Influence of the Location of the cAMP Receptor Protein Binding Site on the Geometry of a Transcriptional Activation Complex in *Escherichia coli*†

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ABSTRACT: The interactions between the cAMP receptor protein (CRP) and RNA polymerase during transcriptional activation at the *Escherichia coli malT* promoter have been analyzed using a combination of footprinting methods. We show that a closed complex is formed at this promoter in the absence of activator and that CRP merely stabilizes the open complex. The α-subunits of the RNA polymerase are involved in this effect as shown by $KMnO_4$ footprinting. The open complex formed in the presence of CRP is structurally identical to the one found at a CRP-independent promoter up-mutant. UV-laser footprinting yields distinct signals for the different protein−DNA interactions within the complex and for interactions between CRP and RNA polymerase. We monitor these signals in promoter variants that place the CRP binding site at different distances upstream of the start site of transcription. Signals within the core promoter region, as well as those located just upstream of the −35 hexamer, are unaffected by the position of the CRP binding site. Contacts of RNA polymerase with the upstream promoter region change in a mutant RNA polymerase containing a truncated α-subunit. We conclude that at least one of the α-subunits of RNA polymerase binds to DNA upstream of the −35 hexamer and that this interaction is unaffected by the position of the CRP binding site. We discuss models that account for the different activities of CRP in transcriptional activation as a function of promoter geometry.

A large number of *Escherichia coli* promoters are activated by the cAMP-CRP[1] complex (called CRP in this article). The molecular mechanism of activation has been intensely studied and represents a paradigm for the interactions between an activator and RNA polymerase (Kolb et al., 1993a; Busby & Ebright, 1994). Despite the numerous experiments performed with this model system, some of the molecular details of activation remain elusive. Transcriptional activation implies that the interactions between CRP and the RNA polymerase modify the properties of RNA polymerase in such a way as to increase the rate of transcriptional initiation. In this article, we investigate the details of RNA polymerase−promoter contacts and the modifications induced by CRP on these interactions using a combination of footprinting techniques.

One of the most intriguing features of transcriptional activation by CRP is the observation that CRP can activate transcription when placed at many different positions upstream of the transcription start site. The only obvious requirement appears to be a correct helical phasing between CRP and the RNA polymerase (Gaston et al., 1990; Ushida & Aiba, 1990).

Mostly on the basis of genetic criteria, CRP-activated promoters have been divided into two classes (Ebright, 1993). Class II promoters are characterized by a CRP binding site that overlaps the −35 hexamer of the promoter. The prototype of a class II promoter is the *galP1* promoter. Class I promoters possess a CRP binding site centered −61.5 base pairs from the start site of transcription or at integral helical turns further upstream. CRP-activated promoters with a CRP binding site centered at −50.5 have not been observed. By this definition, the class I promoters constitute a heterogeneous collection of promoters with the common feature that the CRP binding site is located on the same face of the double helix upstream of the promoter. The most intensely studied promoter of this type is the *lac* promoter with a CRP binding site centered at position −61.5 with respect to the start site of transcription. It is generally assumed that the mechanism of transcriptional activation is the same at all class I promoters (Zhou et al., 1994). In this article, we examine the structural basis of this assumption by analyzing the contacts within the activation complexes formed at the *malT* promoter (CRP binding site at −70.5) and at variants of this promoter placing the CRP binding site further upstream.

The understanding of the mechanism of CRP-dependent transcriptional activation has been considerably advanced in the past few years by the study of mutants of CRP or of RNA polymerase subunits that possess altered activation properties. Mutants of CRP that bind to the target site on the DNA, but fail to activate transcription (positive control or pc mutants), have been used to show that a particular region (activating region I, ARI) located at the surface of the known structure of the CRP dimer is responsible for activation at *lac*-type promoters (Bell et al., 1990; Eschenlauer & Reznikoff, 1991; Williams et al., 1991; Zhou et al., 1993). The same region is involved in CRP−RNA polymerase contacts at class II promoters, but two additional activating regions, ARII and ARIII, interact with RNA polymerase at these promoters (West et al., 1993; Attey et

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[1] Abbreviations: cAMP, cyclic adenosine 3′:5′-cyclic monophosphate; CRP, cAMP receptor protein; wt, wild type

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al., 1994; Williams et al., 1996).

Activating region I of CRP is thought to make direct protein−protein interactions with the RNA polymerase α-subunit (Ishihama, 1993; Chen et al., 1994; Tang et al., 1994; Murakami et al., 1996); mutants of the α-subunit that no longer respond to activation by CRP have been isolated (Igarashi & Ishihama, 1991; Zou et al., 1992). Similar experiments indicate that the C-terminal domain of the σ-subunit is involved in activation by CRP at the *gal* promoter (Kumar et al., 1994).

In order to understand the mechanism of transcriptional activation, it is imperative to know which step of transcriptional initiation is improved by the activator. The most clear-cut mechanism operates at the *lac* promoter, where CRP strengthens RNA polymerase binding to the promoter in the closed complex (increasing K_B) (Malan et al., 1984), thereby increasing promoter occupancy. The major effect of CRP at the *gal* promoter is acceleration of the rate of isomerization to the open complex (increase in k_2) (Herbert et al., 1986). The *malT* promoter of *E. coli* is an interesting example of a class I promoter; CRP acts mainly by accelerating promoter escape (Menendez et al., 1987). The molecular mechanism underlying this activity remains unknown.

The structural basis for the mechanism of transcriptional activation has been analyzed by various footprinting techniques, mostly for promoters where the CRP binding site is centered around position −41.5 and −61.5. Much less is known about promoters where the CRP binding site is located further upstream and direct protein−protein contacts may be more difficult to establish. Different footprinting reagents yield specific structural information about the complex. The occupancy and extent of interaction of the protein with the DNA can be assessed by DNase I (Brenowitz et al., 1986) or hydroxyl-radical footprinting (Dixon et al., 1991). Melted regions of the DNA are detected by OsO_4 or $KMnO_4$ (Sasse-Dwight & Gralla, 1991). Important phosphate contacts can be deduced by ethylation interference experiments (Wissmann & Hillen, 1991). We make extensive use of UV-laser footprinting because this technique can detect small distortions of the DNA and is therefore very sensitive to small structural changes within a nucleoprotein complex (Buckle et al., 1991; Engelhorn et al., 1995). By combining this information with signals from more conventional footprinting methods, we obtain a detailed structural characterization of the complex.

EXPERIMENTAL PROCEDURES

General Methods and Reagents. Standard methods of molecular biology were used unless otherwise specified (Sambrook et al., 1989). DNA oligonucleotides used as primers and for the construction of synthetic promoter sequences were synthesized on a Perkin-Elmer ABI 392 synthesizer. *E. coli* RNA polymerase holoenzyme was purchased from Sigma. Reconstituted wild type (wt) and α-235 RNA polymerase holoenzymes were prepared as described in Igarashi and Ishihama, 1991. CRP is purified according to a standard protocol (Ghosaini et al., 1988).

Plasmids. The plasmid carrying the *malTp1* promoter is constructed by the insertion into the polylinker of the pBluescriptII SK^+ vector (Stratagene) of an *Eco*RI/*Hind*III fragment that includes the original *malTp1* promoter sequence from −122 to +89 (Cole & Raibaud, 1986). For the footprinting experiments with the *malT* promoter, the *Sal*I/*Bam*HI fragment of plasmid pOM35 (Raibaud et al., 1991) has been recloned into the polylinker of pBluescriptII KS^+. The *malTp1* and *malT* variants that differ in the location of the CRP site were constructed as described in Déthiollaz et al. (1996). In order to restore the doublet of thymines that is present in the wt sequence 8.5 base pairs downstream of the 2-fold axis of the CRP binding site, a single site substitution was introduced by a mutagenic primer using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene). The detailed sequences of all constructions are shown in Déthiollaz et al. (1996). All plasmids were purified with the plasmid midiprep kit of Quiagen (in order to avoid any exposure of the DNA to UV before laser irradiation), and their sequence has been verified by dideoxy sequencing (Sanger et al., 1977).

