Location of the *Escherichia coli* RNA polymerase α subunit C-terminal domain at an FNR-dependent promoter: analysis using an artificial nuclease

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Abstract The *Escherichia coli* FNR protein is a global transcription regulator that activates gene expression via interactions with the RNA polymerase α subunit C-terminal domain. Using preparations of *E. coli* RNA polymerase holoenzyme, specifically labelled with a DNA cleavage reagent, we have determined the location and orientation of the C-terminal domain of the RNA polymerase α subunit in transcriptionally competent complexes at a class II FNR-dependent promoter. We conclude that one α subunit C-terminal domain binds immediately upstream of FNR, and that its position and orientation is the same as at similar promoters dependent on CRP, another *E. coli* transcription activator that is related to FNR. In complementary experiments, we show that the second α subunit C-terminal domain of RNA polymerase can be repositioned by upstream-bound CRP, but not by upstream-bound FNR.

Key words: FNR; RNA polymerase; Alpha subunit C-terminal domain; FeBABE reagent; *Escherichia coli*

1. Introduction

All transcription in *Escherichia coli* is catalysed by a single, multi-subunit RNA polymerase that has the subunit composition $\alpha_2\beta\beta'\sigma\omega$ [1]. Promoter recognition is primarily assured by the σ subunit, which recognises and binds to promoter −10 and −35 elements, and the C-terminal domains of the α subunits (αCTD), which bind to upstream promoter sequences [2]. Previous studies have shown that the N- and C-terminal domains of the RNA polymerase α subunits can function independently and are joined by a 20 amino acid flexible linker [3]. The flexibility of this linker allows great variation in the location of αCTD binding at different promoters (reviewed in [4]).

In recent work, a method to determine experimentally the location and orientation of αCTD at promoters has been developed [5–7] using purified α protein engineered to contain a single cysteine residue. The single cysteine is tagged with the DNA cleavage reagent, iron [*S*]-1-[*p*-bromoacetamidobenzyl] ethylenediaminetetraacetate (FeBABE), and the purified, tagged α subunits are used to reconstitute RNA polymerase in vitro. After formation of promoter open complexes with the FeBABE-tagged RNA polymerase, the FeBABE-mediated DNA cleavage activity is triggered and the locations of DNA cleavage give information about the location of αCTD. Comparison of the reactivity patterns due to the reagent attached at residue 273 or 302 allows the deduction of its orientation [7]. These positions were chosen in our recent study, since substitution at these positions had little or no effect upon FNR- or CRP-dependent transcription activation [7].

Transcription initiation at many promoters requires an activator. Most activators bind to specific DNA sequences at target promoters and then recruit RNA polymerase to the promoter. In many cases, this recruitment requires direct contacts between the activator and either the α or σ subunits of RNA polymerases [2]. In *E. coli*, a large group of transcription activators (known as class II activators) bind to DNA sites that overlap the target promoter −35 hexamer element. At such promoters (known as class II promoters), the activator makes a direct contact with the RNA polymerase σ subunit and αCTD binds somewhere upstream of the activator (Fig. 1) [8,9]. In this study we report experiments with the *E. coli* regulator of fumarate and nitrate reduction, FNR, a typical class II transcription activator that is a global regulator responsible for the induction of gene expression during oxygen starvation. FNR is an iron–sulphur protein and its activity is contingent on the formation of an iron–sulphur cluster that is stable only in the absence of oxygen (reviewed in [10]). At most FNR-dependent promoters, an FNR homodimer binds to a 22 bp target site centred between bp 41 and 42 upstream of the transcription start point (i.e. position −41.5) and interacts with the RNA polymerase σ subunit (reviewed in [11]). At such promoters, αCTD is positioned upstream of bound FNR such that it interacts with FNR, but, to date, its precise location has not been determined experimentally [12,13]. Here, using the FeBABE reagent, we show that one αCTD binds immediately upstream of FNR in transcriptionally competent complexes at a class II FNR-dependent promoter. We then compared the location of αCTD at a similar promoter dependent on the *E. coli* cyclic AMP receptor protein (CRP). CRP is another global regulator that is related to

