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E.coli RNA polymerase, deleted in the C-terminal part of its α-subunit, interacts differently with the cAMP-CRP complex at the *lac*P1 and at the *gal*P1 promoter

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ABSTRACT

A deletion of the C-terminal part of the α-subunit of RNA polymerase is known to affect differently promoters activated by CRP depending on the location of the CRP binding site at the promoter. When the CRP binding site is located at −61.5, as at *lac*P1 (a type I promoter), activation is strongly impaired while it is not significantly affected at *gal*P1 where CRP binds 41.5 bp upstream of the start of the message (type II promoter). We have investigated the differences in the architecture of the corresponding open complexes by comparing the positioning of holoenzymes reconstituted respectively with native or with truncated α-subunits (containing the first 235 or 256 residues of a) at two 'up' promoter mutants of the *lac*P1 and *gal*P1 promoters (respectively *lac*UV5 and *gal*9A16C). First, the affinity of wild-type RNA polymerase for both promoters is increased by the presence of CRP and cAMP. By contrast, holoenzymes reconstituted with truncated α-subunits, show cooperative binding at the *gal*P1 promoter only. Second, footprinting data confirm these observations and indicate that the truncated holoenzymes are unable to recognize regions of the promoter upstream from position −40. The absence of contacts between the truncated enzymes and CRP at the *lac*P1 promoter can explain the deficiency in activation. At the *gal*P1 promoter, where the CRP site is closer to the initiation site, protein-protein contacts can still occur with the truncated polymerases, showing that the C-terminal part of the α-subunit is not involved in activation.

INTRODUCTION

Positive control of transcription often involves stimulatory protein–protein interactions between regulatory factors and RNA polymerase. Mutants deficient in contacts between the enzyme and the positive activator map either in the genes coding for the different subunits of RNA polymerase or in the activator gene. The most straightforward argument for direct protein-protein contacts between the activator and RNA polymerase involves the isolation of suppressor mutations mapping at a precise locus in one of the RNA polymerase genes which specifically suppress the defect of positive control mutations in the activator or *vice versa* [1,2]. The cAMP-receptor protein, CRP, controls the initiation of transcription of numerous genes [3,4], especially those involved in carbon source utilisation. Mutations defective in activation have been reported at the *crp* locus and in the *rpo* genes. 'Positive control' mutants in *crp* that affect transcription activation without altering the ability of the regulatory protein to recognize its target site have been isolated by different groups [5–8, and R.Ebright, quoted in 8]. They map on an exposed loop between residues 156 and 162 in the C-terminal part of the CRP protein. Mutations in this domain prevent transcription activation at many CRP dependent promoters. For proximal CRP activating sites located around −41 relative to the transcription start, both subunits of the CRP dimer are able to contact RNA polymerase. At those promoters designated as type II, revertants of the positive control mutations such as the double mutant H159L K52N have been isolated [5,7]. This mutant superactivates class II promoters but is totally inactive with respect to the activation of transcription from promoters carrying the CRP site at −61.5 (called type I promoters) [5,7]. Hence, at type II promoters, a second domain may be involved in transcription activation, whereas at type I promoters only the one patch around residue 159 is efficient.

RNA polymerase mutants altered in CRP activation have been isolated and the mutations located in genes coding for three different RNA polymerase subunits, σ [9], β [10,11] and α [39, and R.Ebright, quoted in 41]. Immunochemical [12] and biochemical data support the involvement [13] of the α-subunit in CRP activation at least for the *lac* promoter and other related type I promoters. In fact holoenzymes reconstituted *in vitro* with truncated α-subunits, α-235 or α-256, containing respectively the first 235 and 256 amino acids of the 329 residues of the α protein were perfectly able to initiate transcription at constitutive promoters, but were unable to respond to CRP activation at type I promoters [13]. It was also shown that the *gal*P1 and the pBRP4 promoters, members of the class II promoters where the CRP site is centered at −41.5 and overlaps the −35 region of the promoter, could be recognized in the presence of the CRP-cAMP complex by the α-truncated RNA polymerases and transcribed [14].

As the activation mechanism by CRP-cAMP appears to be different at the *lac*P1 and *gal*P1 promoters, we have decided to test the binding of the reconstituted polymerases to *lac* and *gal* up promoter variants in the presence and in the absence of the CRP-cAMP complex. We show here that cooperative binding takes place at both promoters between the two proteins when an holoenzyme is reconstituted with an entire a-subunit. Cooperativity is maintained when truncated α-subunits are used in the reconstitution and when the enzyme is assayed at the *gal*P1 promoter. Cooperativity is lost when the mutant enzyme is assayed at the *lac*P1 promoter. Footprinting data confirm these results and suggest a plausible interpretation for these findings.

MATERIALS AND METHODS

DNA

The *gal*9A16C promoter contains the A → C change at −16, in order to eliminate the acitity of the *gal*P2 promoter and the G → A change at −9 to enhance the activity of the *gal*P1 promoter [40]. The 203 bp *lac* fragment [15] containing the *lac*UV5 promoter and the 144 bp *gal* fragment containing the *gal*9A16C promoter [40] were purified by polyacrylamide gel electrophoresis. The 130 bp fragment carrying the T7A1 promoter [16] was a gift from Drs. W.Metzger and H.Heumann (Max Planck Institute of Biochemistry, München-FRG). All fragments were labelled using Klenow fragment of DNA polymerase I with α ^{32}P dATP in the presence of the other three dXTPs, unless otherwise specified.