Formation of Nucleoprotein Complexes. Open complexes were formed by incubation for 15 min at 37 °C of 5 nM of plasmid DNA with RNA polymerase (90 nM unless otherwise specified) in transcription buffer at pH 7.8 (20 mM Hepes, 150 mM potassium glutamate, 10 mM magnesium glutamate, and 1 mM EDTA) in a total volume of 20 μL. The KS^+*malT* plasmid was used supercoiled or linearized at the *Xho*I site, i.e., 134 base pairs upstream of the transcription start site. The SK^+*malTp1* plasmid was supercoiled or linearized at the *Xba*I site, i.e., 162 base pairs upstream of the transcription start site. The −80 group of promoter variants was probed only in the supercoiled conformation. When present, CRP was added to a final concentration of 75 nM in the presence of 200 μM cAMP. Nucleoprotein complexes formed with reconstituted RNA polymerase holoenzymes were obtained according to the same procedure but in a smaller volume (10 μL), with 10 nM plasmid DNA and 600 nM reconstituted RNA polymerase.

UV-Laser Footprinting. Nucleoprotein complexes (20 μL) were irradiated in 1.5 mL Eppendorf tubes placed in a water bath at 37 °C. The laser beam was generated by a Spectra-Physics Quanta-Ray GCR Series Nd:YAG laser at a wavelength of 266 nm. A single pulse of 5 ns was delivered onto each sample. After irradiation, the samples were precipitated with 3 volumes of 96% ethanol in a dry ice/ethanol bath. After centrifugation, the pellet was washed with 70% ethanol and resuspended in 10 μL of HE buffer [10 mM Hepes (pH 7.5) and 1 mM EDTA].

For primer extension analysis, these samples were denatured by boiling for 3 min and then chilled on ice for 5 min. Five microliters of a mix containing 150 mM Hepes (pH 7.5), 30 mM $Mg^{2+}SO_4$, 600 μM DTT, each dNTP at 600 μM, and 100 nM labeled primer was added. For the footprints of the entire promoter region, the primer used had the sequence 5′-AGTTAATCACTTCACTGTGG-3′ and hybridizes from +60 to +40; it was labeled at the 5'-end using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase (Biofinex). For the footprints around the CRP site, we used a primer with the sequence 5′-GCGCTTCGTAATTAATGGTT-3′ hybridizing to positions +15 to −4. For primer annealing, the samples were incubated for 3 min at 50 °C and then chilled on ice for 5 min. After addition of 1 unit of T7 DNA polymerase (Pharmacia), the extension reaction was carried out by incubation for 12 min at 37°C. The reaction was terminated by the addition of 3 volumes of 96% ethanol and precipitation

in a dry ice/ethanol bath. After centrifugation, the pellet was resuspended in 15 μL of loading buffer (96% formamide and 20 mM EDTA). One-half of each sample was denatured for 2 min at 100 °C and run on a 10% polyacrylamide denaturing gel in parallel with a sequencing reaction using the same primer.

The gels were fixed in a water/methanol/acetic acid bath (80:10:10), dried, and autoradiographed. Subsequently, they were exposed overnight in a BioRad GS250 Imaging Screen cassette BI. The cassette was scanned in a Molecular Imager, and profiles of each lane were obtained using the Phosphor Analyst program (Biorad). For a quantitative comparison of two lanes, the corresponding profiles were exported into Microcal Origin and superimposed. If necessary, as judged by the comparison of the intensity of reference bands, differences in the amount of material loaded were corrected by a small weight factor.

$KMnO_4$ Footprinting. The oxidation reaction was initiated by the addition of 2 μL of 100 mM $KMnO_4$ to the nucleoprotein complexes (20 μL), prepared as indicated above except that the RNA polymerase concentration was 180 nM. For the footprints with reconstituted RNA polymerase holoenzymes, 10 nM linearized plasmid DNA and 600 nM RNA polymerase were mixed in a total volume of 10 μL; CRP when present was at a concentration of 100 nM. After incubation for 2 min at room temperature, the reaction was stopped by the addition of 2 μL of β-mercaptoethanol. The samples were then precipitated with 3 volumes of 96% ethanol, resuspended in 10 μL of H_2O, and passed through a Sephadex G-25 (Pharmacia) spin column equilibrated with H_2O. The primer extension procedure was identical, as described for the irradiated complexes.

DNase I Footprinting. One microliter of DNase I (FPLC pure, Pharmacia) at a concentration of 1 ng/μL in dilution buffer [10 mM Tris (pH 7.6), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 10% glycerol] was added to the nucleoprotein complexes (20 μL), and the incubation was continued for 1 min at 37 °C. The reaction was stopped by the addition of 80 μL of stop mix [20 mM Tris (pH 7.8), 20 mM EDTA, 250 mM NaCl, 0.5% SDS, and 10 ng/μL sonicated salmon sperm DNA]. After precipitation with 3 volumes of 96% ethanol and 1 μg of pBluescriptII SK^+ carrier DNA, the samples were submitted to a primer extension as described for the $KMnO_4$ and UV-laser footprinting reactions.

In Vitro Runoff Transcriptions. The fragment carrying the −81.5*malT* promoter variant was obtained by a *Hin*fI digestion of the SK^+−81.5*malT* plasmid (Déthiollaz et al., 1996). The fragment carrying the wt promoter was a *Bam*HI/*Hin*dIII digestion product from plasmid $KS^+\Delta$5*malT* (Déthiollaz et al., 1996) that includes the original *malT* sequence from −89 to +90. The fragments were recovered from a native polyacrylamide gel by cutting out the desired band, followed by electroelution of the DNA. The DNA was precipitated, resuspended in H_2O, and passed through a Sephadex G-25 spin column. The DNA concentration was determined spectrophotometrically. The *in vitro* run off transcriptions were performed in a total volume of 20 μL in transcription buffer. The reaction mixtures contained 10 nM −81.5*malT* fragment or 7.5 nM wt *malT* fragment. The nucleotide concentrations were 200 μM for ATP, CTP, and GTP, 20 μM for UTP, 70 nM for [α-^{32}P]UTP (3000 Ci/mmol, NEN Dupont), and 200 μM for cAMP. When present, CRP was at a concentration of 100 nM (−81.5*malT* fragment) or 75 nM (wt fragment). The samples were preincubated for 10 min at 37 °C. The transcription reaction was initiated by the addition of RNA polymerase to a final concentration of 30 nM (−81.5*malT* fragment) or 20 nM (wt fragment). The reaction was terminated after 5 min of incubation by the addition of 60 μL of 96% ethanol and precipitation in a dry ice/ethanol bath. After centrifugation, the pellet was resuspended in 15 μL of 96% formamide and 20 mM EDTA. Seven microliters of each sample was heat-denatured for 3 min at 85 °C and loaded onto a 10% denaturing polyacrylamide gel. The transcripts were 70 nucleotides for the −81.5 variant and 104 nucleotides for the wt promoter.

RESULTS

Structure of the Open Complex at the malTp1 Promoter by UV-Laser Footprinting. The *malT* promoter of *E. coli* is a very weak promoter [only a 2/6 and 3/6 match with the consensus sequences of the −10 and −35 hexamers, respectively (Hawley & McClure, 1983)], and structural information is difficult to obtain. In order to establish a reference for the UV-laser footprinting signals of an open complex, we probed the *malTp1* promoter, an up-mutant that changes the guanine at −12 into thymine and thereby brings the −10 hexamer closer to the consensus sequence (Chapon, 1982). We then verified that an identical structure is formed at the wt promoter, albeit with lower efficiency.