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Abbreviations: αCTD, RNA polymerase α subunit C-terminal domain; FNR, regulator of fumarate and nitrate reduction; CRP, cyclic AMP receptor protein; FeBABE, iron [*S*]-1-[*p*-bromoacetamidobenzyl] ethylenediaminetetraacetate

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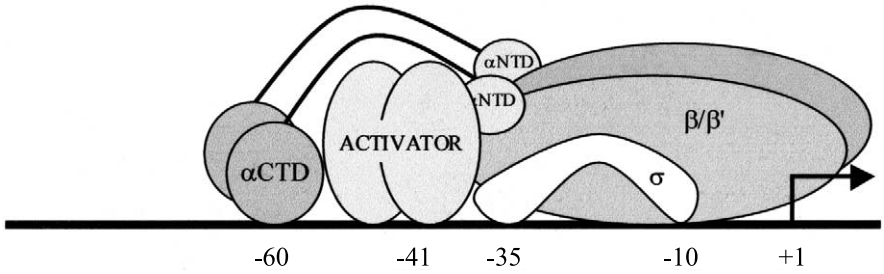

Fig. 1. Class II transcription activation. The figure shows the organisation of activator and RNA polymerase subunits at a typical class II bacterial promoter [8]. The activator is shown as a dimer binding to a target site centred near position −41.5, upstream of the transcription start-point (+1). The different RNA polymerase subunits are indicated, with the two domains of the two α subunits joined by a line that depicts the flexible linker. The two αCTDs are known to be located somewhere upstream of the activator and, for CRP, FNR and several other activators, it is known that at least one αCTD interacts with the activator as shown in the figure.

FNR at the level of primary sequence, and previous studies have shown that CRP and FNR activate transcription by similar mechanisms [14]. Our results show that the location of αCTD is the same at both the class II FNR-dependent and CRP-dependent promoters.

In the last part of the study, we investigated the effects of introducing a second DNA site for FNR upstream of the class II FNR-dependent promoter. Our results show that the location of αCTD is unaffected by FNR binding to the second site. In contrast, when a second DNA site for CRP was introduced upstream of the class II CRP-dependent promoter, the location of αCTD is affected by CRP binding to the second site.

2. Materials and methods

Overexpression and purification of His-tagged α protein containing a single cysteine residue at position 273 or position 302, and conjugation with FeBABE, was performed as described by Lee et al. [7]. RNA polymerase holoenzyme was reconstituted with FeBABE-tagged α subunits using the method of Tang et al. [15], and its activity was checked by electromobility shift assays and in vitro transcription assays as detailed by Lee et al. [7]. The D154A mutant of FNR was purified as described by Wing et al. [13] and CRP was purified using the method of Ghosaini et al. [16].

The promoters used in this work were the semi-synthetic FNR-dependent *FF*(−41.5) and *FF*(−90.5)*FF*(−41.5) promoters, described by Barnard et al. [17], and the related CRP-dependent *CC*(−41.5) and *CC*(−90.5)*CC*(−41.5) promoters, described by Busby et al. [18] (Fig. 2). *FF* and *CC* refer to 22 bp DNA sites for FNR or CRP, respectively, that are centred at position −41.5 or position −90.5. The promoters were cloned on *Eco*RI-*Hin*dIII fragments into the vector plasmid, pSR, and were excised on *Aat*II-*Hin*dIII fragments for footprinting [17]. Purified fragments were labelled at the *Hin*dIII site using [γ-^{32}P]ATP and polynucleotide kinase (for the template strand) or [α-^{32}P]ATP and *E. coli* DNA polymerase Klenow fragment (for the non-template strand). For footprinting, RNA polymerase holoenzyme preparations (50 nM) and FNR D154A (100 nM) or CRP (100 nM) were pre-incubated at 37°C for 10 min with promoter DNA in a reaction volume of 35 μl (20 mM HEPES pH 8.0, 50 mM potassium glutamate, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin). Complexes were then treated with heparin (50 μg/ml final concentration; 1 min at 37°C). DNA cleavage was initiated by the addition of 3 mM sodium ascorbate (final concentration) and 3 mM hydrogen peroxide (final concentration). Samples were incubated at 37°C for 2 min for α302 FeBABE or for 15 min for α273 FeBABE, before the addition of 45 mM EDTA (final concentration)

