Exploitation of a Chemical Nuclease to Investigate the Location and Orientation of the Escherichia coli RNA Polymerase α Subunit C-terminal Domains at Simple Promoters That Are Activated by Cyclic AMP Receptor Protein*

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RNA synthesis in bacteria is due to holo-RNA polymerase (RNAP), which is a multisubunit complex with subunit composition αββ′ω. It is well known that the major factor ensuring “correct” gene expression in bacteria is the efficiency with which RNAP recognizes the promoters of different genes. Although the principal determinants for promoter recognition reside in the RNAP α subunit, at many promoters, the α subunits also play a key role (1–3). Each RNAP α subunit consists of two independently folding domains connected by a flexible linker. The N-terminal domain (αNTD; residues 8–235 in Escherichia coli α) carries determinants for the interaction of the α subunits with the rest of RNAP, whereas the C-terminal domain (αCTD; residues 249–329 in E. coli α) carries determinants for interaction with promoter DNA elements and with certain transcription factors (4, 5). At some promoters, the interaction of αCTD with specific DNA sequence elements (known as UP elements) is essential for optimal transcription.

The C-terminal domain of the α subunit (αCTD) of bacterial RNA polymerase plays an important role in promoter recognition. It is known that αCTD binds to the DNA minor groove at different locations at different promoters via a surface-exposed determinant, the 265 determinant. Here we describe experiments that permit us to determine the location and orientation of binding of αCTD at any promoter. In these experiments, a DNA cleavage reagent is attached to specific locations on opposite faces of the RNA polymerase α subunit. After incorporation of the tagged α subunits into holo-RNA polymerase, patterns of DNA cleavage due to the reagent are determined in open complexes. The locations of DNA cleavage due to the reagent attached at different positions allow the position and orientation of αCTD to be deduced. Here we present data from experiments with simple Escherichia coli promoters that are activated by the cyclic AMP receptor protein.

Initiation. At other promoters, optimal expression depends on activator proteins that function by making a direct contact with αCTD, thereby recruiting RNAP to the promoter (3).

High resolution structures for E. coli αCTD, either free or bound to the UP element DNA, have been obtained (6–8). When bound to DNA, αCTD contacts the minor groove (9, 10). Genetic analyses, together with the structural studies, have identified residues in two helix-loop-helix motifs that are responsible for DNA binding. Thus, Arg261, Asn264, Gly265, Gly266, Lys268, and Ser269 appear to be the crucial α subunit residues that make contact with the DNA minor groove (reviewed in Ref. 3 and see Ref. 8). These residues are part of a small segment of the surface of αCTD known as the 265 determinant. The linker joining αNTD and αCTD contains at least 13 amino acids and appears to be very flexible and unstructured (11). A consequence of this is that αCTD can bind at different locations at different promoters. At promoters where RNAP initiates transcription without the need for an activator protein, one αCTD contacts the DNA minor groove near position −41, upstream of the promoter −35 element (3, 12). In some cases, a distinct segment of the surface of this αCTD, known as the 261 determinant (including α residue Glu261), has been shown to contact domain 4 of the RNAP σ subunit, which docks with the promoter −35 element (13, 14). At activator-independent promoters, the second αCTD may contact UP element DNA or may be free to contact upstream sequences at different locations (3, 12). At activator-dependent promoters, the location of αCTD is dependent on the organization of the DNA site(s) for the activator, and there are great variations in the positioning of αCTD at different promoters (15). For any activator-dependent promoter, a key challenge is to understand how the different determinants of bound αCTD are oriented with respect to the rest of RNAP and other bound proteins.

The role of αCTD has been most intensively studied at E. coli promoters that are dependent on the cyclic AMP receptor protein (CRP; also known as catabolite activator protein). When triggered by cyclic AMP, CRP binds to target sequences at dozens of different promoters and activates transcription by recruiting RNAP to these promoters (reviewed in Ref. 16). CRP is a homodimer that binds to 22-bp sequences at target promoters. Simple CRP-dependent promoters (i.e. promoters that are dependent on binding of a single CRP dimer) fall into two classes (reviewed in Ref. 17). At class I promoters, CRP binds upstream of the RNAP binding determinants (usually centered near position −61, −71, or −81), and the downstream subunit of the CRP dimer makes a direct interaction with αCTD to recruit it, and thereby the rest of RNAP, to the promoter. At class II promoters, CRP binds to a site centered near position −41 and overlaps the promoter −35 region. The upstream

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1 The abbreviations used are: RNAP, RNA polymerase; NTD, N-terminal domain; CTD, C-terminal domain; CRP, cyclic AMP receptor protein; MOPS, 4-morpholinepropanesulfonic acid; oligos, oligonucleotides; αCTD, C-terminal domain of the α subunit; AR1, activating region 1; αNTD, N-terminal domain of the α subunit; FeBABE, [S]-1-[p-bromocetamidobenzy]ethylenediaminetetraacetate.