Proteins

CRP protein was purified from the overproducing strain BS646 by the method of Ghosaini et al. [17]. It was 75% active in DNA binding as measured in gel retardation assays.

RNA polymerases: After isolation of each RNA polymerase subunits $\beta\beta'$ and α as well as truncated a-subunits α-235 and α-256, three different core enzymes containing the wild-type α-subunit or the truncated α-235 or α-256 subunits were reconstituted and purified as previously described [13].

For reconstitution of holoenzymes, the reconstituted core enzymes were mixed with four-fold molar excess of σ^{70} subunit. The RNA polymerases were found to be between 15% and 20% active using a titration with the strong T7A1 promoter at 9 nM in gel retardation assay (described below). The values of dissociation constants given in Table I are not corrected for this measured activity of the enzymes.

Gel retardation assays

4 μl of a mixture of ^{32}P radiolabelled DNA (concentration $\leq$0.2 nM) and cAMP (400 μM) in glutamate buffer (40 mM Hepes pH 8, 10 mM $MgCl_2$, 100 mM K glutamate, 500 μg/ml BSA) with or without CRP protein (30 nM) were gently mixed with a RNA polymerase solution (5−80 nM) in glutamate buffer. The sample was incubated at 37°C for 30 min; 3 μl of an heparin xylene cyanol-blue sucrose solution (150 μg/ml heparin, 0.1% dye, 50% sucrose) in glutamate buffer containing 200 μM cAMP and no BSA was added and the mixture loaded on a 4.5% polyacrylamide gel (38:1 acrylamide bis) in TBE buffer containing 200 μM cAMP as quickly as possible. The gel was run at room temperature at 13 V/cm until the dye had migrated through half of the gel (~2 hr). 200 μM cAMP was present in the gel reservoir at the cathode. The gel was fixed with a mixture of water ethanol-acetic acid (70:20:10) and dried and exposed on a Phosphorimager 400A (Molecular Dynamics). The ratio of free to bound DNA was used to estimate the dissociation constant for RNA polymerase, Kd. Each value given in Table I is the average of ten experimental points. The cooperativity factor is defined as the ratio between dissociation constants determined in the absence and in the presence of CRP. The nature of the competitor and also the CRP concentration can affect the extent of cooperativity observed: phage M13 single-stranded DNA is a milder competitor than heparin; it does not remove CRP from its binary complex. In the presence of M13, the cooperativity factor at the *lac* promoter is increased from 3 to 5. In the case of the strong T7A1 promoter, the specific activity of the labelled DNA fragment was not high enough to directly determine the dissociation constants for the promoter; we used a competition assay with the *lac*UV5 promoter, hence the large errors associated with the values presented in Table I.

Footprinting studies

The 203 bp *lac* fragment labelled with either α^{32}P dATP and Klenow fragment or γ^{32}P ATP and T4 polynucleotide kinase was cut with *Pvu*II restriction endonuclease (Boehringer). All footprinting experiments were performed at 37°C in glutamate buffer with 200 μM cAMP; RNA polymerase was at 75 nM and CRP when added at 40 nM. The methods were those used in Spassky et al. [18] for DNAaseI footprinting studies, in Tullius and Dombroski [19] for cleavage by hydroxyl-radicals, and in Buckle et al. [20] for laser photocrosslinking experiments.

RESULTS

In vitro, the α-truncated RNA polymerases alone recognize three different promoters with the same hierarchy as wild-type RNA polymerase

Intermediate and open complexes of RNA polymerase with promoter DNA can be isolated from free promoter DNA by non denaturing polyacrylamide gel electrophoresis (cf. for example [16,21]). We used this method to determine the affinities of the holoenzymes for three different strong promoters, the phage T7A1 promoter, the *lac*UV5 promoter and the *gal*9A16C promoter, which carries simultaneously a down mutation in the P2 Pribnow box which abolishes *gal*P2 activity and an up mutation in the P1 Pribnow box which increases the efficiency of the *gal*P1 promoter [40]. The radiolabelled DNA promoter fragments were incubated with various concentrations of reconstituted holoenzymes at 37°C for 30 min to reach equilibrium between the free species and open complexes; preformed complexes were challenged with heparin and separated on a 4% native polyacrylamide gel (Fig. 1). No difference in the mobility of complexes formed with either wild-type or α-truncated RNA polymerase was detected under these conditions.

Apparent dissociation constants, Kd, for open complex formation are given in Table I-A. Three conclusions can be drawn:

- The three polymerases show slightly different affinities for the same promoter. Whilst the increase in the mean estimate of the Kd values in general parallels the extent of the deletion of the α-subunit, it is most noticeable in the case of the *gal* promoter.
- All three polymerases exhibit the same hierarchy. They bind preferentially to the T7A1 promoter then to *lac*UV5 and least well to *gal*9A16C.