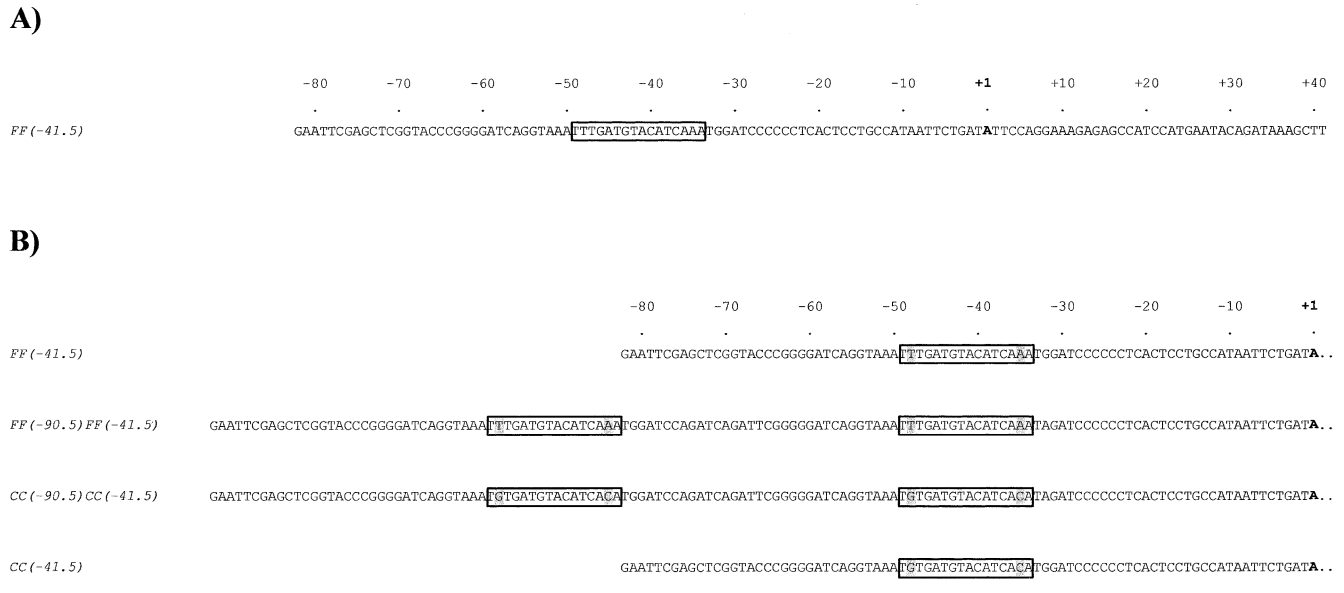

Fig. 2. Promoters used in this work. A: The complete sequence of the *Eco*RI-*Hin*dIII fragment of the *FF*(−41.5) promoter is shown. The DNA site for FNR is boxed, and the transcription start site, +1, is highlighted in bold. B: The sequences of the related *FF*(−41.5), *FF*(−90.5)*FF*(−41.5), *CC*(−41.5) and *CC*(−90.5)*CC*(−41.5) promoters from the *Eco*RI site to the transcription start site, +1, are shown, aligned at the transcription start site. All the promoters are identical to the *FF*(−41.5) promoter (shown in A) downstream of the transcription start site. The DNA sites for FNR and CRP are boxed, and the bases which vary between the different promoters are highlighted in grey.

and 7 mM thiourea (final concentration) to stop the reaction. Modified DNA was extracted with phenol/chloroform and precipitated with ethanol before analysis on a 6% polyacrylamide gel containing 6 M urea. Gels were calibrated with Maxam–Gilbert G+A sequencing ladders, and were processed and scanned using a phosphorimager (Molecular Dynamics) and Bio-Rad Quantity One software.