We formed open complexes *in vitro* by incubating plasmid DNA carrying the *malTp1* promoter with RNA polymerase for 15 min at 37 °C. The samples were irradiated with a 5 ns pulse of 266 nm UV laser light. The energy of one laser pulse is greater than 30 mJ, i.e., in excess of 70 nmol of photons. The number of absorbing molecules is at least one order of magnitude lower than the number of photons delivered, thus ensuring that all bases are excited by the laser pulse. The DNA is recovered by precipitation, denatured, and submitted to a primer extension using T7 DNA polymerase (Buckle et al., 1991). Virtually every base can undergo a photoreaction as can be seen in Figure 1. No photoreaction has occurred in nonirradiated DNA (lane 1) and the T7 DNA polymerase extends the radiolabeled primer to the end of the DNA fragment. Lanes 2 and 3 show the primer extension profiles of DNA irradiated in the absence of protein. The bands correspond to elongation arrests of T7 DNA polymerase at photomodified bases. The nature of the photoreactions remains uncharacterized in most cases. However, this does not limit the usefulness of UV light for footprinting because the signal is constituted by the *change* in photoreactivity upon addition of the protein, irrespective of the nature of the photoreaction.

One of the most prominent and best characterized photoreactions is the formation of pyrimidine dimers (Franklin et al., 1985). However, the photoreactivity is not determined exclusively by the sequence of base pair doublets but rather by a larger sequence context. Not all doublets of thymine (doublets of adenines in the sequencing reaction shown in the figure) react to the same extent; their reactivity is modulated by the surrounding sequence (see for example positions +4, -6, −19, −34, or −60).

When RNA polymerase forms an open complex at the *malTp1* promoter (Figure 1b), we observe the following major modifications of the primer extension pattern. The

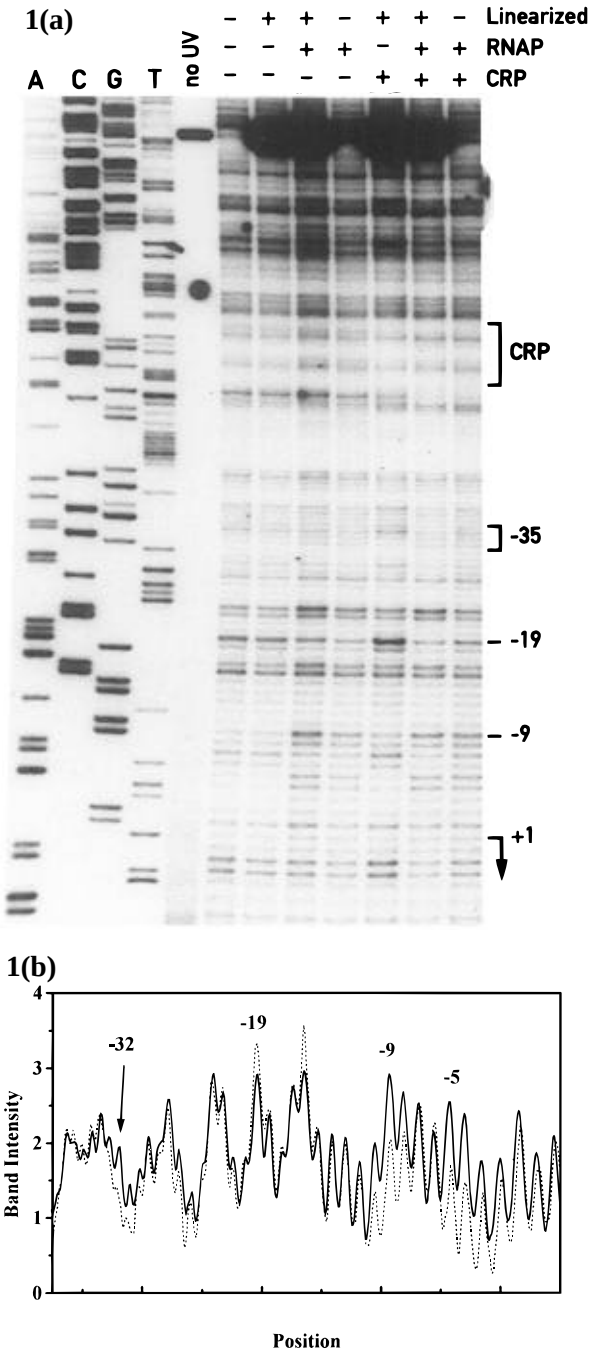


FIGURE 1: (a) Primer extensions on UV-laser-irradiated nucleoprotein complexes at the *malTp1* promoter. The T7 DNA polymerase synthetizes the bottom strand, thus revealing the modifications induced on the top strand. A sequencing reaction using the same primer is shown on the left side. The positions of the CRP binding site and of the -35 hexamer are indicated with brackets on the side of the gel. The transcription start site is indicated by a broken arrow. The plasmid DNA in lanes 3, 4, 6, and 7 has been linearized at the *Xba*I site. RNA polymerase is present in lanes 4, 5, 7, and 8, CRP in lanes 6−8. The sample in lane 1 has not been irradiated. (b) Superposition of the Phosphor Imager profiles obtained for lanes 2 and 8 of Figure 1a: DNA alone (dotted line) and DNA + CRP + RNA polymerase (light line).

intensity of bands at positions −4, −5, −8, −9, −10, and −32 increases and the band at position −19 decreases in intensity. These signals (except the −19 band) arise in regions where the RNA polymerase (particularly the σ-subunit) is in close contact with the DNA (Dombroski et al., 1992). It is likely that the profound modification of the photoreactivity from −10 to −4 results from the melting of

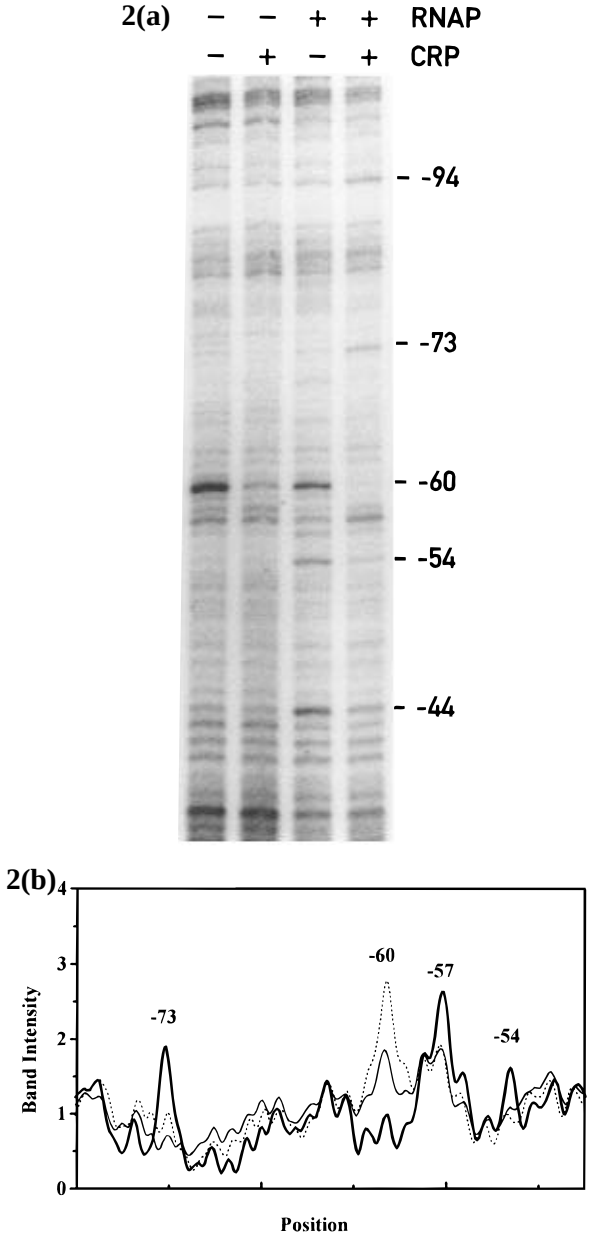


FIGURE 2: (a) UV-laser footprints of nucleoprotein complexes at the CRP site of the *malTp1* promoter. The primer used for the extension hybridizes from +15 up to -4 on the top strand. The DNA is linearized. CRP is added in lanes 2 and 4, and RNA polymerase in lanes 3 and 4. (b) Superposition of the Phosphor Imager profiles obtained for lanes 1, 2, and 4 of Figure 2a: DNA alone (dotted line), CRP (light line), and DNA + CRP + RNA polymerase (heavy line).

the DNA in this region, characteristic of the formation of the open complex (see below). Signals further upstream, at positions −44 and −54 (Figure 2a, lane 3), show that the RNA polymerase covers a large region of DNA [probably via the α-subunit(s) in this upstream region].