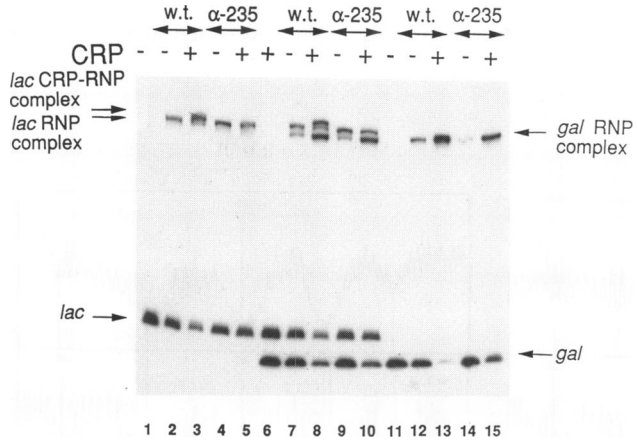

Figure 1. Visualization of complexes between the 203 bp *lac*UV5 and the 144 bp *gal*9A16C promoter fragments and wild-type or α-235 RNA polymerases after heparin challenge. Lanes 1 to 5 contained *lac* DNA; lanes 6–10 *gal* and *lac* DNA; lanes 11–16 *gal* DNA. In lanes 3, 5, 6, 8, 10, 13 and 15, CRP was added at a concentration of 15 nM. Note the absence of CRP-DNA complexes, due to the presence of heparin added before loading the samples on the gel. In lanes 1, 6 and 11, no polymerase was added. Lanes 2, 3, 7, 8, 12 and 13 contained wild-type reconstituted RNA polymerase and lanes 4, 5, 9, 10, 14 and 15, α-235 reconstituted RNA polymerase.

Table I. Apparent dissociation constants Kd for open complex formation at three different promoters with the reconstituted holoenzymes (expressed in 10^{-9} M) in the absence of CRP (A), in the presence of CRP (B) and their ratio (C).

		wild-type	α-256	α-235
A	**−CRP**			
	*lac*UV5	4.9±2	9±3	9.6±4
	gal 9A16C	17±7	20.1± +3.4	55±18
	T7A1*	0.5±0.4	0.8±0.5	1±0.5
B	**+CRP**			
	*lac*UV5	1.8±0.7	7.2±2.5	8.6±2.7
	*gal*9A16C	3.5±1.9	2.5±0.9	8.2±0.4
	T7A1*	0.7±0.5	0.7±0.4	0.8±0.3
C	**Ratio $\frac{-CRP}{+CRP}$**			
	*lac*UV5	~3	~1	~1
	*gal*9A16C	~6	~8	~7

* The apparent dissociation constant for the T7A1 promoter was determined by direct competition assay with the *lac*UV5 promoter.

– The discriminating factor between the three promoters appears to be the same for each RNA polymerase. All polymerases bind the T7 promoter with an affinity 10-fold higher than the *lac* promoter and 20 to 50-fold higher than the *gal* promoter.

In summary, the deletion of parts of the C-terminal segment of the α-subunit affects, in a qualitatively similar manner, the overall association of RNA polymerase with the three promoters.

α-truncated RNA polymerases bind synergistically with the cAMP-CRP complex at the *gal*P1 promoter, but not at the *lac*P1 promoter

Cooperative binding between the cAMP-CRP complex and RNA polymerase at the *lac*P1 and *gal*P1 promoters has been widely supported by a variety of footprinting studies [18,22,24]. Even