3. Results and discussion

3.1. Location of RNA polymerase αCTD at a class II FNR-dependent promoter

In previous work [12,13], we used the *FF*(−41.5) promoter (Fig. 2), which contains a single DNA site for FNR cloned upstream of the *E. coli melR* promoter, as a model class II FNR-dependent promoter, and we reproduced FNR-dependent transcription activation in vitro. To avoid the need to work in oxygen-free conditions, we used the D154A mutant of FNR, which is active in the presence of oxygen [19]. Here, we have exploited two RNA polymerase derivatives, described by Lee et al. [7], that have the DNA cleavage reagent FeBABE specifically attached to either position 273 or position 302 of αCTD, to investigate the location of αCTD in FNR-dependent open complexes formed at the *FF*(−41.5) promoter. The patterns of DNA cleavage, due to the FeBABE-labelled RNA polymerase, on the non-template and template strands of the *FF*(−41.5) promoter are shown in Fig. 3A. Cleavage occurs after the addition of sodium ascorbate and hydrogen peroxide, which generates a short-lived pulse of hydroxyl radicals that emanates from the iron atom of the tethered FeBABE reagent. Because of the instability of hydroxyl radicals, DNA cleavage should occur only in the immediate vicinity of the tethered FeBABE reagent. However, unexpectedly, in this experiment, we observed faint but clear DNA cleavage in the absence of FeBABE-labelled RNA polymerase. Control experiments showed that this cleavage is FNR-dependent and thus we interpret these signals as due to the presence of iron atoms (up to 0.8 per FNR dimer) that remain associated with FNR after iron–sulphur cluster degradation. The position of this cleavage is consistent with the known binding of an FNR homodimer to a single DNA site centred at position −41.5 [13].

Results in Fig. 3A show the locations of cleavage in the ternary complexes of promoter DNA, FNR and FeBABE-labelled RNA polymerase. The locations of cleavage due to FNR are unaltered but supplementary bands appear up-

