRP were varied. (B) A set of promoters where the location of the DNA site for CRP was varied but the UP element remained at position -44.

fragment carrying the DNA site for CRP and the upstream extension to give the CC(−81.5)α(−44) and CC(−82.5)α(−44) promoters. The remainder of the promoter constructs were derived from CC(−97.5)α(−44), which was made by annealing the synthetic oligos 5'-CTAGCTCGAGGTCGACGCATGCT-3' and 5'-CTAGAGCATGCGTCGACCTCGAG-3' and ligating the product into the *Xba*I site of CC(−74.5)α(−44). This linker contains sites for *Xho*I, *Sal*II, *Sph*I and *Xba*I and these sites were exploited to construct the CC(−83.5)α(−44), CC(−85.5)α(−44), CC(−90.5)α(−44), CC(−91.5)α(−44), CC(−95.5)α(−44), CC(−101.5)α(−44) and CC(−105.5)α(−44) promoters.

Derivatives of CC(−61.5)

The CC(−61.5) promoter is a derivative of CC(−72.5) with a consensus DNA site for CRP centred at position −61.5 [18]. CC(−61.5) carries a unique *Bam*HI site immediately downstream of the DNA site for CRP (see Figure 7). To make CC(−61.5)α(−83), the *Eco*RI–*Bam*HI fragment from the CC(−41.5)α(−63) promoter (described in Figure 1 of [17]) was ligated to the *Bam*HI–*Hind*III fragment from CC(−61.5). To make CC(−61.5)CC(−102.5), the *Eco*RI–*Bam*HI fragment from the ML(−82.5) promoter carrying tandem DNA sites for CRP (described in Figure 2 of [19]) was ligated to the *Bam*HI–*Hind*III fragment from CC(−61.5).

Construction of promoter p11G derivatives

The construction of the p11G substitution in the CC(−72.5)α(−46) promoter (NJS4) has been described by Savery et al. [16]. To transfer this substitution to any of the CC(−*n*)α(−44) promoters, the *Eco*RI–*Bam*HI fragment from the corresponding CC(−*n*)α(−44) promoter was ligated to the *Bam*HI–*Hind*III fragment from CC(−72.5)α(−46) carrying the p11G substitution. To transfer this substitution to the CC(−61.5), CC(−61.5)α(−83) and CC(−61.5)CC(−102.5) promoters, PCR was used to amplify sequences upstream of position −28 at each of these promoters by using the primers 5'-ACCTGACGTCTAAGAAACC-3' (which hybridizes upstream of the *Eco*RI site of the pAA121 vector) and 5'-GGGAGATCTTTAAACATCGATCT-3' (which introduces a suitably placed *Bgl*II site downstream of the DNA site for CRP). Each of the PCR products was digested with *Eco*RI and *Bgl*II and, after purification, the *Eco*RI–*Bgl*II fragment carrying upstream sequences was ligated to the *Bam*HI–*Hind*III fragment from CC(−72.5)α(−46) carrying the p11G substitution. This resulted in the construction of p11G derivatives of the CC(−61.5), CC(−61.5)α(−83) and CC(−61.5)CC(−102.5) promoters.

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*crp*⁺ or M182*Δcrp*, and transformants were grown in Luria-Bertani broth supplemented with appropriate antibiotics. β -Galactosidase expression was measured exactly as described previously [16,17] using the Miller [20] method, and β -galactosidase activities were taken to be proportional to the activity of the cloned promoter. The CRP-dependence of any promoter was judged by comparing its activity in the M182*crp*⁺ and M182*Δcrp* backgrounds.

Transcription and footprinting experiments

In vitro studies were performed using purified holo-RNAP and CRP or CRP HL159, purified as described in [21] using the method of Ghosaini et al. [22]. Transcription assays were performed using supercoiled pSR plasmid derivatives carrying the CC(−69.5)α(−44) or CC(−74.5)α(−44) promoters. The assays were performed in 16 μ l volumes using 5 nM DNA, 25 nM holo-RNAP and 100 nM CRP (wild type or HL159 as required) in 20 mM Tris/HCl, pH 8.0/100 mM NaCl/5 mM MgCl₂/0.1 mM EDTA/1 mM dithiothreitol/50 μ g/ml BSA/200 μ M cAMP. Template DNA was pre-incubated with CRP (as required) for 5 min at 37 °C. RNAP was then added and the mixture was incubated for a further 5 min at 37 °C. Transcription was initiated by the addition of 1.5 μ Ci of [α -³²P]UTP, unlabelled UTP to a final concentration of 10 μ M, ATP, GTP and CTP to a final concentration of 200 μ M and heparin to a final concentration of 100 μ g/ml. After a further 5 min incubation, reactions were stopped by the addition of 12 μ l of stop mix (20 mM EDTA/80% de-ionized formamide/0.1% Bromophenol Blue) and heating to 90 °C for 30 s. Samples were analysed by denaturing gel electrophoresis and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software.