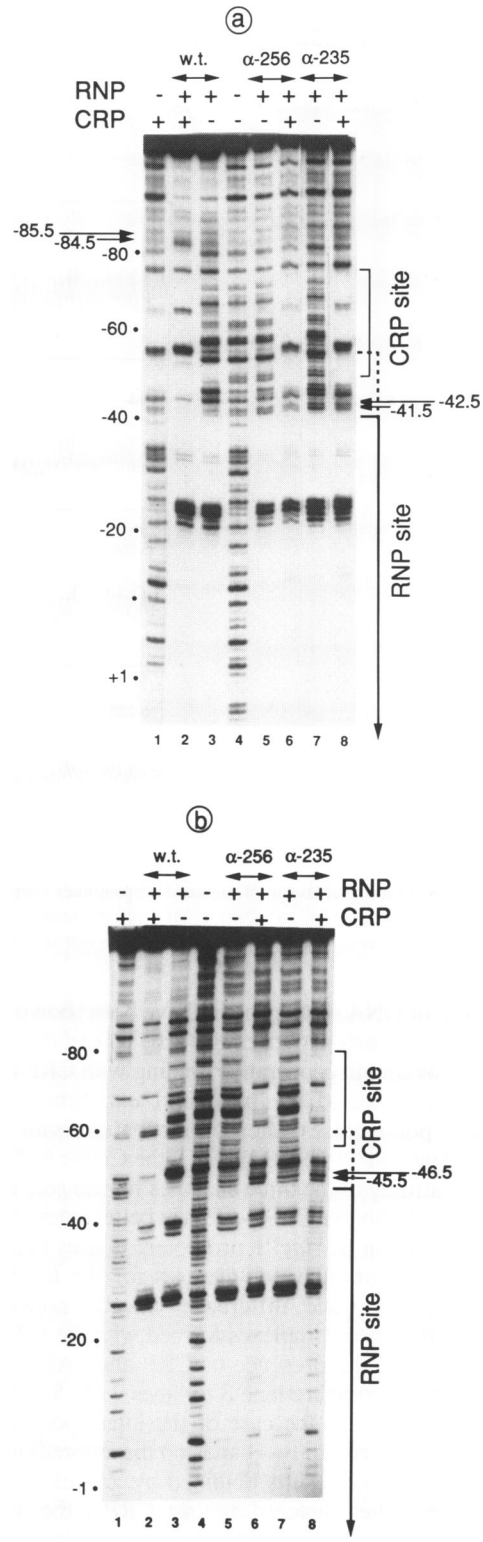

Figure 2. Protection of the *lac*UV5 promoter by the wild-type or mutant RNA polymerases in the absence and in the presence of CRP against DNAaseI cleavage (a: upper strand; b: lower strand). The CRP site is indicated by a thin bracket. The truncated RNA polymerase site is marked by a thick bracket and extends into the downstream region in the direction of the arrow. The additional upstream footprint due to wild type RNA polymerase is indicated by the dashed bracket. When added, the CRP and RNA polymerase concentrations were respectively 40 and 75 nM.

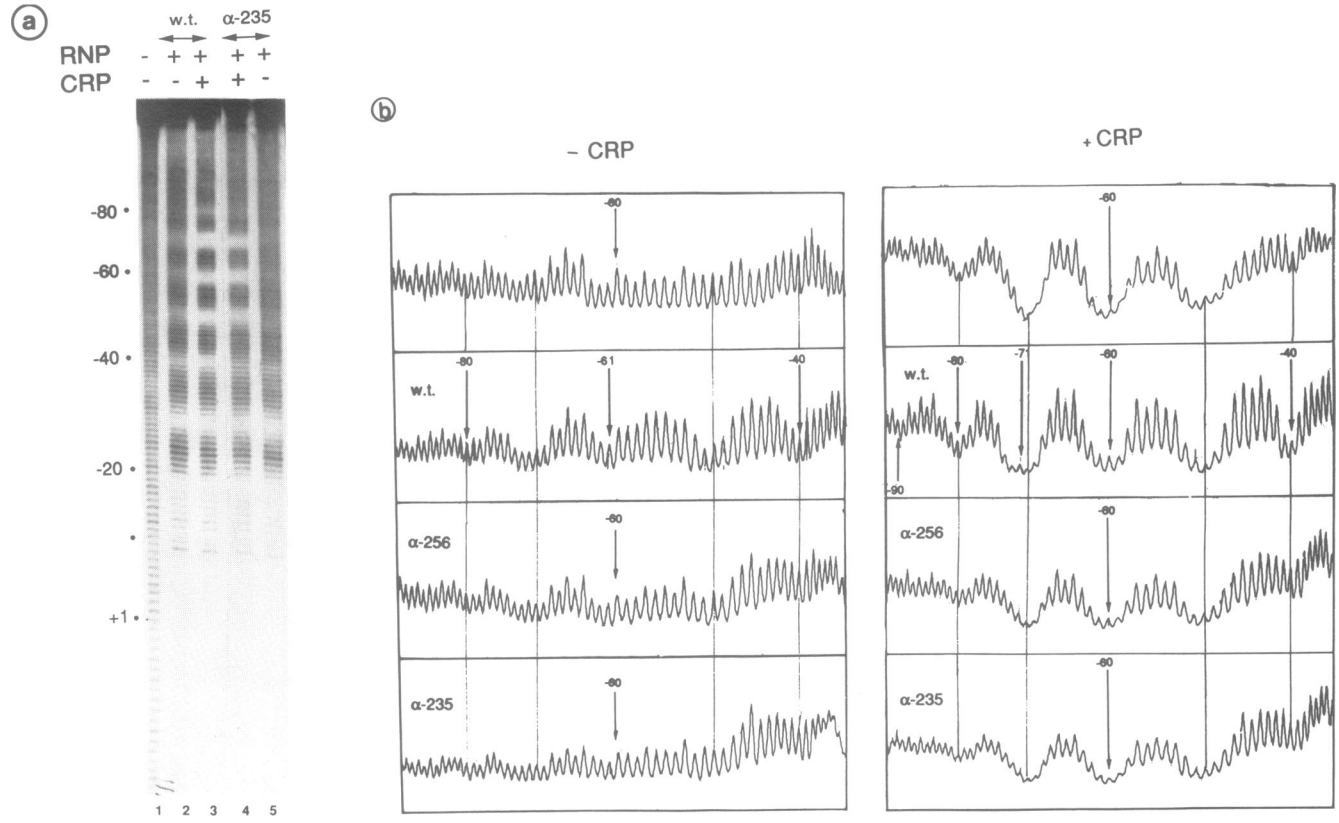

Figure 3. Hydroxyl-radical cleavage of the *lac*UV5 promoter (upper strand) with wild-type or α-235 RNA polymerases in the absence and in the presence of CRP. **a**: autoradiogram of the gel. **b**: ^{32}P scans of an extended sequencing gel from positions −92 to −35, also showing the pattern of the CRP-DNA binary complex and the patterns of the α-256 polymerase DNA complexes without or with CRP.

in the absence of DNA, both proteins have been shown to interact weakly [25,26]. In order to investigate the role of the C-terminal part of the α-subunit in cooperative binding with CRP at different promoters, we repeated the band-shift experiments with the reconstituted polymerases after preincubation with CRP and cAMP. As shown in Table I-B and I-C, the CRP-cAMP complex increases the affinity of the three enzymes for the *gal*P1 promoter by 6 to 8-fold. Although CRP permits a better interaction of the wild-type enzyme at the *lac*P1 promoter, it does not affect the affinity of the α-truncated polymerases for the *lac* fragment.

