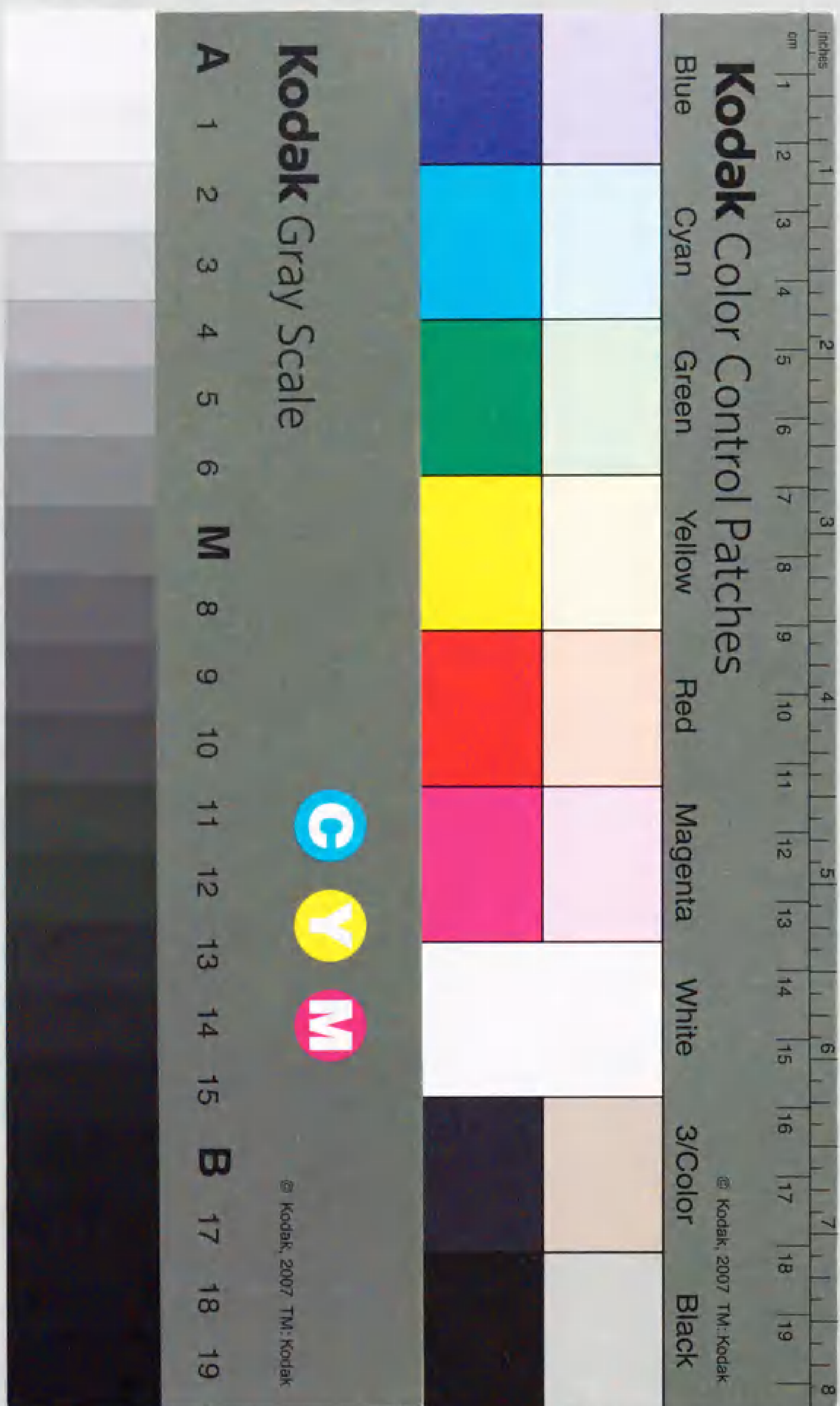

MECHANISMS OF TRANSCRIPTION INITIATION AND ACTION OF ACTIVATOR, CRP IN *ESCHERICHIA COLI*

転写開始複合体形成と調節因子 CRP の作用機構

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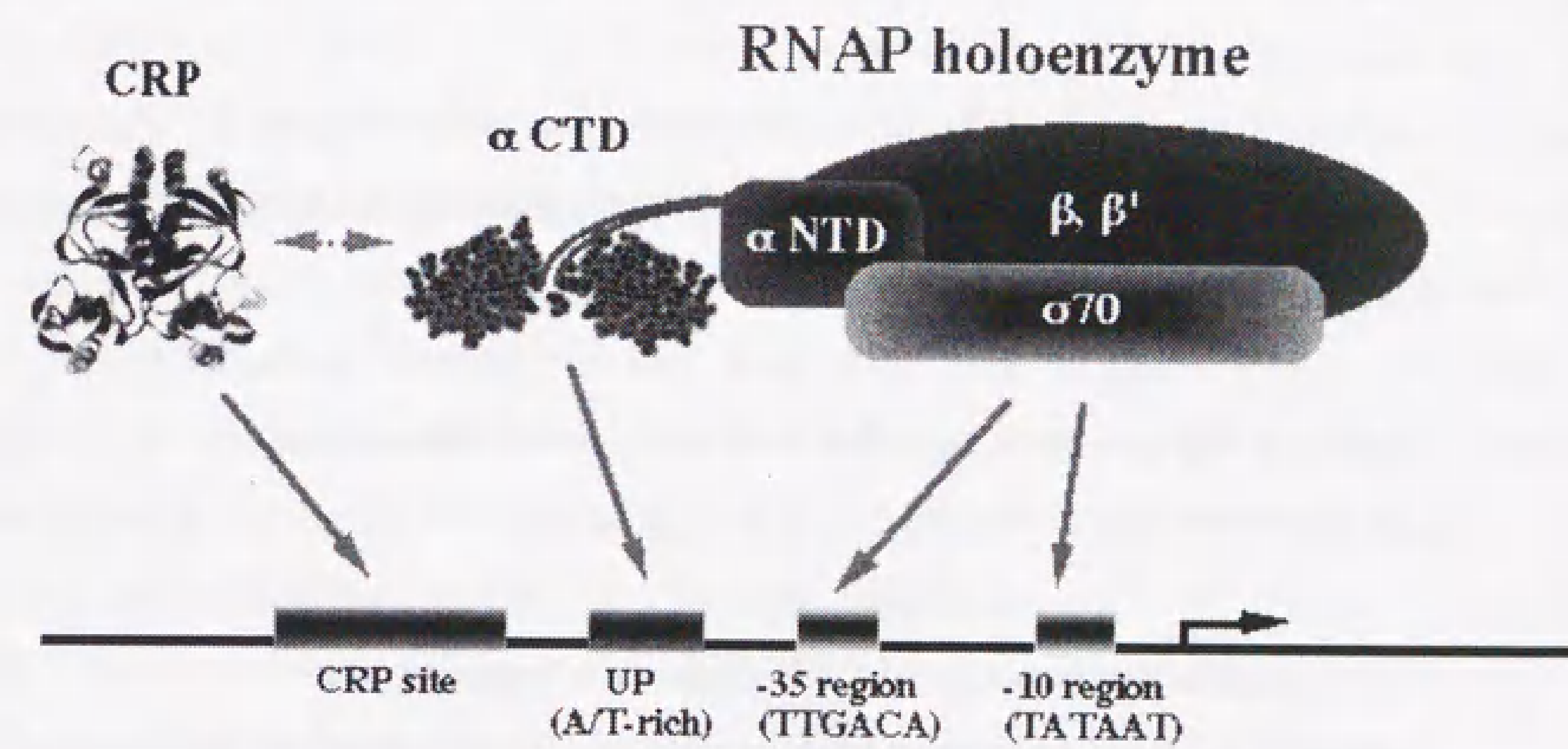

Schematic model for interactions of CRP-RNAP-Promoter.

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TABLE OF CONTENTS

Chapter **Page**

Chapter		Page
1	General Introduction and Outline of this thesis	3
2	Role of CRP in transcription activation at *Escherichia coli lac* promoter: CRP is dispensable after the formation of open complex	13
3	A common role of CRP in transcription activation: CRP acts transiently to stimulate events leading to open complex formation at a diverse set of promoters	33
4	A novel inactive open complex mediated by UP element at *Escherichia coli* promoter	57
5	The complex mechanisms of CRP-dependent transcription activation at *malT* promoter: Partnerships of UP element and CRP	77
	Conclusion and Perspectives	88
	Acknowledgments	90
	References	91

Chapter 1

GENERAL INTRODUCTION

The information in DNA should carry out function as proteins or RNAs at an appropriate time and place in response to environmental conditions. Transcriptional regulation plays a key role in the control of gene expression both in prokaryote and eukaryote. The principle concept of the regulation of transcription has been established by Jacob and Monod in 1960s, from studies of *Escherichia coli lac* operon. The concept that *trans* factor acts to *cis* element to turn on or off the gene is a major advance in our understanding of control of gene expression. Now, we know many *trans* factors to regulate transcription in many organisms, and the domain(s) or site(s) which are responsible for interaction with DNA and/or protein(s) have been revealed. Nevertheless, the nature of these interactions and how they lead to transcription activation and repression are largely unknown. The end of this thesis is how activator stimulate transcription through protein-protein and/or protein-DNA interaction by using a model system of *Escherichia coli* CRP and its target promoters.