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subunit of the CRP dimer makes a direct interaction with αCTD that recruits it to the DNA segment immediately upstream of bound CRP. Although two αCTDs of RNAP could potentially interact with the two subunits of the CRP dimer, a single CRP-αCTD interaction suffices for CRP-dependent transcription activation at both classes of CRP-dependent promoter (18).

The same surface of CRP interacts with αCTD at both class I and class II promoters (17). This has been identified as a surface-exposed loop adjacent to the helix-turn-helix DNA binding motif (activating region 1, AR1; residues 156–164). Analysis of the high resolution crystal structure of a CRP-αCTD-DNA ternary complex shows that AR1 contacts a segment of the surface of αCTD that is located on the opposite face from the 261 determinant (8). This surface, known as the 287 determinant, includes residues 285–289 that had been identified in previous genetic analyses as being the most likely residues in αCTD to make direct contact with CRP (19, 20).

The high resolution crystal structure of a CRP-αCTD-DNA ternary complex, together with genetic and footprint analyses, provides a framework for understanding the arrangement of CRP and RNAP subunits during open complex formation at any CRP-dependent promoter. Notwithstanding this, there is a need for direct methods to find the location and, more importantly, the orientation of the two RNA αCTDs at different promoters with different organizations. A novel approach to this problem was taken by Murakami et al. (21, 22) who exploited the DNA cleavage reagent, iron [S]-1-[p-bromoacetamido]benzylethylenediaminetetraacetate (FeBABE). RNAP was reconstituted with purified α subunits that had been covalently modified with FeBABE, open complexes were formed at different promoters, and the DNA cleaving ability of FeBABE was triggered. In these experiments, the FeBABE reagent was tethered to Cys269, immediately adjacent to the DNA binding 265 determinant, and thus the positions of DNA cleavage at the target promoter reveal the locations of αCTD binding. In this study, we have extended this analysis by attaching the FeBABE reagent either to position 273 or to position 302 of the RNAP α subunit; these positions are located on opposite faces of αCTD (Fig. 1). Our aim was to choose two well separated positions that were removed from the DNA binding surface of αCTD, in order to investigate not only the location of the two

### Table I

**Bacterial strains, plasmids, and promoter fragments**

| Strains       | Brief description                                                                 | Source/Ref. |
|---------------|-----------------------------------------------------------------------------------|-------------|
| DH5α         | E. coli Δlac recA−                                                                 | 23          |
| XL1-Blue     | E. coli recA1 [F’ lacI*]                                                          | 24          |
| BL21 DE3     | E. coli ΔDE3. Encodes T7 RNAP under control of lacUV5 promoter                    | Novagen     |

| Plasmids     |                                                                                   |             |
|---------------|-----------------------------------------------------------------------------------|-------------|
| pSR          | pBR322 derivative containing transcription terminator                              | 25          |
| pHTT7f1NHα   | Plasmid carrying rpoA encoding RNAP α subunit with His6 coding region between codons 1 and 2, under control of a T7 promoter | 26          |
| pHTT7f1NHα 273C | Plasmid carrying rpoA encoding RNAP α subunit carrying single cysteine at position 273 | This work   |
| pHTT7f1NHα 302C | Plasmid carrying rpoA encoding RNAP α subunit carrying single cysteine at position 302 | This work   |
| pHf1α 269A   | Plasmid carrying rpoA encoding RNAP α subunit carrying alanine substitution at position 269 | 27          |

| Promoters    |                                                                                   |             |
|---------------|-----------------------------------------------------------------------------------|-------------|
| CC(−61.5)p12T | CRP-dependent promoter with consensus DNA site for CRP centered at position −61.5 and an improved – 10 hexamer | This work   |
| CC(−41.5)    | CRP-dependent promoter with consensus DNA site for CRP centered at position −41.5 | 28          |
| CC(−69.5) a(−44) | CRP-dependent promoter with consensus DNA site for CRP centered at position −69.5 and UP element immediately downstream | 29          |