DNase I footprinting of proteins bound to labelled *Pst*I–*Hind*III fragments, from pAA121 derivatives carrying the CC(−69.5)α(−44) or CC(−74.5)α(−44) promoters, was performed as in our previous work [16,17]. Samples contained 4 nM end-labelled DNA, 100 nM CRP or CRP HL159 and 500 nM purified holo-RNAP in 20 mM Hepes (pH 8.0), 5 mM MgCl₂, 50 mM potassium glutamate, 1 mM dithiothreitol, 200 μ M cAMP, 500 μ g/ml BSA and 20 μ g/ml herring sperm DNA. Footprinting gels were calibrated with Maxam-Gilbert 'G' sequencing reactions of the labelled fragments.

RESULTS AND DISCUSSION

The function of half a UP element

The starting point for this work was the CC(−72.5) promoter, a semi-synthetic Class I CRP-dependent promoter, carrying a DNA site for CRP centred at position −72.5. Savery et al. [16] described a derivative, NJS4, in which the 22 bp UP element from the *E. coli rrnB* P1 promoter was cloned immediately downstream of the DNA site for CRP, and showed that the effect

Table 1 Effects of UP element segments on promoter activity

The β -galactosidase levels made in M182 and M182*Δcrp* cells containing pRW50 carrying different promoters are listed. 'No promoter' refers to cells containing the pRW50 *lac* expression vector with no promoter insert. Cells were grown aerobically in Luria broth containing 35 μ g/ml tetracycline and were harvested during exponential growth. β -Galactosidase levels were measured by the Miller protocol [20] and are expressed as Miller units. Each assay was performed independently at least three times and the values shown are the averages of these determinations.

Promoter	β -Galactosidase expression (in Miller units) by pRW50 carrying promoters in:	
	M182 <i>crp</i> ⁺ cells	M182 <i>Δcrp</i> cells
No promoter	17	15
CC(−72.5)	198	17
NJS4 [CC(−72.5)α(−46)]	839	32
NJS5	459	30
NJS6	463	21

Table 2 Promoter activity with UP elements at different locations

The β -galactosidase levels made in M182 and M182 Δ crp cells containing pRW50 carrying different promoters are listed. Cells were grown aerobically in Luria broth containing 35 μ g/ml tetracycline and were harvested during exponential growth. β -Galactosidase levels were measured by the Miller protocol [20] and are expressed as Miller units. Each assay was performed independently at least three times and the values shown are the averages of these determinations.

Promoter	β -Galactosidase expression (in Miller units) by pRW50 carrying promoters in:	
	M182crp ⁺ cells	M182 Δ crp cells
CC(−66.5) α (−40)	81	45
CC(−68.5) α (−42)	500	47
CC(−70.5) α (−44)	1570	63
CC(−71.5) α (−45)	1415	60
CC(−72.5) α (−46)	850	40
CC(−74.5) α (−48)	60	52
CC(−75.5) α (−49)	41	38
CC(−76.5) α (−50)	35	34

of the UP element was to increase CRP-dependent activation. We have constructed two derivatives, NJS5 and NJS6 (Figure 1), in which only the upstream and downstream parts of the *rrnB* P1 UP element were inserted into CC(−72.5). The activities of these promoters were determined by cloning them into the broad host-range *lac* expression vector, pRW50, and compared with the

activities of CC(−72.5) and NJS4. Results presented in Table 1 confirm that expression from CC(−72.5), NJS4, NJS5 and NJS6 is CRP-dependent, and that the UP element in NJS4 results in a 4–5-fold increase in transcription. Both the upstream and downstream halves of the UP element, in NJS5 and NJS6 respectively, cause a clear, albeit lesser, stimulation of CRP-dependent expression. This result is consistent with the suggestion that the two α CTDs bind to adjacent positions on the 22 bp UP element, and that each α CTD makes an independent contribution to promoter strength (see [5] for a fuller discussion of this point; see also [23]). The observation that the *rrnB* P1 UP element has a 'modular' structure is in sharp contrast to the situation with most other protein-binding promoter elements. For example, even short deletions into the 22 bp DNA site for CRP completely suppress the binding of CRP [24].