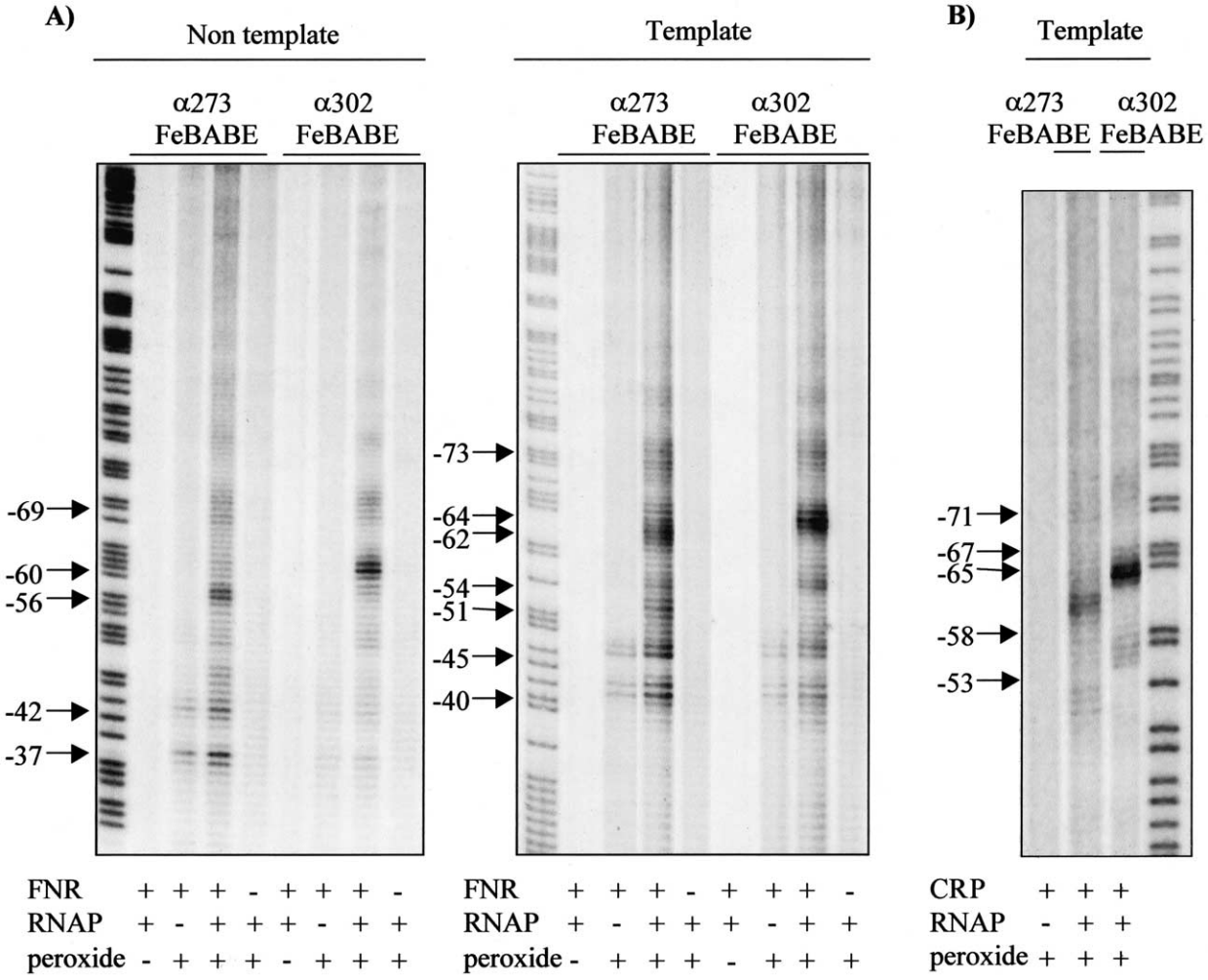

Fig. 3. DNA cleavage at the *FF*(−41.5) and *CC*(−41.5) promoters. A: The figure shows a phosphorimager scan of a gel on which DNA cleavage patterns at the *FF*(−41.5) promoter were analysed. Results are shown for both the template and the non-template strand. The gels were calibrated using Maxam–Gilbert G+A sequencing ladders. DNA fragments were incubated with 100 nM FNR D154A and 50 nM RNA polymerase reconstituted with αCTD tagged with FeBABE at position 273 or position 302, as indicated. B: The figure shows a phosphorimager scan of a gel on which DNA cleavage patterns at the *CC*(−41.5) promoter were analysed.

stream. The major cleavage sites, which must be due to the FeBABE reagent attached to αCTD, are found near position −60. We interpret these signals as being due to one αCTD binding just upstream of the bound FNR, as at other class II activator-dependent promoters (Fig. 1). For both DNA strands, cleavage due to FeBABE located at residue 273 of the RNA polymerase α subunit occurs 4–5 bp downstream of cleavage when FeBABE is located at residue 302. Since residues 273 and 302 are located on opposite faces of αCTD (Fig. 4), this allows us to determine the orientation of binding of this αCTD at the *FF*(−41.5) promoter. Previous analysis has identified opposite faces of αCTD containing the 261 and 287 determinants, which can make interactions with activators and different parts of RNA polymerase [20,21]. To explain the cleavage patterns in Fig. 3A, we suppose that, in open complexes formed at the FNR-dependent *FF*(−41.5) promoter, the αCTD located immediately upstream of the bound FNR is oriented with the face containing the 287 determinant pointing towards FNR, and with the face containing the 261 determinant pointing away from FNR (as illustrated in Fig. 4B).

In a recent study [7], Lee et al. used an identical approach to determine the location and orientation of αCTD in CRP-dependent open complexes formed at the *CC*(−41.5) promoter, a derivative of *FF*(−41.5) carrying two base changes that convert the DNA site for FNR into a DNA site for CRP (Fig. 2) [22]. Since CRP and FNR appear to activate transcription by similar mechanisms, we compared the location of αCTD in activator-dependent complexes at the *CC*(−41.5) and *FF*(−41.5) promoters. Results in Fig. 3B show that the pattern of DNA cleavage with FeBABE-labelled RNA polymerase at *CC*(−41.5) is very similar to that observed at *FF*(−41.5). In particular, the differences in DNA cleavage due to tethering FeBABE at residue 273 or residue 302 of α are very similar at the two promoters. Thus, we can conclude that the location and orientation of αCTD immediately upstream of the activator at the *FF*(−41.5) and *CC*(−41.5) promoters is very similar, if not identical. Note that, in the experiment with CRP, no significant DNA cleavage is observed in the absence of FeBABE-labelled RNA polymerase. This is because, unlike FNR, CRP contains no iron atoms and hence the complication of FeBABE-independent signals is avoided.