### Table II

**Primer used to engineer cysteine codons in plasmids encoding α**

| Primer name | Primer sequence (5′–3′ sequence) | Uses                                                                 |
|-------------|----------------------------------|----------------------------------------------------------------------|
| D10885      | GCTGATCCTACTCGTCAG               | Used with D21316 to prepare Ala302 fragment for cloning, and with mutagenic primers to create megaprimers for introduction of Cys273 and Cys302 codons |
| D21316      | CAACCATTCGCTGGCTAACTG            | Used with D10885 to prepare Ala302 fragment, and with megaprimers to introduce Cys273 and Cys302 codons |
| D25330      | CCCGAAATTCCCATGAGAAGATGGAGGCGCCAATCGCAGCCGTC | Mutagenic primer used with D25335 to introduce Ala176 codon |
| D25331      | CGCAGCAGTGATGCGCTAGAGGCCACCCCTGAGG | Mutagenic primer used with D25332 to introduce Ala131 codon |
| D25332      | GGGCCGCTCATCTTCCTCC              | Used with D25331 to introduce Ala131 codon |
| D25333      | CCTCGGTGACCCGCGCTAGAGGCCAGCTCAGC | Mutagenic primer used with D25337 to create megaprimer for introduction of Ala54 codon |
| D25334      | CAGATCACGTGCTGCGGC               | Used with megaprimer to introduce Ala54 codon |
| D25335      | GGCTGAGCTACATCGC                 | Used with D25330 to introduce Ala170 codon. Also used as a sequencing primer |
| D25387      | GGATAACAATTTCCCCCTCTAGACC        | Used with D25333 to create megaprimer for introduction of Ala54 codon |
| D25612      | GGCCGCTATCCCTGCTC                | Sequencing primer |
| D25613      | AAAATCGGATGCTGACC                | Sequencing primer |
| D25617      | CGCCCTTTAACAGCATGTGCTATCCATATCAG | Mutagenic primer for generation of Cys273 codon |
| D26543      | AAATCTCTAATTCTGTTACATTAAAGACGCTCGG | Mutagenic primer for generation of Cys302 codon |
RNAP αCDTs but also their orientation when bound at different CRP-dependent promoters. Thus, we present data for complexes at simple class I and class II CRP-dependent promoters, as well as at a CRP-independent derivative.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Promoter Derivatives**—Bacterial strains, plasmids, and promoter derivatives used in this study are listed in Table I. EcoRI-HindIII fragments carrying promoter derivatives were cloned in vector plasmid pSR. Primers used in the mutagenesis of RNAP α-subunit were listed in Table II. The CCCT/–615 promoter was generated by PCR from the CCCT/–615 promoter and oligonucleotide 5’-TGCTATAATTCT-3’. The template DNA was pSR/lacUV5 (0.2 μM). The positions of the transcript initiated at the lacUV5 promoter (123 nucleotides), and the plasmid-encoded transcript, RNA I (108 nucleotides), are indicated. RNAP concentrations were 8 nM (lanes 1 and 4–6), 16 nM (lanes 2 and 7), or 2 μM (lanes 3 and 8).

**Construction of Plasmids Encoding αααα**—To overexpress and purify α protein, plasmid pHTT7f1NHαααα was used. This plasmid carries the rpoA sequence with cysteine codons at positions 54, 131, 176, and 269. Thus, using PCR, these were changed to alanine codons, prior to the insertion of single cysteine codons at desired locations. For the PCR, mutagenic oligos were used together with flanking oligos, and the resulting PCR fragments were cloned into pHTT7f1NHαααα vector DNA, cut with the appropriate restriction enzymes. Oligos D25330 and D25335 were first used to replace codon Cys176, with the resulting PCR fragment being cloned into pHTT7f1NHαααα using EcoRI and HindIII sites. Next, oligos D25331 and D25332 were used to replace codon Cys131 with the resulting PCR fragment being cloned into pHTT7f1NHαααα using PmlI and EcoRI sites. In the next step, meqamprimer PCR was used to remove codon Cys176. The meqamprimer fragment was generated using oligos D25333 and D25335, and then the meqamprimer and oligo D25334 were used to add codon Ala131. The resulting PCR product containing Cys176 was cloned into the pHTT7f1NHαααα using XbaI and PmlI sites. Finally, a derivative of pHTT7f1NHαααα, encoding rpoA with no cysteine codons, was made by cloning a DNA fragment from plasmid pHTT1e carrying an Ala176 codon (27) using oligos D10885 and D21316 and HindIII and BamHI sites. This derivative was then used as the template for the construction of mutants carrying single cysteine codons at positions 273 or 302 in the rpoA gene, using meqamprimer PCR. Mutagenic oligos D25817 or D25818, respectively, were used together with oligo D10885 to generate the meqamprimer fragments, and then the meqamprimer and oligos D21316 were used to add the single cysteine codons. Resulting PCR fragments were cloned into pHTT7f1NHαααα using HindIII and BamHI sites. The sequences of all plasmids were checked using automated sequencing with the sequencing primers, D25612 and D25613 (University of Birmingham Genomics Facility).

**Reconstitution of RNAP Derivatives**—Preparation of inclusion bodies containing β, β’, or σ70 subunits from strains XL1-Blue (pLH12β), BL21 DE3 (pT7β’), and BL21 DE3 (pMKSe2), respectively, and reconstitution of RNAP using these inclusion bodies was performed as described in Tang et al. (26). The reconstituted holoenzyme was then purified based on the methods of Tang et al. (26) and Igarashi and Ishihama (31). Briefly, the reconstituted holoenzyme was diluted 2.5-fold with dilution buffer (10 mM Tris/HCl, pH 7.9, 100 μM EDTA, 5% (v/v) glycerol) and subjected to DEAE-Sepharose chromatography and Ni2+-nitrilotriacetic acid-agarose affinity chromatography (Amersham Biosciences). The DEAE-Sepharose column was pre-equilibrated with 10 mM Tris/HCl, 7.9, 100 mM NaCl, 5% (v/v) glycerol, and the bound holoenzyme was eluted with a linear gradient of 0–250 mM imidazole in storage buffer. Fractions containing holoenzyme were pooled, and buffer was exchanged into storage buffer and concentrated using spin columns (Vivascience). Samples were stored at –20 °C after the addition of 50% (v/v) glycerol.