Transcription initiation

RNA polymerase (RNAP) recognizes promoter elements and starts a transcription through a complex process involving direct protein-DNA contacts and protein-protein interactions. Transcription initiation is assumed to be composed of three major steps (see Figure 1-3) (McClure, 1985). Firstly, RNAP binds reversibly to promoter DNA to form a closed complex that is transcriptionally inactive. Secondly, the closed complex is converted to an open complex in which a short region of DNA around the transcription start site is unwound. The open complex is able to produce a series of short RNAs abortively in the presence of ribonucleotides. The third step is the promoter clearance in which RNAP escapes from the promoter with a loss of σ factor to form a stable elongation complex. The efficiency of each of these steps may be subject to regulation by transcriptionl activator or repressor proteins. Further subdivision and intermediates may be warranted by many kinetic studies (deHaseth *et al.*, 1998). Recently, another model, a branched pathway in transcription initiation, has been

suggested from a study of the λ P_R promoter (Kubori and Shimamoto, 1996). In this model, each RNAP molecule has a certain probability of becoming irreversibly trapped in carrying out abortive synthesis or of escaping this fate and entering the elongation mode. It is unclear how the two different models can be reconciled. It is required further evidence of various postulated intermediates for the elucidation of the complex process of transcription initiation.

RNA polymerase, and promoter in Escherichia coli

Escherichia coli RNAP holoenzyme is composed of four protein subunits, with a composition $\alpha_2\beta\beta'\sigma$(Burgess, 1969). The core enzyme with the subunit structure $\alpha_2\beta\beta'$ contains all of the catalytic machinery required for the synthesis of an RNA chain, whereas σ subunit confer promoter specificity to core enzyme. The various species of σ subunits each specify initiation selectivity for specific classes of genes. The σ^{70}, the primary sigma factor in *E. coli* involves the expression of most of the housekeeping genes. The σ^{70} of RNAP holoenzyme makes sequence-specific contacts with two hexamer promoter elements located around 10 and 35 bp upstream from the transcription start site. The conserved regions 2.4 and 4.2 of the σ^{70} are responsible for the recognition of –10 and –35 region, respectively (Gross *et al.*, 1996). Homologies between promoter region formulated so-called consensus sequence: TATAAT at –10 and TTGACA at –35. The distance between the –10 and –35 regions (spacer) is ordinarily 17 bp. The similarity of the –10 and –35 regions to consensus sequence, and the length of the spacer are major determinant for promoter strength (McClure, 1985). In addition with this, recent works have shown that several strong promoters contain a third *cis*-acting promoter module; the upstream (UP) element, which is located upstream of the core promoter (-35 and -10 elements), and which is an A/T-rich sequence (Ross *et al.*, 1993). The promoter strength is greatly increased by UP element. The C-terminal domain of RNAP α subunit (αCTD) makes direct contacts with UP element and is responsible for UP-dependent stimulation of promoter activity. The α subunit consists of two structural domains connected by a flexible linker (Blatter *et al.*, 1994). The N-terminal domain is mainly involved in RNAP assembly and basal transcription (Igarashi and Ishihama, 1991). The αCTD functions both as a DNA binding domain to UP element and as the target for a number of transcription factors to activate or repress the

transcription (Busby and Ebright, 1994; Ishihama, 1993). The three-dimensional structure of the αCTD contains a spatial arrangement of four helices (Figure 1-1) (Jeon *et al.*, 1995).

cAMP receptor protein (CRP)

The cyclic AMP receptor protein (CRP; also known as CAP, catabolite gene activator protein) of *E. coli* is one the best-characterized transcription factor (Botsford and Harman, 1992; Kolb *et al.*, 1993a). When complexed with its allosteric effector cAMP, CRP participates in a wide regulatory network as a global transcription factor both activating and repressing a large set of genes. It is known that the reduction in the intracellular concentrations of both cAMP and CRP causes "catabolite repression", which is independent on the operon specific repressor and is one of the mechanisms of so-called "glucose effect"(Ishizuka *et al.*, 1993). The protein is a dimer of two identical subunits composed of 209 amino acids (Aiba *et al.*, 1982). The larger N-terminal domain is responsible for cAMP binding and dimerization. The smaller C-terminal domain contains a helix-turn-helix DNA binding motif. The helix-turn-helix motifs of the two subunits insert into successive major groves of a DNA that is bent by 90° (Figure 1-2). The consensus DNA sequence of CRP-binding site is 22 bp palindromic sequence: 5'-AAATGTGATCTAGATCACATTT-3', where the two TGTGA motifs are relatively well conserved at different promoters.