3.2. Location of αCTD at complex FNR- and CRP-dependent promoters

At CRP-dependent class II promoters, only one of the two

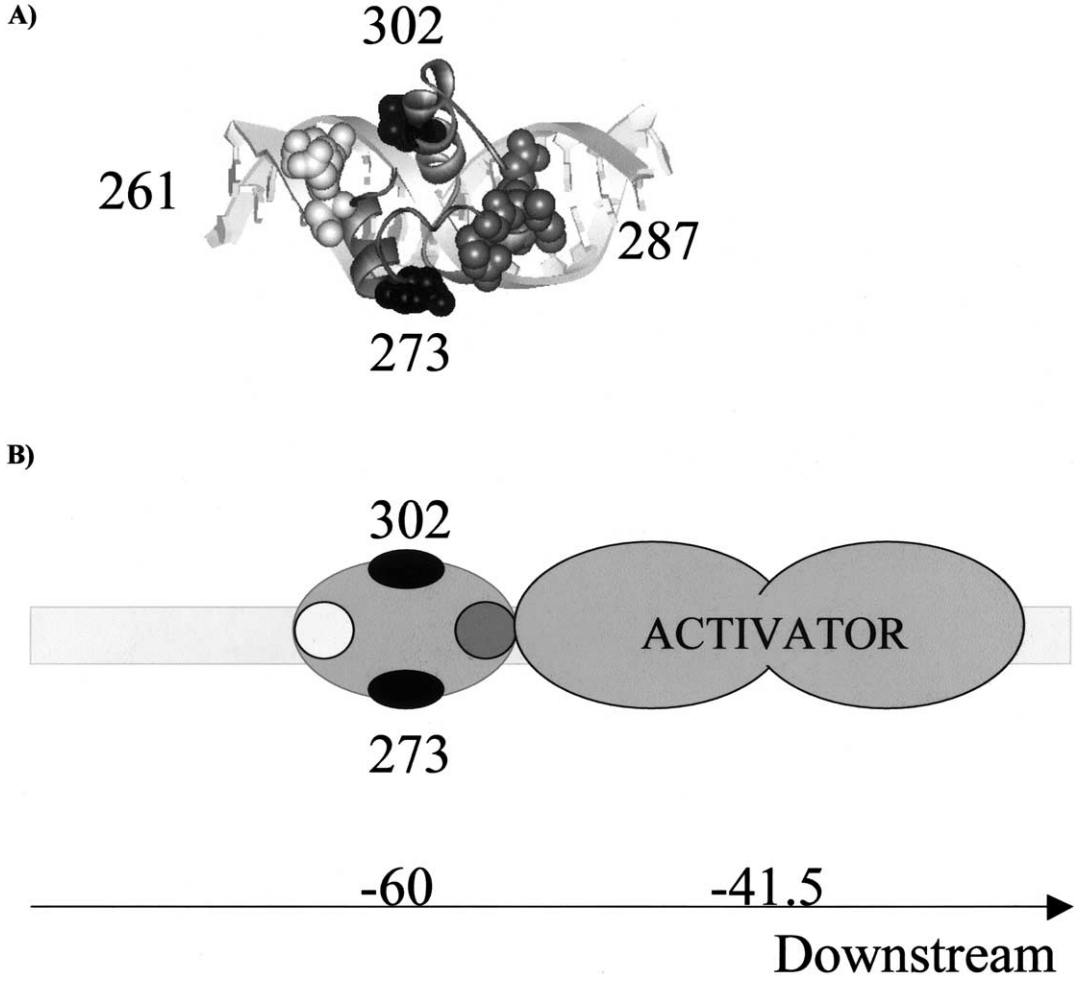

Fig. 4. Models of αCTD. A: Models of RNA polymerase αCTD bound to DNA, adapted from Benoff et al. [26] to show the locations of residues 273 and 302, which were tagged with FeBABE, and the 261 and 287 determinants [14]. B: Sketch to illustrate the juxtaposition of αCTD bound immediately upstream of FNR or CRP at the *FF*(−41.5) and *CC*(−41.5) promoters. The light