**Fig. 2. In vitro transcription with derivatized RNAP**. The figure shows a PhosphorImager scan of a gel on which RNA transcripts produced in a typical multiround transcription experiment were analyzed. The experiments used purified RNAP, reconstituted either with wild type (WT) unmodified α subunits or with α subunits conjugated with FeBABE at position 273 (273FeBABE) or position 302 (302FeBABE) as indicated. The template DNA was pSR/lacUV5 (0.2 μM). The positions of the transcript initiated at the lacUV5 promoter (123 nucleotides), and the plasmid-encoded transcript, RNA I (108 nucleotides), are indicated. RNAP concentrations were 7 μM (lanes 1 and 2), 8 μM (lanes 2 and 7), or 16 μM (lanes 3 and 8).
In Vitro Transcription Experiments—To show that reconstituted RNAP derivatives were transcriptionally active, transcription assays were carried out according to Savery et al. (19). Reactions were initiated by the addition of the RNAP derivative and were terminated after 15 min at 30 °C by the addition of 25 μl of stop solution (7 M urea, 1% SDS, 10 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). Products were analyzed on 6% acrylamide gels containing 7 M urea. Gels were processed and then scanned using a PhosphorImager (Amersham Biosciences) and Quantity One software (Bio-Rad).

DNA Cleavage by FeBABE—DNA templates for footprinting were obtained using cesium chloride preparations of plasmids carrying the promoter. AatII-HindIII fragments were purified and labeled at the HindIII end with either [γ-32P]ATP and T4 polynucleotide kinase (for the template strand) or α-32P]ATP and E. coli DNA polymerase Klenow fragment (for the non-template strand). RNAP holoenzyme was mixed with promoter DNA in a reaction volume of 35 μl (20 nM HEPES, pH 8.0, 50 mM potassium glutamate, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin), and incubated for 10 min at 37 °C. After 10 min, complexes were treated with heparin (50 μg/ml final concentration) for 1 min at 37 °C. DNA cleavage was then initiated by the addition of 3 mM sodium ascorbate and 3 mM hydrogen peroxide. Samples were incubated for at least 2 min at 37 °C before cleavage was stopped by the addition of EDTA and thiourea to final concentrations of 45 and 7 mM, respectively. Modified DNA was extracted with phenol/chloroform and precipitated with ethanol and then analyzed on a 6% polyacrylamide gel containing 6 M urea. Gels were calibrated with Maxam-Gilbert G, C, A sequence ladders. Gels were processed and then scanned using a PhosphorImager (Amersham Biosciences) and Quantity One software (Bio-Rad).

RESULTS

Construction of RNA Polymerase Conjugated with FeBABE—Models of aCTD bound to DNA are shown in Fig. 1. Contact with the minor groove of the DNA target is due to residues of the 265 determinant (pink), and the 281 determinant (yellow) and 287 determinant (green) are displayed on opposite faces of aCTD so that they could interact with neighboring proteins bound to adjacent sites on the DNA. The aim of our work was to devise a method to allow experimental determination of the orientation of binding of RNAP aCTD in tran-
scriptionally competent complexes at any promoter. Thus, for any case, we could investigate whether the 287 determinant was pointing upstream or downstream (and vice versa for the 261 determinant). To do this, we exploited the DNA-cleaving agent, FeBABE, which can be tethered to specific residues in the RNAP α subunit by engineering derivatives carrying single cysteine residues.

Starting with plasmid pH7T7f1NHα, a T7 vector for overexpression of RNAP α carrying a hexahistidine tag, a derivative was first engineered that encoded cysteine-free α, in which all the naturally occurring cysteine residues were replaced with alanine residues. By using this construct, further derivatives, pH7T7f1NHα 273C and pH7T7f1NHα 302C, encoding α with single cysteines, either at position 273 or position 302, were then constructed by replacing the glutamic acid residues at positions 273 or 302 with cysteines. These two positions in αCTD (Fig. 1) and because alanine substitution at residue 302, open complexes were formed at the RNAP. This uncertainty is principally due to variations in transcriptional activities between different preparations. However, the clarity of the FeBABE cleavage patterns, discussed below, argues that any variations in transcriptional activities between different preparations are unlikely to affect substantially our conclusions.