The changes induced by binding of CRP to its recognition site map to the boundaries of the CRP binding site (Figure 2a, lane 2). The major change is a 2-fold decrease in intensity of the band at −60. Bases −61 and −62 on the template strand of the primer extension are thymines. The signal is therefore most likely due to the formation of a thymine dimer. When both proteins are present on the DNA, we observe a superposition of the two separate patterns, except at certain positions in the region upstream of the promoter (Figure 2a, lane 4). The band at −60 disappears

completely whereas new bands appear at positions −57, −73, and −94 (Figure 2b). Since these UV-footprinting signals are present only in the ternary complex, they must be the consequence of an interaction (direct or indirect) between the two proteins (see Discussion). In contrast, the contacts of RNA polymerase with the region just upstream of the core promoter (signals at −44 and −54) are not (or very little) affected by CRP.

Structure of the Open Complex at the malT Promoter by UV-Laser Footprinting. The wt *malT* promoter possesses a very low basal transcriptional activity but is activated several hundred-fold by CRP (Déthiollaz et al., 1996). As expected, the footprinting patterns of the DNA alone are identical for the *malT* (Figure 3a, lanes 2 and 3) and *malTp1* (Figure 1) promoters, except at position −12, the site of the base substitution. The modifications of photoreactivity caused by the ternary complex (Figure 3b) are identical for both promoters, i.e., increases at −4, −5, −9, −10, and −32 and a decrease at −19. In the absence of CRP, however, most of these signals are lost. The scans of the lanes representing the RNA polymerase−promoter complex and the promoter alone superimpose exactly in the region downstream of position −18. RNA polymerase is clearly incapable of forming a stable open complex at the *malT* promoter in the absence of the activator. However, the signals at −32 and −19 are conserved. We interpret the data to mean that RNA polymerase can form a closed complex at the wt *malT* promoter but needs the activator to stabilize an open complex. In the upstream region, all signals that were observed in the ternary complex at the *malTp1* promoter (increases at −44, −54, −57, −73, and −94 and a decrease at −60) are conserved at *malT*. Independent of the particular interpretation, we can conclude that CRP stabilizes a structure at the *malT* promoter that is identical to the structure of the open complex formed at the *malTp1* promoter. Neither supercoiling nor an increase in the RNA polymerase concentration drives the reaction toward the formation of the open complex at *malT* in the absence of CRP (Figure 3a, lanes 6 and 7).

DNA Melting at the malTp1 and malT Promoters. In order to confirm the identity of the open complexes detected by UV-laser footprinting, we investigated the extent of DNA melting in the different complexes using $KMnO_4$. This compound reacts specifically with thymine residues in single-stranded regions of DNA. Figure 4 shows that in both promoters the region from −10 to +3 is melted in complexes that we termed "open complexes" in the previous paragraphs. The nucleotides at positions −9 and +1 possess the strongest reactivity toward oxidation by $KMnO_4$. These experiments confirm directly the interpretation of the UV-laser footprints. CRP is dispensable for the stabilization of the open complex at the *malTp1* promoter (Figure 4a, lanes 2 and 3) but is required at the *malT* promoter (lanes 5 and 6). The extent of DNA opening at the *malTp1* promoter is greater than that at the *malT* promoter (compare the intensity of the signals at −9 and at +1 for the two promoters). A small amount of DNA opening can be observed at the *malT* promoter even in the absence of activator (Figure 4b).

$KMnO_4$ Footprints with α-235 RNA Polymerase. The C-terminal domain of the α-subunit of the RNA polymerase makes direct contacts with a specific region of CRP at certain promoters (Ishihama, 1993). Truncated versions of the α-subunit that lack the domain of interaction with CRP have been characterized. These C-terminally truncated α-subunits can be used for reconstitution of a holoenzyme competent for transcription (Igarashi & Ishihama, 1991). The reconstituted RNA polymerases, wt or α-truncated, are slightly less active than the purified holoenzyme, and higher concentrations have to be used for footprinting experiments

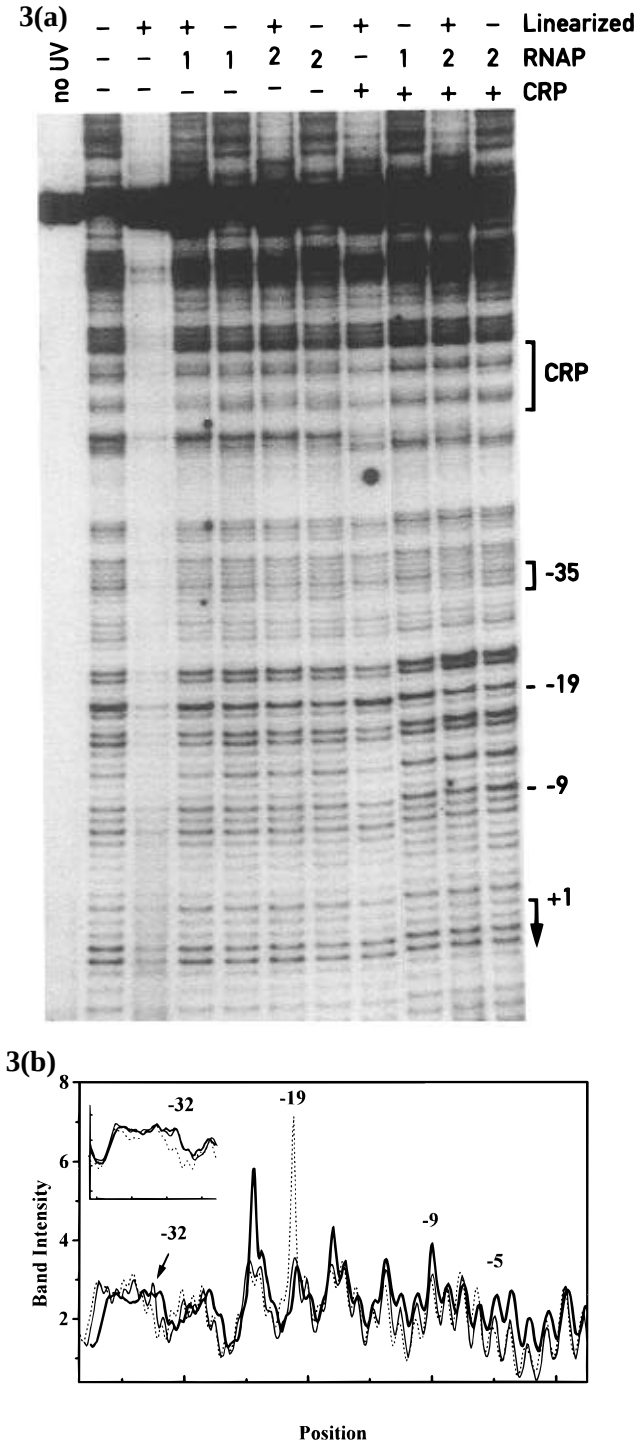
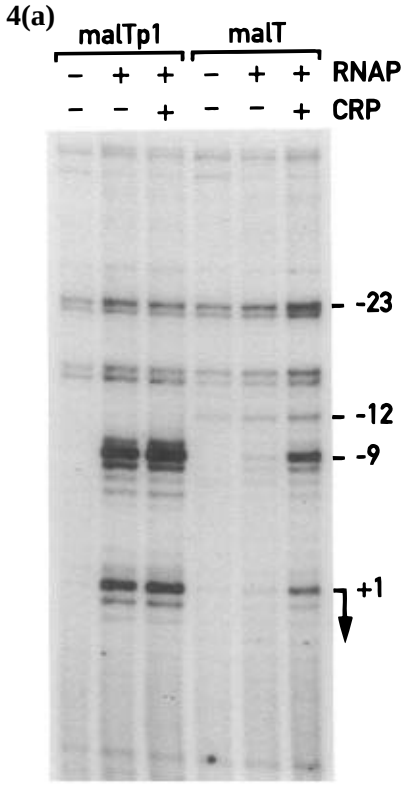


FIGURE 3: (a) UV-laser footprints of nucleoprotein complexes at the *malT* promoter. The plasmid has been linearized at the *Xho*I site in lanes 1, 3, 4, 6, 8, and 10. The RNA polymerase concentration is 90 nM in lanes 4, 5, and 9, and 180 nM in lanes 6, 7, 10, and 11. CRP is present in lanes 8−11. The sample in lane 1 has not been irradiated. (b) Superposition of the Phosphor Imager profiles obtained for lanes 2, 7, and 11 of Figure 3a: DNA alone (dotted line), DNA + RNA polymerase (light line), and DNA + CRP + RNA polymerase (heavy line). The scans around position −32 have been realigned (inset).