Alterations in UP element location

It was possible that the UP element in the NJS4 promoter was not optimally placed. We therefore constructed a series of short insertions and deletions that altered the location of the UP element (Figure 2A). To facilitate presentation, these and subsequent promoter derivatives have been named by the convention suggested by Lloyd et al. [17], reflecting the location of the DNA site for CRP and the UP element: thus the NJS4 promoter was renamed CC(−72.5) α (−46). Insertions and deletions were introduced immediately downstream of the UP element, and the activities of the resulting promoters (listed in Figure 2A) were determined after cloning in pRW50 (Table 2). The measurements

Figure 3 Activity of different promoters

β -Galactosidase activities measured in M182crp⁺ Δ lac and M182 Δ crp Δ lac cells carrying different Class I CRP-dependent promoters cloned into the *lac* expression vector pRW50 are shown. The different promoters all carried a UP element located at position −44 and a DNA site for CRP centred at the positions shown on the x-axis. Cells were grown exponentially in Luria-Bertani broth containing 35 μ g/ml tetracycline, and β -galactosidase expression was measured by the Miller protocol [20]; activities are expressed in Miller units of β -galactosidase activity. Each value is the mean $\pm$ S.D. of at least three independent assays. Cells carrying the vector pRW50 with no promoter insert contain less than 25 units of β -galactosidase activity.

in the Δcrp background show that, regardless of its location, stimulation of promoter activity by the UP element is minimal. This shows that our previous report [16], that the *rrnB* P1 UP element was unable to relieve the CRP-dependence of this promoter, was not an artefact due to inappropriate positioning of the UP element. Interestingly, the measurements in *crp*⁺ cells revealed that two of the newly constructed promoters, CC(−70.5) α (−44) and CC(−71.5) α (−45), showed a higher level of CRP-dependent transcription than the starting CC(−72.5) α (−46) promoter, in spite of the fact that the DNA site for CRP had been moved from its 'preferred' location [18]. The more active of these new promoters, CC(−70.5) α (−44), was retained for further study.

Stringent spacing requirements for activation by CRP

At Class I CRP-dependent promoters, the CRP dimer can be centred near positions −61.5, −71.5, −81.5 or −91.5 and, irrespective of its location, CRP makes the same contact with α CTD (reviewed in [11,12]). In previous studies [18,25], it was found that, in the absence of a UP element, CRP was most active when located at position −61.5, and gradually became less efficient as it was displaced upstream. We reasoned that we might find a different situation in the presence of a UP element, since promoter activity will be greatest when α CTD is able to make optimal contacts with both CRP and UP element DNA. Thus, by deleting and inserting DNA segments between the UP element and CRP site at the CC(−70.5) α (−44) promoter, we created a family of 20 promoters with the DNA site for CRP centred at locations from position −105.5 to position −61.5 (Figure 2B). The activity of each of these promoters was determined after cloning in pRW50. The results shown in Figure 3, from assays in the *crp*⁺ strain, show that, when located at position −69.5 or −70.5, CRP is able to activate transcription many fold better than when at other locations. CRP-dependent activity is maximal when the DNA site for CRP is located at position −69.5; thus, at the CC(−69.5) α (−44) promoter, CRP and α CTD appear to be optimally placed to interact with each other. To check that the observed activity of the CC(−69.5) α (−44) promoter was not an artefact, we performed three controls. First, using the Δcrp background, we checked that activity is completely dependent on CRP (Figure 3). Secondly, we established that activation of the CC(−69.5) α (−44) promoter is dependent on activating region 1 of CRP (which is known to interact with α CTD during transcription activation): the HL159 substitution in CRP, which completely inactivates activating region 1 of CRP [21], reduces CRP-dependent activation of the CC(−69.5) α (−44) promoter (and the other promoters in this series) by more than 10-fold (E. C. Law, unpublished work). Thirdly, we constructed a derivative of the CC(−69.5) α (−44) promoter carrying the p11G mutation that alters the −10 hexamer element from CATAAT to CGTAAT. We previously showed that this single substitution completely suppresses the activity of the *melR* promoter and its derivatives [16,19]. In the present work, we found that the p11G substitution completely suppresses the activity of the CC(−69.5) α (−44) promoter (results not shown), eliminating the possibility that the observed activity is due to the chance creation of an alternative promoter.