**Binding of αCTD at a Class II Promoter Activated by CRP**

The different promoters that we have investigated are illustrated in Fig. 3. The first promoter that we studied is a simple class II CRP-dependent promoter, CC(−41.5), that carries a consensus DNA site for CRP centered at position −41.5. This promoter was chosen because previous footprinting studies had clearly shown that both αCTDs bind upstream of bound CRP (33). One αCTD protects the DNA minor groove near position −60, and its 287 determinant interacts with AR1 of CRP (19). The second αCTD protects the DNA minor groove near positions −70 and −80. By using purified CRP and purified RNAP, reconstituted with α subunits carrying FeBABE at residue 273 or residue 302, open complexes were formed at the CC(−41.5) promoter using end-labeled DNA fragments. Fig. 4A shows the patterns of DNA cleavage revealed by gel and PhosphorImager analysis after the nuclease activity of FeBABE had been trig-
tered by the addition of H$_2$O$_2$ and sodium ascorbate. A single cluster of DNA cleavages near position −60 was observed with both the template and the non-template promoter strand. We interpret these signals as due to one αCTD binding to the minor groove just upstream of bound CRP, in agreement with previous footprinting results (33). For both DNA strands, cleavage due to FeBABE located at residue 273 occurs 4–5 bp downstream of the sites of cleavage due to FeBABE located at residue 302. The locations of the different cleavage sites on the two strands are illustrated in Fig. 4B. The simplest interpretation of these data, illustrated in Fig. 4C, is that αCTD binds to the minor groove near position −60 and is oriented such that the 287 determinant points downstream and the 261 determinant points upstream. The 287 determinant would thus be well placed to interact with AR1 in the upstream subunit of the CRP dimer, in agreement with previous footprinting data (33), genetic studies (19), and the CRP-αCTD-DNA co-crystal structure (8).

In control experiments, we checked that the observed signals were dependent on the addition of H$_2$O$_2$ and sodium ascorbate, and that no signals were seen in the absence of CRP. Surprisingly, we found no clear strong signal from FeBABE attached to the second αCTD, perhaps by conventional footprinting studies to bind further upstream (33). Previous studies have shown that this second αCTD is not required for CRP-dependent activation at CC(−41.5) (18), and it is possible that its location is not sufficiently fixed to generate a strong signal.

**Binding of αCTD at a Class I Promoter Activated by CRP.** Site for CRP at Position −61.5—Next, we turned our attention to the simple class I CRP-dependent promoter, CC(−61.5), a derivative of CC(−41.5) with the consensus DNA site for CRP moved to position −61.5 (28). Previous studies with CC(−61.5), and the related lac promoter, had shown that one αCTD protects the DNA minor groove near position −41 (34), and its 287 determinant interacts with AR1 of the downstream subunit of the CRP dimer (20), whereas the second αCTD binds somewhere upstream (12). In this study we used the p12T derivative of the CC(−61.5) promoter, in which the −10 hexamer has been changed from 5′-CATATAA-3′ to 5′-TATAATT-3′ (Fig. 3). We found that this change to a consensus −10 hexamer improves the occupancy of promoter open complexes in *vitro*. In addition, the p12T substitution allows open complex formation in the absence of CRP, and thus it is possible to compare the positioning of αCTD in the absence and in the presence of CRP.

Fig. 5A shows the patterns of DNA cleavage by FeBABE-tagged RNAP, revealed by gel and PhosphorImager analysis, in open complexes at the CC(−61.5)p12T promoter in the absence of CRP. The DNA cleavage pattern shows a series of groups of bands, separated by 10–11 bp, suggesting that αCTD binds successively to the minor groove along one face of the promoter DNA. The strongest DNA cleavage is found near position −41, and we interpret this as due to the binding of one of the two αCTDs. For both DNA strands, cleavage due to FeBABE conjugated to residue 302 occurs 4–5 bp downstream of the sites of cleavage due to FeBABE conjugated to residue 273. The locations of the different cleavage sites on the two strands are illustrated in Fig. 5B. The simplest interpretation of these data, illustrated in Fig. 5C, is that this αCTD binds to the minor groove near position −41 and is oriented such that the 261 determinant points downstream and the 287 determinant points upstream. The 261 determinant would thus be well placed to interact with domain 4 of the RNAP σ subunit in agreement with previous data (13, 14).

The results in Fig. 5A argue that, at the CC(−61.5)p12T promoter in the absence of CRP, αCTD can also bind to promoter DNA near positions −52, −62, and −72. The signals at these positions are weaker than those near position −41, and we suggest that they are due to the second αCTD being able to visit different sites at different times. The signals due to RNAP reconstituted with α subunits tagged with FeBABE at residue 273 and 302 are similar. This suggests that the orientation of this αCTD on the DNA is not fixed.

In the next set of experiments, we investigated the patterns of DNA cleavage by FeBABE-tagged RNAP in open complexes at the CC(−61.5)p12T promoter in the presence of CRP. In preparatory experiments, we found that CRP activated this promoter by 3–4-fold in vivo and that both CRP-dependent and CRP-independent activity were reduced to background levels if the −10 hexamer was changed from 5′-CATATAA-3′ to 5′-TG-