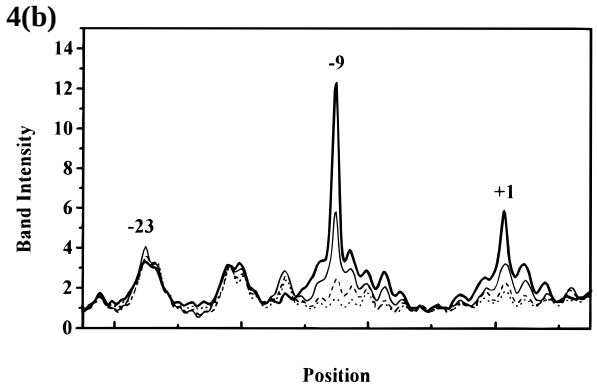


FIGURE 4: (a) $KMnO_4$ footprints of open promoter complexes at the *malT* and *malTp1* promoters on linearized DNA: lanes 1-3, *malTp1* promoter (5 nM); and lanes 4−6, *malT* promoter (5 nM). Samples in lanes 2, 3, 5, and 6 are supplemented with 180 nM RNA polymerase. CRP (75 nM) is present in lanes 3 and 6. The band at −23 can be used as a reference; the band at −12 indicates the position of the substitution. (b) Superposition of the Phosphor Imager profiles for lanes 2 and 4−6 of Figure 4a: DNA alone, *malT* promoter (dotted line); DNA + RNA polymerase, *malT* promoter (dashed line); DNA + CRP + RNA polymerase, *malT* promoter (light line); and DNA + RNA polymerase, *malTp1* promoter (heavy line).

(Ross et al., 1993). The reconstituted wt RNA polymerase induces a similar pattern in response to oxidation by $KMnO_4$ as does the native wt RNA polymerase (Figure 5, lanes 2 and 3). When we use the α-235 RNA polymerase, no open complex is formed at the *malT* promoter (lanes 4 and 5). The stabilizing effect of CRP is lost, suggesting that the α-subunits are involved in the CRP-mediated activation, most likely by a direct interaction. Both polymerases lead to open complex formation at the CRP-independent *malTp1* promoter (lanes 6 and 7).

DNase I Footprints at the malT and malTp1 Promoters. In order to precisely localize the two proteins on the promoter, we performed DNase I footprinting experiments.

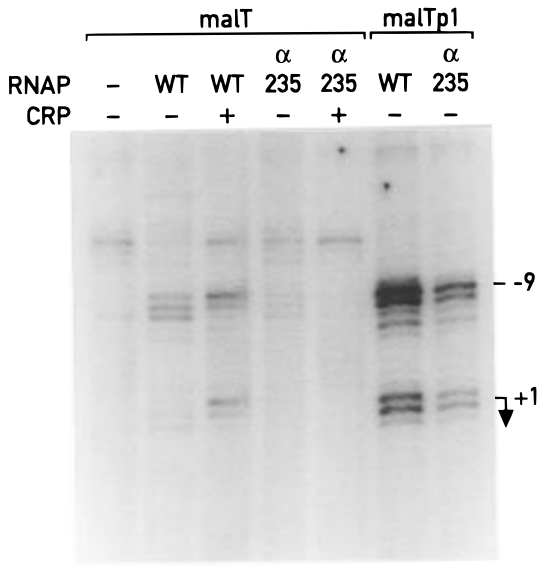


FIGURE 5: $KMnO_4$ footprints with reconstituted RNA polymerases at the *malT* and *malTp1* promoters: lanes 1−5, *malT* promoter (10 nM); and lanes 6 and 7, *malTp1* promoter (10 nM). Samples are supplemented with reconstituted wt RNA polymerase (600 nM) in lanes 2, 3, and 6 or reconstituted α-235 RNA polymerase (600 nM) in lanes 4, 5, and 7. CRP (100 nM) is present in lanes 3 and 5.

Open complexes were formed under the same conditions as in the previous experiments, i.e., using the plasmid-borne promoter. As a consequence, we could not label the DNA directly, and contrary to the most common way of performing DNase I footprint analysis, the DNase I digestion products were identified by primer extension.

At the *malTp1* promoter, the region from −44 up to at least +10 is efficiently protected by RNA polymerase (Figure 6, lane 2 compared to lane 1). The precise upper and lower limits of the footprint are difficult to establish because they arise in regions [poly(A) tracts] that are almost insensitive to DNase I even in the absence of protein (Drew & Travers, 1984). In the presence of CRP (lane 3), the pattern of RNA polymerase protection remains unmodified, with the notable exception of the appearance of hyperreactive bands at −54 and −65.

At the *malT* promoter, the degree of protection is significantly lower, especially with RNA polymerase alone (lane 6 compared to lane 4). Despite the quantitative difference between *malT* and *malTp1*, qualitatively, the patterns are identical in the presence of the activator, suggesting that the structure of the transcription complex is the same for both promoters. There is a good correlation between the amount of open complex formed, as judged by $KMnO_4$, and the extent of protection against DNase I.

DNase I Footprints with α-235 RNA Polymerase. It has been proposed that CRP correctly positions the α-subunit on the DNA and thereby activates transcription (Busby & Ebright, 1994). This model can be verified by determining the location of the α-subunits at the *malTp1* promoter and at variants of the promoter that move the CRP binding site further upstream or downstream. We first determined the location of the α-subunits by comparing the protection pattern obtained with the α-235 RNA polymerase (Figure 7a, lanes 3 and 4) to the one characteristic of the reconstituted wt polymerase at the *malTp1* promoter (lanes 1 and 2). The footprints of the native RNA polymerase and of the

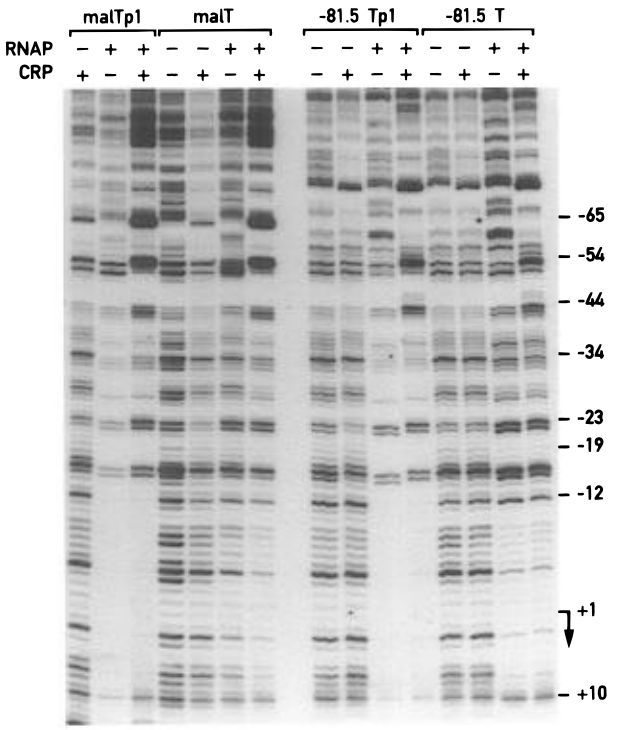
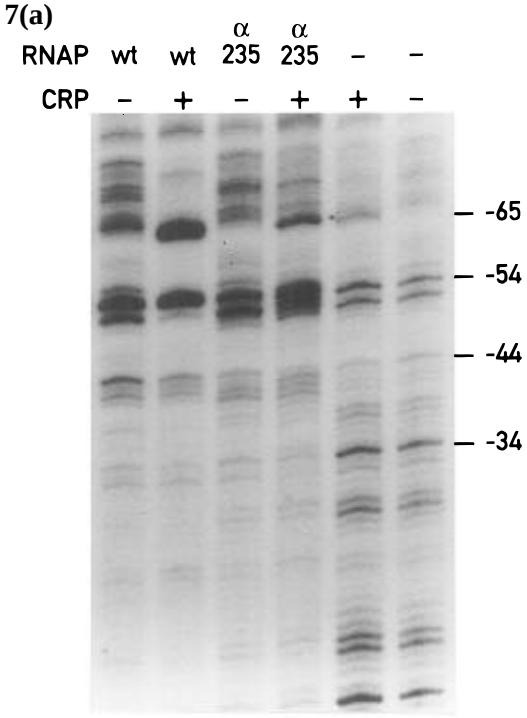