Furthermore, a subtle difference can be noticed in the migrations of the open complexes formed with the wild-type and mutant enzymes in the presence of CRP and cAMP at the *lac* promoter (Fig. 1; compare lane 3 to lanes 2, 4, 5 and also lane 8 to lanes 7, 9, 10). In the case of the intact polymerase, the final complex gives rise to two bands on the autoradiogram (lane 3). We confirmed the results obtained by Zinkel and Crothers [27] using antibodies directed against CRP : the upper band contains the CRP protein and the lower band does not. In the case of the α-truncated enzymes, only the lower band which does not contain the CRP protein can be detected on the gel. These experiments were carried out in the presence of heparin which is known to rapidly and completely dissociate the CRP-DNA binary complex. Hence in the ternary complex formed with the α-truncated RNA polymerases, at the *lac* promoter, CRP can be completely chased by heparin whereas the wild-type enzyme partially protects CRP against this challenge. In the case of the *gal*P1 promoter carried by the 144 bp fragment, only one band is observed on the autoradiogram irrespective of the presence of CRP in the polymerase-promoter complex. When present in the incubation mixture, CRP was detected in this single band of complex on the gel by revelation with antibodies directed against CRP.

The extent of binding synergy between the various polymerases and CRP at the *lac* and *gal* promoters is confirmed by a direct competition assay between the two promoters. In the absence of CRP, all polymerases bind better to the *lac* fragment than to the *gal* fragment (Fig. 1, lanes 7 and 9); in the presence of CRP, with the truncated polymerases, a better binding at *gal* is observed (Fig. 1, lane 10) consistent with the absence of cooperativity found at the *lac* promoter. As explained in Materials and Methods, the nature of the competitor affects the extent of cooperativity observed. The results however are quite clear: positive cooperativity always occurs at the *gal*P1 promoter, independent of the C-terminal part of the α-subunit but is only observed with the intact wild-type enzyme at the *lac* promoter. These results are perfectly consistent with observations [13] indicating that the mutant RNA polymerases did respond to CRP activation at the *gal* promoter (and more generally at type II promoters), but not at the *lac* promoter (at type I promoters).

Upstream promoter regions are not recognized by the truncated RNA polymerases in contrast to the wild-type enzyme

Since the activation of transcription by CRP (a kinetic process) and the occurence of positive interactions between the two proteins in the open complex (an equilibrium process) go hand in hand, we have studied in greater detail the structure of those

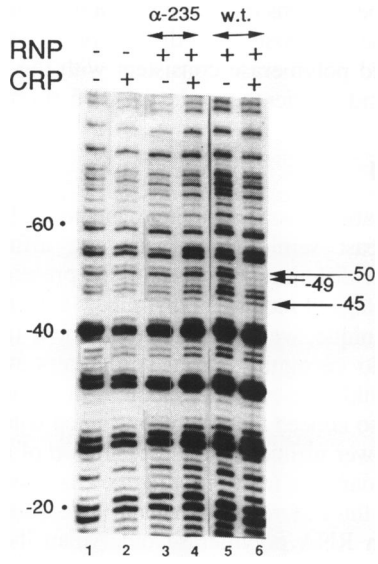

Figure 4. Primer extension pattern on the *lac*UV5 upper strand promoter fragment after laser irradiation of open complexes formed with wild-type or α-235 RNA polymerases in the absence and in the presence of CRP.

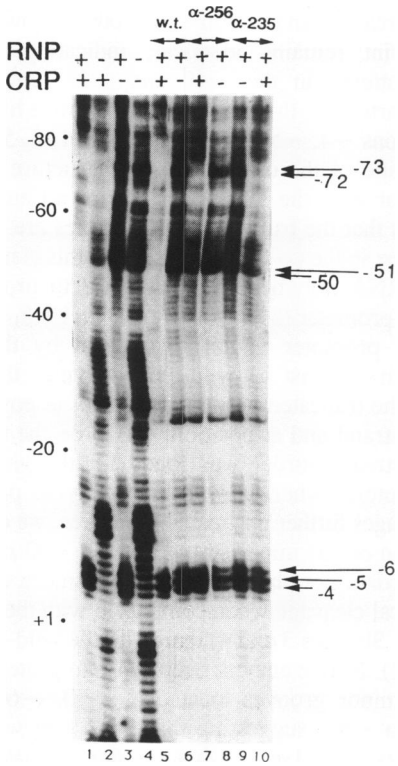

Figure 5. Copper phenanthroline reaction on the *lac*UV5 lower strand with wild-type or mutant RNA polymerases in the absence and in the presence of CRP. In lanes 1 and 3 wild-type native RNA polymerase was added, whilst in lanes 5 and 6 wild-type reconstituted RNA polymerase was used.