- TAAT-3′ (p11G). Similarly, in *vitro* experiments, only background levels of DNA cleavage were observed with the CC(−61.5)p12T promoter carrying the p11G mutant −10 hexamer (data not shown). Fig. 6 shows the patterns of DNA cleavage revealed by gel and PhosphorImager analysis after the nuclease activity of FeBABE had been triggered. The patterns of cleavage observed in the presence of CRP are clearly different to those in the absence of CRP, although DNA cleavage is still seen on both strands near position −41. We ascribe this cleavage by FeBABE attached to one αCTD that is
sandwiched between bound CRP and domain 4 of the RNAP σ subunit. Previous studies have suggested that the 287 determinant of this aCTD interacts with AR1 of the downstream subunit of bound CRP, whereas its 261 determinant interacts with domain 4 of the RNAP σ subunit (see Ref. 13 and reviewed in Ref. 17). Our results support these conclusions: for both DNA strands, sites of cleavage due to FeBABE conjugated to residue 302 occur 4–5 bp downstream of the sites of cleavage due to FeBABE conjugated to residue 273. Thus, as with aCTD bound near this location at the CC(−61.5)p12T promoter in the absence of CRP, the simplest interpretation of the data is that aCTD binds to the minor groove and is oriented such that the 261 determinant points downstream and the 287 determinant points upstream (Fig. 5C). However, our results indicate that this aCTD is shifted 1–2 bp upstream by the binding of CRP. This is shown in Fig. 7, where patterns of DNA cleavage with RNAP reconstituted with α subunits tagged with FeBABE at position 302 were analyzed with and without CRP.

Comparison of results in Fig. 5A and Fig. 6 shows that the binding of CRP to the CC(−61.5)p12T promoter suppresses DNA cleavage by the FeBABE reagent near positions −52, −62, and −72. However, cleavage appears near position −81, which corresponds to the first free minor groove on the same face of the DNA upstream of bound CRP. We suppose that this results from binding of the second RNAP α subunit at this site. The signals due to RNAP reconstituted with α subunits tagged with FeBABE at residue 273 and 302 are similar, suggesting that the orientation of this aCTD on the DNA is not fixed.

Binding of aCTD at a Class I Promoter Activated by CRP. Site for CRP at Position −69.5—In the final part of our study, we wanted to investigate the location of aCTD at a promoter with CRP bound further upstream. In previous work, we studied several class I CRP-dependent promoters with the DNA site for CRP located upstream of position −61.5, and we noted that open complex formation in vitro was inefficient. Thus, we have exploited the CC(−69.5)pα(−44) promoter, which is a derivative of CC(−41.5) carrying an UP element upstream of the −35 region and the DNA site for CRP centered at position −69.5 (Fig. 3). We found previously that expression from this promoter is dependent on CRP and that transcription activation can be reproduced in vitro (29).

Fig. 7. Effect of CRP on aCTD bound near position −41 at the CC(−61.5)p12T promoter. A, the figure shows a PhosphorImager scan of a gel run to analyze DNA cleavage due to the binding of FeBABE-tagged RNAP in the absence or presence of CRP (100 nM). Lanes 2 and 3 show results for the template strand, and lanes 4 and 5 show results for the non-template strand. DNA fragments were incubated with 50 nM RNAP reconstituted with α subunits carrying FeBABE at position 302 (lanes 2 and 4), or with 100 nM CRP and 50 nM RNAP reconstituted with α subunits carrying FeBABE at position 302 (lanes 3 and 5). Lanes 1 and 6 show Maxam-Gilbert sequence ladders that were used for the calibrations shown alongside the gel. B, model of DNA showing the minor groove locations of DNA cleavage near position −41 due to RNAP reconstituted with α subunits carrying FeBABE at position 302 (red) or RNAP reconstituted with α subunits carrying FeBABE at position 273 (blue), in the absence of CRP. C, model of DNA showing the minor groove locations of DNA cleavage near position −41 due to RNAP reconstituted with α subunits carrying FeBABE at position 302 (red) or RNAP reconstituted with α subunits carrying FeBABE at position 273 (blue), in the presence of CRP.

Fig. 8. FeBABE-mediated DNA cleavage at the CC(−69.5)pα(−44) promoter. The figure shows a PhosphorImager scan of a gel on which DNA cleavage patterns due to the binding of FeBABE-tagged RNAP, in the presence of CRP, were analyzed. Lanes 1–3 show results for the non-template strand, and lanes 5–7 show results for the template strand. DNA fragments were incubated with no protein (lanes 3 and 7), 100 nM CRP and 150 nM RNAP reconstituted with α subunits carrying FeBABE at position 302 (lanes 1 and 5), or 100 nM CRP and 150 nM RNAP reconstituted with α subunits carrying FeBABE at position 273 (lanes 2 and 6). Lane 4 shows a Maxam-Gilbert sequence ladder that was used for the calibrations shown alongside the gel.
many different methods (3). Perhaps the most direct method is that described by Naryshkin et al. (12), who incorporated a photo-activatable cross-linking reagent at different locations in the lacUV5 promoter. After formation of transcriptionally competent complexes and activation of the reagent, they determined which RNAP subunits were cross-linked by the reagent located at different positions. The alternative approach, taken by Ishihama and co-workers (21, 22, 35, 36), exploits RNAP carrying a DNA cleavage reagent specifically located in αCTD. In this work, we have developed Ishihama’s method so that the orientation of αCTD, as well as its location, can be determined experimentally. We focused on 3 simple CRP-dependent promoters, so that we could cross-check our results with data from other studies. Our conclusions are summarized in Fig. 9.