FIGURE 6: DNase I footprinting reactions: Lanes 1−3, *malTp1* promoter; lanes 4−7, *malT* promoter; lanes 8−11, *malTp1* promoter variant with a CRP site centered at −81.5; and lanes 12−15, *malT* promoter variant with a CRP site centered at −81.5. The concentration of DNA in each sample is 5 nM. The RNA polymerase concentration is 120 nM in lanes 2, 3, 6, 7, 10, 11, 14, and 15. The CRP concentration is 75 nM in lanes 1, 3, 5, 7, 9, 11, 13, and 15.

reconstituted wt RNA polymerase are identical. Using the α-235 RNA polymerase, we obtain efficient protection downstream of base pair −37 but we lose protection between positions −38 and −44 (Figure 7b). The most straightforward interpretation of this result agrees with earlier findings for other promoters (Kolb et al., 1993b; Ross et al., 1993; Landini & Volkert, 1995) and indicates that the α-subunit of RNA polymerase binds to the DNA directly upstream of the −35 hexamer. The upper limit of the α-subunit binding site cannot be precisely mapped because of the low reactivity upstream of −44. However, since the hyperreactivity at −54 is lost as a result of the truncation of the α-subunit, we conclude that the binding site probably extends to or beyond this position. The UV signals that were previously identified at −44 and −54 are therefore most likely due to the binding of the α-subunit(s) to this segment of DNA.

Even though the protection pattern between positions −40 and −54 is altered slightly when CRP is added to the binary complex containing the truncated version of RNA polymerase, the two hyperreactive bands at −54 and −65 no longer appear. Loss of these hypersensitive sites indicates that the interaction between CRP and RNA polymerase is lost or that the nature of the interaction is different (see Discussion).

Positioning of the RNA Polymerase on the Promoter when the CRP Binding Site Is Moved. Does the α-subunit(s) maintain its contact with CRP, or does it remain bound to the DNA site upstream of −35 when the CRP binding site is moved? We have functionally characterized a series of *malT* and *malTp1* promoter variants where the CRP binding site is centered around −60, −70, and −80. Peaks of maximal transcriptional activation occur *in vivo* when CRP



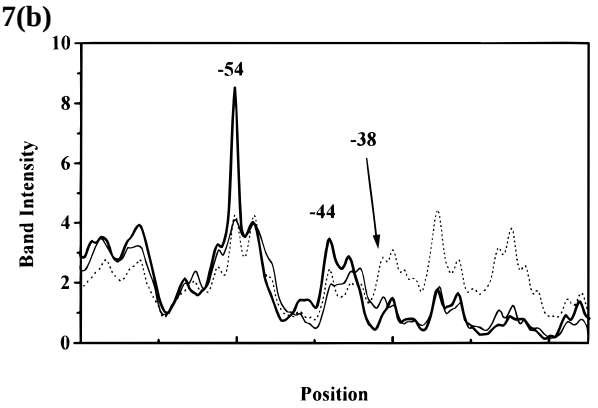


FIGURE 7: (a) DNase I footprints with reconstituted RNA polymerase holoenzymes at the *malTp1* promoter: lanes 1 and 2, reconstituted wt RNA polymerase (600 nM); and lanes 3 and 4, reconstituted α-235 RNA polymerase (600 nM). CRP (75 nM) is added in lanes 2, 4, and 5. (b) Superposition of the Phosphor Imager profiles for lanes 1, 3, and 6 of Figure 7a: DNA alone (dotted line), DNA + reconstituted α-235 RNA polymerase (light line), and DNA + reconstituted wt RNA polymerase (heavy line).

is bound at positions −60.5, −71.5 and −81.5 (Déthiollaz et al., 1996). Runoff transcriptional analysis confirms the CRP-mediated transcriptional activation *in vitro* for the wt promoter and the −81.5 variant (Figure 8). We focus our attention on the −80 variants since the potential binding site for the α-subunit(s) is modified in the −60 and −70 variants.

Displacement of the CRP binding site does not result in the appearance of new characteristic signals on the UV-laser footprints (Figure 9a). Introduction of additional nucleotides in the sequence does, of course, change the photoreactivity pattern in the region where the substitution has occurred, but the presence of the RNA polymerase and/or CRP does not further modify the basal reactivity noted for the DNA alone. Consequently, we focus on the signals already described for the wt promoter at −60, −57, −54, and −44.

At the wt promoter, the disappearance of the UV signal at −60 is testifying to an interaction between CRP and the

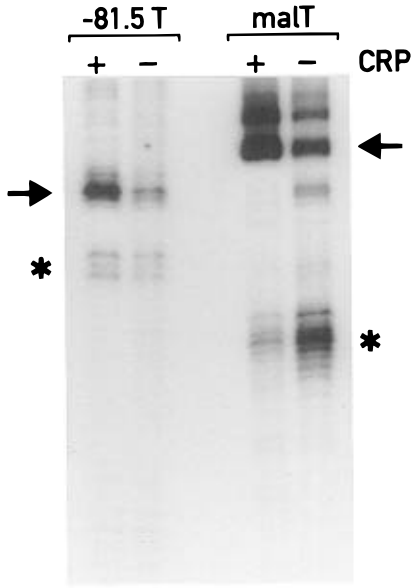


FIGURE 8: *In vitro* runoff transcriptions at the wt *malT* promoter and at the −81.5 *malT* variant. The respective transcripts are identified by the arrows. Transcription is carried out in the presence or absence of CRP as indicated. The lanes can be normalized with respect to the second CRP-independent weaker transcript indicated by an asterisk. The different concentrations are as indicated in Experimental Procedures.

α-subunit of the RNA polymerase. This modification of photoreactivity is no longer observed on a UV-laser footprint of the *malTp1* promoter in the presence of an α-235 RNA polymerase (data not shown). An identical photoreactivity pattern has been described for the *lac* promoter (Kolb et al., 1993b), at the same position with respect to the CRP binding site. What is the fate of this signal when the CRP site is moved further upstream? Since the signal at −60 is directly related to the location of the CRP binding site, it should move in parallel with the displacement of the center of the CRP site. It is indeed always observed 10.5 base pairs downstream of the 2-fold axis of the CRP binding site, i.e., at −69, −71, and −73 for the −79.5, −81.5, and −83.5 variants, respectively (Figure 9a). The effect induced by CRP alone on this signal is the same for the wt promoter and for the variants, i.e., a 2-fold decrease in intensity (Figure 9b). However, the complete disappearance of the band, characteristic of the ternary complex, is no longer observed in the −80 group of promoter variants. The secondary signal at −68 (lane 8) is expected to be the counterpart of the signal at −57 at the wt promoter. However, whereas the signal is present in the ternary complex at the wt promoter, it is absent at the −81.5 variant (compare Figures 2b and 9b). Even though the transcriptional activation persists at the −81.5 variant *in vitro* and *in vivo*, the geometry of this complex is different from those found at the *lac* and wt *malT* promoters (see Discussion for possible explanations).