CRP-dependent promoters

The CRP-binding sites lie at different locations relative to the transcription start site of various promoters. Promoters in which CRP alone is sufficient for activation can be divided into two groups: Type I (also referred to as Class I) promoters, where the CRP-binding site is located upstream of the –35 region; and Type II (Class II) promoters, where the CRP-binding site overlaps the –35 region (Aiba *et al.*, 1989; Ebright, 1993). The *lacP1* and *malT* promoters are examples of type I promoter, with a CRP-binding site at positions –61.5 and –70.5, respectively. The *galP1* promoter is a prototype type II promoter with a CRP-binding site at position at –41.5. The CRP-binding site lies well upstream (typically more than –90) in several CRP-dependent promoters.

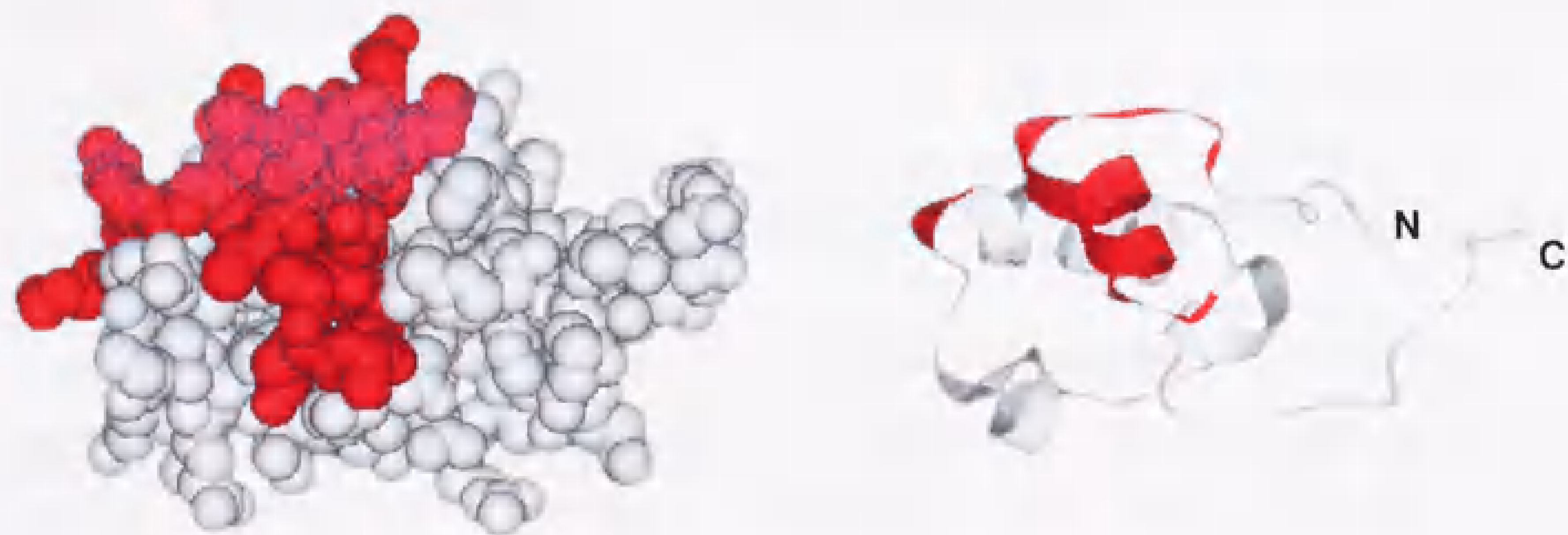

Figure 1-1. The solution stracture of the C-terminal domain of α subunit (α CTD) of E. coli RNA polymerase (Joen *et al.*, 1995). The amino acid residues in contact with CRP and UP element are shown in red (position 261, 262, 265, 268, 298,and 299 a.a.).