In vitro studies on ternary RNAP–CRP–promoter complexes

In vitro studies have been exploited to study the juxtaposition of RNAP and CRP in open complexes at the CC(−69.5) α (−44) promoter. As a control, the CC(−74.5) α (−44) promoter, which is hardly activated by CRP (Figure 3), was included in this study.

Figure 4 Transcription from the CC(−69.5) α (−44) and CC(−74.5) α (−44) promoters

Analysis of transcripts made *in vitro* from supercoiled pSR plasmid carrying the CC(−69.5) α (−44) (lanes a–c) and CC(−74.5) α (−44) (lanes d–f) promoters is shown; transcripts initiating at CC(−69.5) α (−44) and CC(−74.5) α (−44) run to a strong terminator and give the band shown with an arrow. Also shown is the RNA I transcript from the pSR vector that is used as an internal control. The origin of the faint extra band seen in lane b is unknown. To make the transcripts, template DNA was incubated with holo-RNAP (as described in the Experimental section) either without CRP (lanes a and d), with wild-type CRP (lanes b and e) or with CRP carrying the HL159 substitution (lanes c and f).

Figure 4 shows the result of a transcription experiment using the CC(−69.5) α (−44) and CC(−74.5) α (−44) promoters cloned upstream of an efficient terminator in a supercoiled plasmid. The results show that transcription initiation at CC(−69.5) α (−44), but not CC(−74.5) α (−44), is clearly activated by CRP. Moreover, this activation is suppressed by the HL159 substitution in CRP that inactivates activating region 1 of CRP [21]. Thus, as expected, CRP-dependent activation of CC(−69.5) α (−44) is dependent on interaction between activating region 1 of CRP and α CTD.

DNase I footprinting was then used to investigate the architecture of open complexes at the CC(−69.5) α (−44) promoter. The results in Figure 5 (lanes b–g) show that, together, RNAP and CRP give a clear footprint, which is typical of an open complex (we know that this complex is competent in transcription initiation from the results shown in Figure 3). Alone, CRP protects its site centred at position −69.5, and, alone, RNAP only protects the UP element (this protection is due to recognition of the UP element by α CTD). This result is consistent with the proposition that, at the CC(−69.5) α (−44) promoter, the DNA sites for CRP and α CTD are adjacent and suitably juxtaposed to allow interactions to occur. Further evidence for this comes from the effect of the HL159 substitution in CRP: this substitution, which stops the interaction between activating region 1 of CRP and α CTD, causes the de-protection of bases at the junction of

Figure 5 DNase I footprint analyses of complexes formed at the CC(-69.5)α(-44) and CC(-74.5)α(-44) promoters

Shown is an autoradiograph of a sequence gel run to analyse products after DNase I cleavage of complexes formed between DNA fragments carrying the CC(-69.5)α(-44) (lanes b-g) and CC(-74.5)α(-44) (lanes i-n) promoters, purified CRP (w.t. CRP) or CRP HL159 and RNA polymerase (RNAP) as indicated. Lanes a and h show Maxam-Gilbert sequencing reactions that were used to calibrate the gel. The vertical bars indicate regions of protection by αCTD, CRP and the rest of RNA polymerase. The location of the transcript start point, +1, is indicated.

the footprints due to CRP and αCTD (Figure 5). Figure 5 (lanes i-n) also shows a footprinting experiment with the CC(-74.5)α(-44) promoter. The footprints show that both CRP and αCTD of RNAP bind to their respective sites, but there is no evidence for interaction between them, and the extended downstream protection that is characteristic of open-complex formation is not seen.

Positioning of αCTD at promoters with the DNA site for CRP at position -61.5

Previous analyses of footprints of RNAP bound at different promoters suggest that the two αCTDs each cover ≈ 11 bp and

bind adjacent to each other on the same side of the promoter DNA [3-5]. Thus, we suppose that, at the CC(-69.5)α(-44) promoter, the UP element is perfectly placed to interact with the two αCTDs of RNAP bound at the promoter. Additionally, the CRP dimer centred at position -69.5 must be perfectly placed so that activating region 1 can interact with the more upstream αCTD when it is bound to the UP element (Figure 6A). Hydroxyl radical footprints of the ternary RNAP-CRP-CC(-69.5)α(-44) promoter complex confirm this (E. C. Law, unpublished work). However, this raises the question of how the two αCTDs are organized at Class I CRP-dependent promoters where the DNA site for CRP is centred at position -61.5 (such as the *E. coli lac* promoter). In this situation, there is, presumably,