The signals at −44 and −54 are conserved irrespective of the presence or absence of the activator or the location of the CRP binding site (Figure 9a). We attribute these signals to the interaction of the α-subunit(s) with DNA (see above). From the present data, it is impossible to decide whether only one or both of the α-subunits are bound to the DNA. Nevertheless, at least one of the α-subunits remains bound to the DNA upstream of the −35 hexamer for all positions of the CRP binding site.

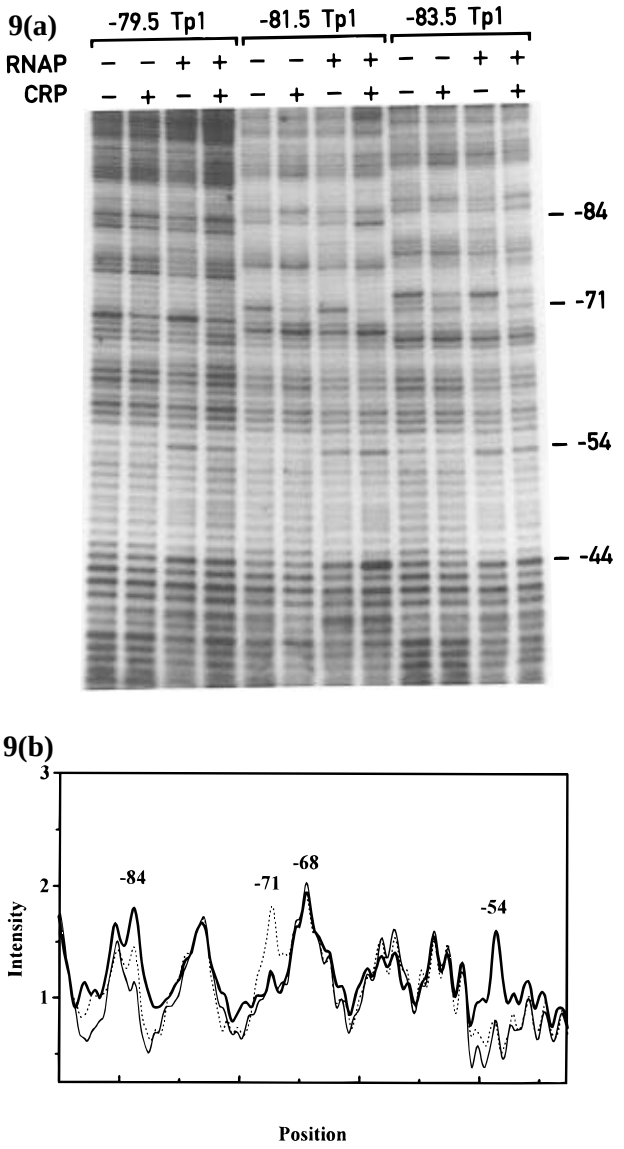


FIGURE 9: (a) UV-laser footprints at *malTp1* promoter variants with a CRP site located at positions −79.5, −81.5, and −83.5. DNA (supercoiled), RNA polymerase, and CRP concentrations are identical to the former UV-laser footprints. CRP is present in lanes 2, 4, 6, 8, 10, and 12. RNA polymerase is present in lanes 3, 4, 7, 8, 11, and 12. (b) Superposition of the Phosphor Imager profiles obtained for lanes 5, 6, and 8 of Figure 9a: DNA alone (dotted line), CRP (light line), and DNA + CRP + RNA polymerase (heavy line).

A band at −84, the counterpart of the signal at −73 of the wt promoter, is observed at the −81.5 promoter variant. It is characteristic of the ternary complex and demonstrates that the interaction with the RNA polymerase affects the DNA conformation within the upstream end of the CRP binding site. It is interesting to note that this upstream interaction is conserved, whereas the interaction downstream of the CRP binding site is lost.

Similar conclusions can be drawn from DNase I footprinting experiments (Figure 6). For the −81.5 promoter variant, no extension of the RNA polymerase protection pattern is observed in the presence of CRP. This argues against any large scale repositioning of the α-subunit mediated by the activator. Some minor changes of the digestion pattern are, however, induced by CRP (lanes 11 and 15). For instance, we observe a strong increase in the reactivity of the −44 band and the appearance of two additional bands at −46

and −47. A strong hyperreactivity appears at −76 in the ternary complex, the counterpart of the −65 hyperreactivity at the wt promoter. Taken together, the data strongly suggest that the α-subunit is not repositioned on the DNA when the CRP binding site is moved. An interaction, direct or indirect, is however maintained between the two proteins.

DISCUSSION

In the past few years, genetic and biochemical experiments have identified a surface-exposed loop of CRP (ARI) that is involved in transcriptional activation. Its target on the RNA polymerase is the C-terminal domain of the α-subunit (Ishihama, 1993). Only one of the two α-subunits is suggested to be responsible for the interaction with CRP (Zou et al., 1994). The consequences of this interaction for the structure of the initiating RNA polymerase, however, remain unknown. We have analyzed the structural changes provoked by the activator on transcriptional complexes at variants of the *malT* promoter. We heavily rely on UV-laser footprinting because the ensuing photoreactions are complete within microseconds, whereas typical rearrangement of a nucleoprotein complex would proceed on the millisecond time scale (Hockensmith et al., 1991). The structural signals thus obtained therefore represent a true frozen equilibrium.

Contacts within the Transcriptional Activation Complex. The possible interactions that could lead to transcriptional activation in our system are (i) direct protein−protein contacts, (ii) conformational changes transmitted by the DNA (Ryu et al., 1994), and (iii) additional contacts with other DNA segments such as an interaction between the DNA upstream of the CRP site and the back side of the RNA polymerase (Schultz et al., 1991; Déthiollaz et al., 1996). The first type of interaction has no direct consequences for the photoreactivity of DNA following UV irradiation. However, indirect effects could certainly be observed due to distortions of the DNA located between the interaction partners. The second and third types of interactions directly involve the DNA molecule and can be detected by UV-laser footprinting.

A series of signals characteristic of the ternary complex formed by the DNA, CRP, and the RNA polymerase at the *malT* and *malTp1* promoters have been identified. We group these signals into four categories. (i) The signals from −4 to −10 and at −32 correspond to the interaction of RNA polymerase with the −10 and −35 hexamers; our data clearly show that CRP acts at the *malT* promoter by stabilizing the open complex. (ii) the signal at −19 arises probably from an untwisting of the linker DNA (deHaseth & Helmann, 1995) when RNA polymerase is bound to the −10 and −35 hexamers (forming a closed or open complex); this signal is due to the formation of a thymine dimer and is therefore expected to be very sensitive to local variations in DNA geometry (Pehrson & Cohen, 1992). (iii) the signals at −44 and −54 are most likely caused by the binding of the α-subunit(s) of RNA polymerase to DNA upstream of the −35 hexamer. (iv) The signals at −57, −60, −73, and −94 appear only in the ternary complex and not in the binary complexes, they reflect therefore the interactions (direct and/or indirect) between CRP and the RNA polymerase.

We have mapped the binding site for the α-subunit at the *malT* promoter by comparing DNase I footprints of the wt holoenzyme and RNA polymerase containing a truncated α-subunit. The mutant RNA polymerase shows protection only downstream of position −37, whereas the footprint of wt enzyme extends at least to −54 (when taking into account sites of DNase I hypersensitivity). The region covered by the α-subunit is very AT-rich, which is consistent with the base composition of the previously described binding sites for the α-subunit (Ross et al., 1993). The DNase I hypersensitivity at −54 suggests a DNA bend toward the protein, thereby widening the minor groove that is facing toward the outside of the bend (Brukner et al., 1995). The UV-laser signals in the same region (−44 and −54) are a consequence of binding of the α-subunit(s) to DNA and could be due to a crosslink between the α-subunits and DNA.