open complexes (at *lac* wild-type and at *lac*UV5) where deletions of the α-subunit affect positive cooperativity. At the *lac* wild-type promoter, RNA polymerase does not correctly position itself in the absence of CRP [14,42]. By contrast, the presence of CRP allows occupancy of the *lac*P1 promoter, giving an extensive protection against DNAaseI cleavage from −80 to +20 interrupted by some hypersensitive bands [14,22,23]. The cooperativity between α-truncated polymerases and CRP is lost at the *lac* promoter, consequently it was expected that binding of CRP at its target in the −60 region would not lead to the occupancy of *lac*P1 by the mutant enzymes. Such was indeed the case. We were unable to obtain a footprint of the α-truncated polymerase at wild-type *lac* promoter in contrast with the wild-type enzyme which, in conjunction with CRP and cAMP, gave an extensive protection from −80 to +20 interrupted by several hypersensitive bands.

The *lac*UV5 promoter is able to bind the mutant RNA polymerase independent of the presence of CRP-cAMP. We reasoned that the lack of cooperativity at the UV5 promoter should result from the loss of productive interactions between the two proteins and could probably be visualized on the promoter DNA at the hinge region between the binding sites of the two proteins. We therefore tested the binding of wild-type and mutant holoenzymes at *lac*UV5 promoter in the presence and in the absence of CRP and cAMP by different techniques: DNAaseI footprinting [18] (Fig. 2), hydroxylradical footprinting (iron EDTA [19] (Fig. 3) or copper-phenanthroline reactants [28] (Fig. 5)) and laser crosslinking [20] (Fig. 4). None of these techniques were able to detect any difference in the pattern of wild-type and mutant RNA polymerases downstream of −38, indicating that crucial recognition patterns in the −10 and −35 regions were not visibly modified. However, noticeable modifications appeared upstream of -38 with the α-truncated RNA polymerases both in the presence and in the absence of CRP-cAMP at *lac*UV5.

In the absence of CRP, wild-type RNA polymerase covers about 70 bp, protecting the upper strand against DNAaseI cleavage from positions −49.5 to positions +19.5 (Fig. 2a, lane 3) and the lower strand from positions −54.5 to positions +16.5 (Fig. 2b, lane 3). Upstream of position −20, strong hypersensitive bands appear. They are phased with the helical pitch of the DNA.

The two α-truncated polymerases do not show the same extent of protection in the extreme upstream part of the footprint; the protection actually stops at −39.5 on the upper strand (Fig. 2a, lanes 5 and 7) and at −43.5 on the lower strand (Fig. 2b, lanes 5 and 7) with no further modification downstream. The phosphodiester linkages at −41.5 and −42.5 (upper strand) and −45.5, −46.5 remain sensitive to DNAaseI cleavage. Surprisingly, the same phosphodiester bond at −41.5 remains sensitive to DNAaseI on the *gal*P1 promoter and on the *malT*P1 promoter (data not shown). These results appear rather striking since DNAaseI is a bulky enzyme and phosphates alkylated at −39.5, −38.5, −37.5 and −36.5 are known to interfere with RNA polymerase binding [29,30]. In summary, the α-truncated RNA polymerases are unable to protect the final upstream helical turn of the promoter from DNAaseI cleavage in contrast to wild-type enzyme and this prompted us to suspect that the mutant enzymes had lost important contacts with the phosphates near the −40 region.

To test this hypothesis, we performed a hydroxyl-radical cleavage with both types of enzymes (Fig. 3a). The protection pattern of the polymerase complexes (Fig. 3a, lanes 2 and 5) is essentially divided into two domains (as proposed by Schickor et al. [16]), the melting domain with a complete protection of the non template strand from −8 to +16 and the recognition domain upstream of −14 with a series of protected sugars every 10−11 nucleotides, indicating that the enzyme faces towards one

side of the DNA. This last domain contains six subdomains which exhibit different protection by the wild-type enzyme against hydroxyl-radical cleavage, the strongest protection being observed at −20, −30 and −40, this becoming progressively weaker at −50, −60 and −70 (Fig. 3a, lane 2; Fig. 3b, row 2). For the mutant polymerases, however, only the two most downstream regions −20 and −30 are protected (Fig. 3a, lane 5; Fig. 3b, rows 3 and 4) : this is consistent with the loss of contacts in the −40 region.

In order to obtain more information about those RNA polymerase subunits which make contacts in the −40 region, we also carried out protein-DNA crosslinking experiments with both enzymes, using a UV laser (Fig. 4). Identification of photochemical products or potential protein DNA-contacts was performed by primer extension assay [20]. A faint band at position −45 seen with the wild-type enzyme (Fig. 4 lane 5) is absent in the mutant (lane 3). This band could represent a contact between RNA polymerase and G-46 in the major groove of DNA. We have not yet identified the subunit which makes this putative contact.