and dark grey circles depict the 261 and 287 determinants respectively. The positions of activator and αCTD binding, and the direction of transcription, are indicated below the diagram.

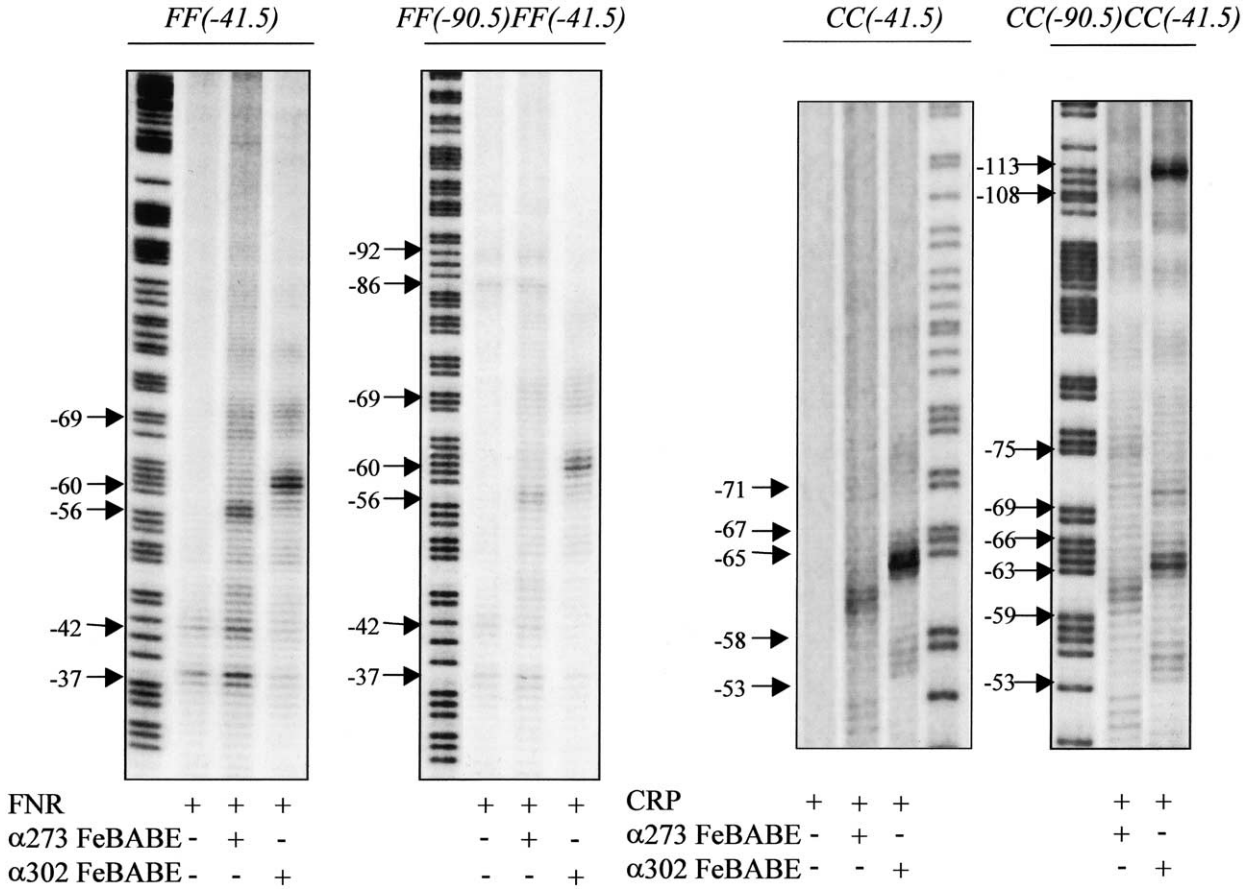

Fig. 5. DNA cleavage at promoters with single or tandem bound FNR and CRP. The figure shows phosphorimager scans of gels on which patterns of DNA cleavage at the *FF*(−41.5), *FF*(−90.5)*FF*(−41.5), *CC*(−41.5) and *CC*(−90.5)*CC*(−41.5) promoters was analysed. Experiments were performed as in Fig. 3.