The UV-laser footprinting band at −60 of the *malTp1* promoter disappears in the ternary complex with the wt RNA polymerase (Figure 2) but persists with the α-235 mutant enzyme (data not shown). A similar observation has been made for the *lac* promoter (Kolb et al., 1993b) which also possesses a doublet of thymines at the same distance from the CRP binding site (position −49 in this case). The revised crystal structure of CRP (Parkinson et al., 1996) shows a secondary kink in the DNA at this position. An increase of the kinking angle in the ternary complex, in a direction unfavorable for the formation of a thymine dimer, would explain the disappearance of the band. At both promoters, the signal depends on an intact α-subunit which has previously been taken as evidence for a direct protein-protein contact. We therefore propose that very similar protein−protein contacts are established at the *lac* and *malT* promoters.

When the CRP binding site is moved one helical turn further upstream (−81.5 promoter variant), we no longer observe the decrease in band intensity of the characteristic UV-footprinting band in the ternary complex. The direct interaction between the α-subunit and CRP may be no longer possible at this distance. Alternatively, an interaction of a different nature (not detectable by footprinting) may persist. A recent model (Zhou et al., 1994) proposed an explanation for the maintenance of the interaction between CRP and the RNA polymerase when the CRP site is moved. It is motivated by the observation that the α-subunit of the RNA polymerase is composed of two domains: a C-terminal domain that interacts with the activator and an amino-terminal domain that interacts with the large subunits of RNA polymerase. The two domains are connected via a flexible linker of at least 13 amino acids. When the CRP site is moved further upstream, the interaction with the activator could be preserved by way of a more extended conformation of the linker. According to this model, the signals at −44 and −54, landmarks for the binding of the α-subunit(s) to DNA, should either disappear or be displaced when the CRP site is moved. This is clearly not the case; at least one of the α-subunits remains bound to this region of the DNA.

Additional changes of the photoreactivity upstream of the CRP binding site (−73 and −94 at the *malT* promoter) suggest that a second type of interaction may be formed in the ternary activation complex. Binding of the far upstream DNA to the back side of RNA polymerase could explain both signals. This more compact conformation would increase DNA bending around CRP (presumably provoking a stronger reactivity of the base at −73) and lead to a conformational change of the DNA around −94 (indirectly

or directly via a contact with RNA polymerase). Evidence for the involvement of the upstream DNA had already been obtained at the *lac* promoter by singlet oxygen footprinting (Buckle et al., 1992) and was deduced from the effect of removal of this DNA on *in vitro* transcriptional activation (Déthiollaz et al., 1996). The increased DNA bending in such a ternary complex explains the UV-laser signal at −60, located at a secondary kink in the DNA (Parkinson et al., 1996), as well as the compaction of the ternary complex (Zinkel & Crothers, 1991) relative to the RNA polymerase−promoter complex. CRP has recently been shown to favor the binding of *lac* repressor to the operator by stabilizing secondary interactions of the repressor with nonspecific DNA, structurally analogous to our proposed back side contacts with RNA polymerase (Vossen et al., 1996). Interestingly, the ternary complex at the −81.5 variant is also characterized by an increase in the intensity of the signal at −84, the counterpart of the signal at −73 at the wt promoter. Signals further upstream are not observed; however, the sequences in this region (and therefore the potential photoreactivity) are different, and no comparison can be made. This type of interaction is largely independent of the location of the CRP binding site and easily explains almost equal transcriptional activation of the −70.5 and the −81.5 promoters.

Possible Mechanisms of Transcriptional Activation. Can a unique structure and mechanism account for transcriptional activation by CRP, despite the different promoter geometries? A direct contact between the α-subunit(s) and CRP certainly exists at the *lac* promoter. We observe the equivalent footprinting signals at *malT*. The mode of interaction is therefore most likely the same even though different α-subunits (and/or different kinetic steps) may be involved at the two promoters. Additional far upstream interactions may also participate in activation (Figure 10a).

Two different models could account for the transcriptional activation at the −81.5 promoter variant. If transcriptional activation remains due to a direct contact between the α-subunits and CRP, at least one of the α-subunits has to move when the CRP binding site is relocated. Our data, however, show that the binding of the α-subunit(s) to the DNA is unaffected by the location of the CRP binding site. Possibly one of the two α-subunits remains bound to the DNA upstream of the −35 hexamer, whereas the other subunit makes a contact with ARI of CRP (Figure 10b). The regions within the C-terminal domain of a single α-subunit that are responsible for DNA binding and transcription activation overlap (Jeon et al., 1995) or are very close (Gaal et al., 1996). Indeed, CRP and the UP element of the *rrnB*P1 promoter contact the same surface of the α-subunit (Murakami et al., 1996). Assigning different roles to the two α-subunits avoids the incompatibility caused by the large spatial separation of the corresponding interaction domains: ARI is relatively far from the DNA, whereas the interaction patch on the α-subunit is very close to the DNA.

Alternatively, both α-subunits could remain bound to the DNA upstream of the −35 region irrespective of the location of the CRP binding site (Figure 10c). Such a model most easily explains the different footprinting patterns for the wt promoter and the −81.5 variant. The UV-laser signals due to RNA polymerase−CRP interactions are only observed for the wt. DNase I footprints show no signals in the −65 region at the −81.5 promoter variant, demonstrating that this region

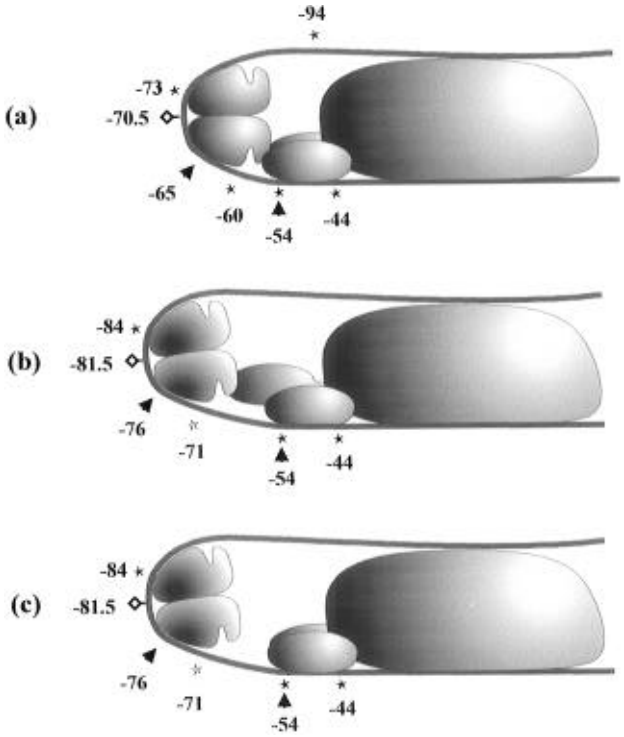


FIGURE 10: Models for the geometry of transcriptional activation complexes at variants of the *malT* promoter. The RNA polymerase is represented by the large ellipse. The C-terminal domains of the α-subunits are represented by small ellipses. The dimer of CRP is shown binding at sites centered (diamond sign) at −70.5 or −81.5. Sites of DNase I hyperreactivity in the ternary complex are indicated by arrows. UV-laser footprinting signals pertaining to binding of the α-subunits or to interaction with CRP are marked with a star. The hollow star indicates that this signal is due to CRP and does not further change in the ternary complex: (a) geometry at the *malT* promoter, (b) activation at the −81.5 promoter by direct protein−protein contacts, and (c) indirect activation at the −81.5 promoter.

is not occupied by a protein (in particular the α-subunit). Finally, changes in the UV footprints upstream of the center of the CRP binding site (−73, −94, and −84, respectively) would be a natural consequence of additional contacts of upstream DNA with the back side of RNA polymerase. Interactions of this far upstream DNA with RNA polymerase have never been shown directly, but a study of the physical interactions and sequence requirements in this region of the promoter is underway.

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