In the presence of CRP and cAMP, the cooperative binding of CRP and the wild-type enzyme at the *lac* UV5 promoter is accompanied by various changes in the footprint which do not occur, either with RNA polymerase alone, or with CRP alone. With DNAaseI (Fig. 2), one observes a better protection of the hinge region between the two proteins (phosphodiester bonds at −46.5 on the upper strand and at −49.5 on the template strand are almost insensitive to DNAaseI attack), and a more extended footprint in the upstream part of the CRP site at least on the upper strand, with the protection of phosphates at −81.5, −82.5 and the appearance of two hypersensitive bands at −84.5 and −85.5 (Fig. 2a, lane 2). This observation is supported by the iron-EDTA cleavage pattern where two additional regions around −80 and −90 seem to be protected (Fig. 3a, lane 3); in addition, the protection around −70 is now much more intense and more extended than the protection due to CRP alone (Fig. 3b, +CRP row 2).

The *lac* template strand is particularly sensitive to the attack by another hydroxyl-radical reagent, the copper-phenanthroline complex which produces strong cleavages in the −70 region (−72, −73), in the −50 region (−52, −50, −49), in the −35 region (−35 to −28) and in the Pribnow box (Fig. 5, lane 4) [28]. RNA polymerase alone protects the −35 region and the Pribnow box, producing three hypersensitive bands at −4, −5, −6 in the single-stranded region (lanes 3, 6, 8 and 9), whereas CRP alone renders the −75 to −55 region inaccessible to the reagent (lane 2). If the combined footprint for CRP and polymerase were simply the sum of the footprints of each protein, then the −50 region should remain highly sensitive to cleavage. However, the whole region is protected by the presence of both proteins (lanes 1 and 5). In contrast, the mutant polymerases and CRP produce a pattern which is simply the sum of the individual CRP and polymerase footprints. Further evidence for a conformational change of the −50 region is given in Fig. 4. Following irradiation by the UV laser we observed that the presence of both wild type polymerase and CRP inhibits the formation of the thymine dimer between T-50 and −51 on the template strand (Fig. 4, lane 6) [20].

All the conformational changes observed in the −50 and upstream regions require the presence of both CRP and wild-type RNA polymerase: none of them occur with the mutant enzymes. All the patterns observed with a truncated polymerase and CRP are the juxtaposition of the individual patterns of CRP and α-C deleted polymerase consistent with the idea that both proteins bind independently to the *lac*UV5 promoter.

DISCUSSION

The gel retardation assay is a rapid method which allows to compare at least semi-quantitatively the affinity of RNA polymerases for different promoters in the presence of heparin. It has the advantage of requiring only minute amounts of material. Using this technique, we have shown that the α-truncated RNA polymerases do recognize strong promoters with the same hierarchy as wild-type enzyme, albeit with a somewhat lower affinity. We also noticed that the reconstituted wild-type enzyme had a 5-fold lower affinity than a preparation of native enzyme taking into account the fraction of active enzymes. We have no explanation for this differential behaviour unless additional factors associated with RNA polymerase other than its five subunits ($\alpha_2\beta\beta'$ and σ) might enhance or stabilize open complex formation. However, we have observed no difference in the footprinting patterns of reconstituted and native wild-type enzymes using all the methods described above.

In contrast, the α-truncated polymerases produce a differential footprint for the open complex when compared to the wild-type enzyme. The defect is localized in the upstream region of the promoter (upstream from position −38), but the downstream part of the footprint remains identical, indicating that crucial recognition patterns in the −10 and −35 regions are not modified. In particular, the copper phenanthroline hypersensitive bands at positions −4, −5, −6 (Fig. 5 lanes 1, 3, 5−10) which are characteristic of the unwound DNA structure of the open complex appear with the same intensity for all enzymes. This result suggests that the truncated holoenzymes are able to open the DNA duplex in the (−10 to +1) region; this is not surprising since σ^{70} of RNA polymerase is supposed to promote strand opening of the promoter (34). However, at *lac*UV5, the upstream region of the promoter is not recognized by the truncated polymerases in contrast to wild-type enzyme; the DNAaseI protection of the truncated polymerases stops at position −45.5 on the lower strand and at position −41.5 on the upper strand (the same upstream border was found for the *gal*9A16C and *malT*p1 promoters) whereas wild-type enzyme protects eight phosphate linkages further upstream. Moreover, we observed that the −40 region on the upper strand and the −43 region on the lower strand (data not shown) were much more accessible to hydroxyl-radical cleavage when complexed with the α-C deleted enzymes (Fig. 3b rows 3 and 4) than with the wild-type enzyme (Fig. 3b row 2). Native enzyme seems also to protect albeit only partially the minor grooves located at −50, −60, −70 and probably up to −80, suggesting that DNA can wrap partially around the enzyme and establish some non-specific contacts in the conditions reported here. No such wrapping is detectable with the truncated enzymes.