Our experiments with the class I CC(−61.5) promoter were performed with the p12T derivative that permits CRP-independent promoter activity. Thus, our study is directly comparable with that of Naryshkin et al. (12) who used the lacUV5 promoter. Recall that the lac promoter has a single DNA site for CRP centered at position −61.5 and that the UV5 mutation permits CRP-independent activity. Our conclusion that αCTD binds near positions −41, −52, −62, and −72 in open complexes in the absence of CRP is in perfect agreement with the data from Naryshkin et al. (12). Our results suggest that one αCTD is fixed near position −41, whereas the other αCTD can “dance” on the DNA between the other three sites. Our data show that the orientation of the promoter-proximal αCTD is such that the 261 determinant is directed toward the promoter −35 element. This is in perfect agreement with recent results from Chen et al. (13) and Ross et al. (14), which suggest that the 261 determinant of the promoter-proximal αCTD can interact with domain 4 of the RNAP σ subunit bound to a promoter −35 element.

Our data with the CC(−61.5)p12T promoter show that, in the presence of CRP, the promoter-proximal αCTD remains near position −41 and is sandwiched between bound CRP and σ, whereas occupation of the sites near positions −52, −62, and −72 is lost, and αCTD binds upstream of bound CRP. Again, this result is identical to that found by Naryshkin et al. (12). Our data show experimentally that the promoter-proximal αCTD remains oriented with its 261 determinant pointing downstream and its 287 determinant pointing upstream. Thus, it can make the simultaneous interactions with AR1 of CRP and domain 4 of σ that have been described (8, 13, 20). Interestingly, the promoter-distal αCTD, which binds upstream of bound CRP, does not appear to adopt a fixed orientation. Note that this αCTD is not essential for CRP-dependent activation (18). Some previous studies (37, 38) have presented evidence that RNAP can make contact with the DNA upstream of bound CRP at class I promoters. Results presented here and by Naryshkin et al. (12) argue that these contacts are most likely due to the “spare” αCTD.

Our experiments with a second class 1 promoter, the CRP-dependent CC(−69.5)α(−44) promoter, yielded the unexpected result that the two αCTDs bind on each side of the CRP dimer, just downstream near position −52, and just upstream near position −90. Law et al. (29) had interpreted their footprint data with this promoter to suggest that both αCTDs bind downstream of CRP. However, their data did show upstream protection, and our present result argues that this was likely due to αCTD. Interestingly, our experiments suggest that both αCTDs adopt a defined orientation with their 287 determinants pointing toward CRP, such that they could interact with AR1. Further experiments will be needed to establish whether interactions involving both CRP subunits contribute to transcription activation, and whether this arrangement is found at other...
class I promoters where CRP binds further upstream than position −61.5. A potential problem with such experiments is that CRP-dependent transcription activation cannot be reproduced in vitro, and thus it will probably be essential to use promoters with improved −10, −35, or UP element sequences. It will be particularly interesting to find other cases where interactions with an upstream activator are sufficiently strong to displace αCTD from its location near position −41 and disrupt its interaction with the RNA polymerase complex.

The third promoter that we studied was the class II CRP-dependent promoter, CCl(−41.5), where it was already known that both αCTDs are located upstream of bound CRP (33). Our results show that one αCTD is located from the second αCTD, we conclude that it is mobile and is not anchored at any particular site (at least in our conditions). The αCTD at position −60 is bound in a fixed orientation, such that its 287 determinant points toward CRP. This provides experimental support for the interaction between the 287 determinant of CRP and AR1 of the upstream subunit of the CRP dimer proposed by Savery et al. (19).

In conclusion, although the studies described here were restricted to simple promoters that are activated by CRP, the methodology we have described could be applied to any promoter where open complexes can be formed efficiently in vitro. Thus, Boucher et al. (39) recently used our constructs to study the position of αCTD at a promoter that is activated by the Bordetella pertussis BvgA protein. One advantage of this approach is that it can give signals due to αCTD binding that makes little or no contribution to promoter activity, and thus cannot be detected by genetic means. Because it is unlikely that structural information can be obtained for every activator-RNA polymerase interaction, we suggest that, in many systems, this methodology will provide a useful complement to genetic studies.