Figure 6 Organization of the RNAP subunits and CRP at different Class I CRP-dependent promoters

The suggested organization of CRP and RNAP subunits in transcriptionally competent complexes at the different promoters discussed is shown: (A) CC(−69.5)α(−44); (B) CC(−61.5); (C) CC(−61.5)α(−83); (D) CC(−61.5)CC(−102.5).

insufficient space to accommodate both αCTDs ‘side-by-side’, if each αCTD requires 11 bp of DNA, and, thus, we suggest that one of the two αCTDs must be displaced to another face of the promoter DNA (Figure 6B; such an arrangement is consistent with previous footprint analyses of this type of promoter [26,27]). Here, we have investigated the possibility that one of the αCTDs can be displaced to make interactions upstream of the bound CRP dimer. To do this, we started with the CC(−61.5) promoter [a derivative of CC(−72.5) with a consensus DNA site for CRP centred at position −61.5] and inserted either a UP element or a second DNA site for CRP just upstream of the DNA site for CRP at position −61.5. Figure 7 shows the base sequences of the two derivatives, CC(−61.5)α(−83) and CC(−61.5)CC(−102.5). Table 3 lists the activities of CC(−61.5) and the two derivatives in both the *crp*⁺ and Δ*crp* backgrounds. The results show that CRP-dependent activation is increased by both the upstream UP element and the upstream second DNA site for CRP. Since both UP elements and CRP stimulate transcription initiation by contacting αCTD, we conclude that at least one αCTD can be displaced and can reach upstream of the DNA site for CRP at position −61.5 (Figures 6C and 6D). To eliminate the possibility

Table 3 Activity of derivatives of CC(−61.5)

The β-galactosidase levels made in M182*crp*⁺ and M182Δ*crp* cells containing pRW50 carrying different promoters are listed. ‘No promoter’ refers to cells containing the pRW50 *lac* expression vector with no promoter insert. Experiments were performed as in Tables 1 and 2.

Promoter	β-Galactosidase expression (in Miller units) by pRW50 carrying promoters in:	
	M182 <i>crp</i> ⁺ cells	M182Δ <i>crp</i> cells
No promoter	13	12
CC(−61.5)	540	20
CC(−61.5)α(−83)	1100	44
CC(−61.5)CC(−102.5)	1700	47

that observed increases in activity were due to the chance creation of an alternative promoter, we constructed derivatives of the CC(−61.5)α(−83) and CC(−61.5)CC(−102.5) promoters containing the p11G mutation. In both cases, this single substitution,

Figure 7 Base sequences of derivatives of the CC(−61.5) promoter

The sequences of CC(−61.5) and derivatives from the upstream *Eco*RI site (GAATTC) to the transcript start (+1) is shown in each case. The −10 hexamer is singly underlined and the DNA sites for CRP are identified by double underlining. The UP element in the CC(−61.5)α(−83) promoter is boxed.

which alters the –10 hexamer element to CGTAAT, completely suppresses promoter activity (results not shown).

Conclusions

At bacterial promoters, the α CTDs of RNAP can contribute to transcription initiation by interacting both with promoter DNA and with transcription activators. Owing to the flexible linker joining α CTD to the rest of RNAP, there is considerable flexibility in the possible location of both promoter elements and activators that function by contacting α CTD [9]. Moreover, the two α CTDs of RNAP can be positioned independently [6,19]. This study has shown that, with α CTD anchored at a UP element, there is a single most-favoured location for CRP. Surprisingly, at the promoters discussed here, the optimal location for CRP is position –69.5, and not position –61.5 as at the *E. coli lac* promoter. Our logic suggests that, at Class I CRP-dependent promoters where the DNA site for CRP is centred at position –61.5, one of the two α CTDs must be unable to bind to the same face of the promoter as CRP and the other α CTD. Several studies have shown that the *lac* promoter activity is particularly sensitive to base changes between the DNA site for CRP and the –35 region [28–30]. In previous work, Joung et al. [31] showed that transcription initiation at the *lac* promoter could be enhanced by a second upstream-bound CRP molecule. Our present observation that CRP-dependent activation at the CC(–61.5) promoter can be enhanced by either a second upstream-bound CRP or a UP element, suggests that the ‘displaced’ α CTD may be sufficiently flexible to contact DNA or activators upstream of the bound CRP dimer at position –61.5 (Figure 6). It will be interesting to investigate the organization of α CTD at other Class I CRP-dependent promoters (e.g. at the *malT* promoter, see [32]).

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