Consequently, the truncated enzymes have lost the ability to bind to promoter regions upstream from position −38. Two alternative explanations can be proposed: 1) the C-terminal part of α-subunit makes contacts with the upstream region of the promoter; 2) the C-terminal deletion of α induces a conformational change in RNA polymerase which makes the truncated holoenzyme unable to bind to the upstream part of the promoter. No definitive argument can yet be advanced in favour

of either hypothesis. In particular, no contact has been demonstrated yet between the α-subunit and any part of the promoter and the most upstream contact obtained by a rather indirect crosslinking method lies at −46, −47 [31]. Undoubtely the most straightforward explanation of our results would be that the α-subunit contacts the *lac*UV5 DNA in the −40 and −45 regions. Furthermore, the positioning of the σ-subunit seems to be perfectly correct in the -35 region by all footprinting criteria. The recognition helix of the helix-turn-helix motif of s^{70} is supposed to make specific contacts with the base pairs of the DNA in the major groove from positions −32 to −36 [36, 37]. If the σ-subunit were also contacting the adjacent minor groove, at −40 instead of α, one good candidate to perform this task would be the non-recognition helix of the HTH motif. However, it is difficult to imagine how the specific contacts in the major groove could be achieved by the recognition helix when the non-specific contacts of the anchoring helix are inexistent. This weakens the argument for an indirect effect of the deletion.There is presently no reason to reject the simpler idea that the C-terminal part of the α-subunit contacts DNA in the −40 and −45 regions.

The differences between wild-type and truncated polymerases are more striking in the presence of the cAMP-CRP at the *lac*UV5 promoter. Wild-type enzyme and CRP bind cooperatively to the strong *lac*UV5 promoter whereas the truncated enzymes show exactly the same affinity in the presence or absence of CRP (Table I). Moreover, immunoblotting experiments and the appearance or disappearance of the upper band characteristic of the open complex containing CRP at *lac* are consistent with a single explanation: CRP in the ternary open complex with the mutant enzyme is totally chased by heparin whereas it is partially resistant in the complex with wild-type RNA polymerase. The cooperative binding of CRP and the wild-type enzyme at the *lac*UV5 promoter is accompanied by significant changes in the footprint with respect to the two binary complexes: 1) the DNAaseI footprints of RNA polymerase and CRP overlap (Fig. 2; and [18]), moreover, at least on the upper strand, an extension of the footprint of the CRP site is observed up to position −84.5; 2) a more intense protection of the −40, −70 and −80 regions to hydroxyl-radical cleavage is observed (Fig. 3); 3) CRP and RNA polymerase induce a conformational change of the −50 region, which can be detected by the copper-phenanthroline reagent (Fig. 5) or after UV irradiation (Fig. 4; and [32]).

All the changes reported above indicate a conformational change of the DNA at the borders of the CRP site (−70, −80 and −50) and suggest a more extensive deformation of the DNA around the CRP-wild-type RNA polymerase complex. This explanation is consistent with the increase in bending angle reported to take place in the ternary complex and interpreted in a similar way by Zinkel and Crothers [27]. Buckle et al. have also interpreted an increased reactivity at position −46 to singlet oxygen occuring only in a ternary active complex, as an increase in bending allowing better contacts between the two proteins [32].

α-C deleted RNA polymerases provide good controls for those suggestions. Indeed all the signatures of an active ternary complex are lost when these altered enzymes are substituted for wild-type RNA polymerase. In this case, all cooperativity of binding of the two proteins is suppressed. DNAaseI and copper phenanthroline footprints of both proteins are now separate suggesting that CRP and the truncated RNA polymerase do not share any significant contact on the DNA. The loss of a direct contact between CRP and the C-terminal part of an α-subunit is the most likely source of those differences. In this case, the contact in the CRP dimer should involve the exposed loop around residues 158−162 of the subunit positioned closer to the promoter start at *lac*UV5 since this is the location of the positive control mutations affecting type I promoters [5−7; and R.Ebright, unpublished results].

This proposal is further supported by the discovery of point mutations between residues 261 and 270 of the α-subunit which prevent CRP activation at *lac* ([39, and R.Ebright quoted in 41]. Point mutations are less likely than large deletions to cause substantial rearrangements in the ternary structure of the protein. Alternative (indirect) models are less probable than a direct contact model involving the C-terminal part of α and the 156−162 loop of CRP at *lac*. This loop is located on the three dimensional structure of the co-crystal at 15 Å from the −50 region of the promoter (cf. for example Fig. 2 in [7]). Hence, our proposal that the C-terminal domain of α should span from this CRP contact site to the −50 −40 region of the promoter where it could contact the DNA in the open complex.

At the *gal*P1 promoter, a member of type II promoters, both the wild-type and the α-truncated RNA polymerases bind cooperatively with the CRP protein with a synergistic ratio around 6−8. The truncated enzymes also show a shorter DNAaseI footprint than the wild-type enzyme and protection against hydroxyl-radical cleavage does not extend further upstream from position −42 (lower strand) (data not shown). As the CRP site is centered at −41.5 this still allows contacts of the truncated enzyme with the downstream subunit.

Another important feature of the *gal*P1 promoter is that upstream curved sequences differentially affect the kinetic step at which CRP accelerates the rate of formation of an open complex [40]. However, the overall increase in affinity of RNA polymerase for the *gal*P1 promoter due to CRP binding is roughly the same whether those sequences are present or absent. This point illustrates the limits of a purely static approach for delineating the operational contacts taking place between RNA polymerase and an activator at a given promoter. At type I promoters, the truncation of the C-terminal part of the α-subunit affects the modulation of 'on' rate constants by CRP; this is reflected in changes in cooperativity of binding of CRP and RNA polymerase. At type II promoters we can only conclude that the precise nature of the interactions between CRP and the truncated and wild-type polymerases, and their role in the activation mechanism, remain to be determined but that the C-terminal part of the α-subunit of RNA polymerase is not involved in this process.

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