RNA polymerase αCTDs interacts with the activator [14,23]. Studies with the *CC*(−41.5) promoter have shown that transcription activation can be enhanced by the introduction of a second upstream DNA site for CRP located at position −90.5, and that this enhancement is due to a direct contact between the upstream-bound CRP and the 'spare' αCTD [18,24]. Interestingly, parallel studies with the *FF*(−41.5) promoter, and with a derivative carrying a second upstream DNA site for FNR at position −90.5, have shown that upstream-bound FNR at this location has very little effect upon transcription activation [17]. Thus, we used the two preparations of RNA polymerase, tagged with FeBABE at either position 273 or position 302 of αCTD, to compare the location of αCTD in FNR-dependent open complexes formed at the *FF*(−41.5) and *FF*(−90.5)*FF*(−41.5) promoters and in CRP-dependent open complexes formed at the *CC*(−41.5) and *CC*(−90.5)*CC*(−41.5) promoters. The results (Fig. 5) show that the position of αCTD in FNR-dependent open complexes at the *FF*(−41.5) promoter is not affected by upstream-bound FNR at the *FF*(−90.5)*FF*(−41.5) promoter. Note that DNA cleavage due to the iron atoms in FNR bound at both position −41.5 and position −90.5 is clearly seen. In contrast to the situation at the *FF*(−90.5)*FF*(−41.5) promoter, movement of αCTD to bind adjacent to the upstream-bound CRP at the *CC*(−90.5)*CC*(−41.5) promoter is clearly seen.

3.3. Conclusions

In transcriptionally active complexes at class II CRP-dependent promoters, one αCTD of RNA polymerase binds to the minor groove near position −61, immediately upstream of the CRP homodimer, and makes an interaction that contributes to activation (Fig. 1) [14]. In transcriptionally active complexes at class II FNR-dependent promoters, it is known that αCTD makes an interaction with the upstream subunit of the bound FNR homodimer [12], but there is little direct biochemical evidence concerning its location. In this report, we use a tethered DNA cleavage reagent to show that the location and orientation of αCTD, immediately upstream of bound FNR at a class II FNR-dependent promoter, is the same as its location and orientation upstream of bound CRP at a class II CRP-dependent promoter. Thus, in both cases, αCTD is positioned to make direct interactions with the cognate activator as predicted by previous genetic and structural studies [21,25,26]. Interestingly, in this work, and in the previous study by Lee et al. [7], clear signals due to the second αCTD of RNA polymerase were not detected. Lee et al. speculated that the second αCTD may not be associated with the DNA strongly enough to result in a second signal. However, a second signal, which must be due to FeBABE reagent attached to the second αCTD, is clearly seen at the *CC*(−90.5)*CC*(−41.5) promoter, which carries tandem bound CRP molecules. We suppose that this signal is due to recruit-

ment of the second αCTD of RNA polymerase to the promoter DNA by the upstream-bound CRP. This is consistent with previous data showing that tandem CRP molecules bound at position −90.5 and position −41.5 make independent contacts with the two RNA polymerase αCTDs and thus activate transcription synergistically [23,24]. This 'co-operation' between activators is not found with tandem FNR molecules bound at the *FF*(−90.5)*FF*(−41.5) promoter [17], and this is consistent with the results presented here, which show that upstream bound FNR is unable to reposition αCTD. Thus, although the mechanisms of transcription activation by CRP and FNR are similar in many respects, there is a fundamental difference in the way that tandem bound CRP molecules or tandem FNR molecules interact with RNA polymerase at promoters.

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