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REFERENCES

1. Gross, C., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tuppy, J., and Young, B. (1998) Cold Spring Harbor Symp. Quant. Biol. 63, 141–155
2. Ebert, R. (2000) J. Mol. Biol. 304, 687–698
3. Gourse, R. L., Ross, W., and Gaal, T. (2000) Mol. Microbiol. 37, 687–695
4. Blatter, E. E., Ross, W., Tang, H., Gourse, R. L., and Ebert, R. H. (1994) Cell 78, 889–896
5. Ebert, R., and Busby, S. (1995) Curr. Opin. Genet. Dev. 5, 197–203
6. Jeon, Y. H., Negishui, T., Shirakawa, M., Yamazaki, T., Fujita, N., Ishihama, A., and Kyogoku, Y. (1995) Science 270, 1495–1497
7. Gaal, T., Ross, W., Blatter, E. E., Tang, H., Jia, X., Krishnan, V. V., Assa-Munt, N., Ebert, R. H., and Gourse, R. L. (1996) Genes Dev. 10, 16–26
8. Benoff, B., Yang, H., Lawson, C. L., Parkinson, G., Liu, J., Blatter, E., Ebert, R. W., and Ebert, R. H. (2002) Science 297, 1562–1566
9. Ross, W., Ernst, A., and Gourse, R. L. (2001) Genes Dev. 15, 491–506
10. Yasuno, Y., Yamazaki, T., Tanaka, Y., Kodama, T., Matsumura, K., Katahira, M., Ishihama, A., and Kyogoku, T. (2001) J. Mol. Biol. 306, 213–235
11. Jeon, Y. H., Yamazaki, T., Tomono, T., Ishihama, A., and Kyogoku, Y. (1997) J. Mol. Biol. 267, 953–962
12. Naryshkin, N., Revyakin, A., Kim, Y., Mekler, V., and Ebert, R. H. (2000) Curr. Opin. Genet. Dev. 10, 601–611
13. Chen, H., Tang, H., and Ebert, R. H. (2003) Mol. Cell 11, 1621–1633
14. Ross, W., Schneider, D. A., Paul, B. J., Mertens, A., and Gourse, R. L. (2003) Genes Dev. 17, 1293–1307
15. Heshui, Y., and Busby, S. (1998) Curr. Opin. Microbiol. 1, 152–159
16. Kolb, A., Busby, S., Buc, H., Garges, S., and Adhya, S. (1993) Annu. Rev. Biochem. 62, 749–789
17. Busby, S., and Ebert, R. H. (1999) J. Mol. Biol. 293, 199–213
18. Lloyd, G. S., Niu, W., Tebbutt, J., Ebert, R. H., and Busby, S. J. W. (2002) Genes Dev. 16, 2557–2565
19. Savery, N. J., Lloyd, G. S., Rainz, M., Gaal, T., Ross, W., Ebert, R. H., Gourse, R. L., and Busby, S. J. W. (1998) EMBO J. 17, 3439–3447
20. Savery, N. J., Lloyd, G. S., Busby, S. J. W., Thomas, M. S., Ebert, R. H., and Gourse, R. L. (2002) J. Bacteriol. 184, 2273–2280
21. Murakami, K., Kimura, M., Owens, J. T., Meares, C. F., and Ishihama, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1709–1714
22. Murakami, K., Owens, J. T., Belyaeva, T. A., Meares, C. F., Busby, S. J. W., and Ishihama, A. (1997) EMBO J. U. S. A. 16, 1124–11278
23. Jesse, J. (1986) Annu. Rev. Biochemistry 55, 275–297
24. Boucher, E., and Gourse, R. L., and Ishihama, A. (1997) Biochemistry 36, 1119–1127
25. Kolb, A., Kotlarz, D., Kusano, S., and Ishihama, A. (1995) Nucleic Acids Res. 23, 819–826
26. Tang, H., Severinov, K., Golffarin, A., and Ebert, R. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4902–4906
27. Tang, H., Severinov, K., Golffarin, A., Fenyo, D., Chait, B., and Ebert, R. H. (1994) Genes Dev. 8, 3038–3047
28. Gourse, R. L., Bell, A., Kolb, A., Buc, H., and Busby, S. (1990) Cell 62, 733–743
29. Law, E. C., Savery, N. J., and Busby, S. J. W. (1999) Biochemistry 37, 415–423
30. Greiner, D. P., Miyake, R., Moran, J. K., Jones, A. D., Negishi, T., Ishihama, A., and Meares, C. F. (1997) Bioconjugate Chem. 8, 44–48
31. Igarashi, K., and Ishihama, A. (1995) J. Mol. Biol. 255, 1015–1022
32. Lee, D. J., Wing, H. J., Savery, N. J., and Busby, S. J. W. (2000) Mol. Microbiol. 37, 1633–1640
33. Belyaeva, T., Bown, J., Fujita, N., Ishihama, A., and Busby, S. (1996) Nucleic Acids Res. 24, 2242–2251
34. Kolb, A., Igarashi, K., Ishihama, A., Livigne, M., Buckle, M., and Busby, S. (1996) Nucleic Acids Res. 24, 2242–2251
35. Ozoline, O. N., Fujita, N., and Ishihama, A. (2000) J. Biol. Chem. 275, 1119–1127
36. Ozoline, O. N., Fujita, N., and Ishihama, A. (2001) Nucleic Acids Res. 29, 40–49
37. Buckle, M., Buc, H., and Travers, A. A. (1992) EMBO J. 11, 2619–2625
38. Eichenberger, P., Dethiollaz, S., Fujita, N., Ishihama, A., and Geisselmann, J. (1996) Biochemistry 35, 15302–15312
39. Boucher, P. E., Maris, A. E., Yang, M.-S., and Stibitz, S. (2003) Mol. Cell 11, 163–173