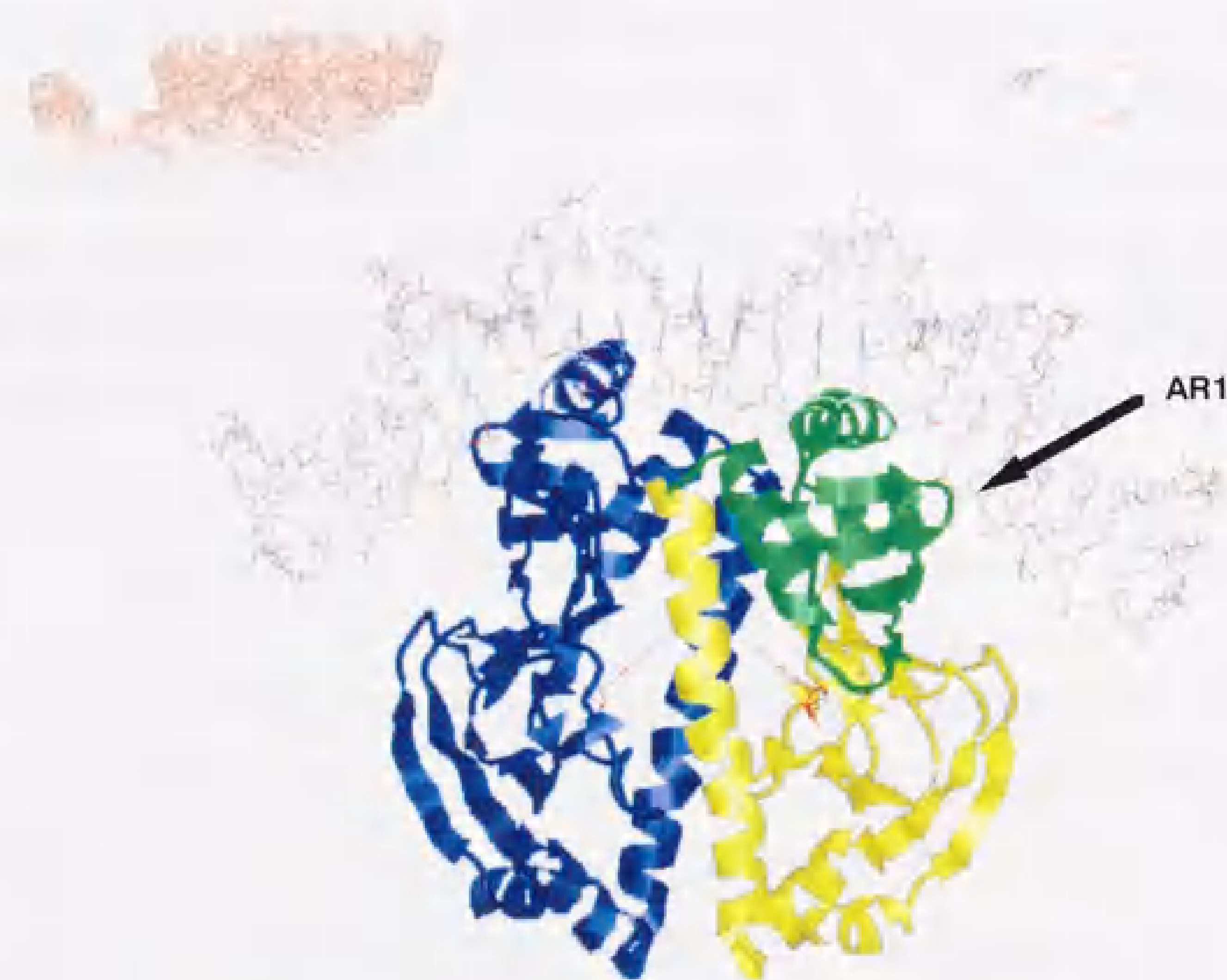

Figure 1-2. The skelton stracture of the CRP-DNA complex (Schults *et al.*, 1991). The figure shows the ribon structure of two CRP monomers with the target DNA. The N-terminal domain and the C-terminal domain of a monomer are shown in yellow and green,respectively. The other monomer is shown in blue. The arrow head represents the activating region 1 (position 156-164 a.a.).

These promoters require a regulon specific activator in addition to CRP for transcription activation (Type III or Class III). The example of the type III promoter is *araBAD* promoter in which CRP binds around -94 and acts as a coactivator of AraC (Lobell and Schleif, 1991). CRP also acts as a repressor or corepressor in several promoters. A simple example is the *cya* promoter, where CRP alone inhibits the transcription by binding a target site located within promoter (Aiba, 1985). A more complex mechanism of repression is found in the operons that are coordinately regulated by CRP and CytR (Sogaard-Andersen and Valentin-Hansen, 1993). An example is the *deo* promoter, where the CRP-binding sites are located at –41 and –94. CRP is required both for the transcription activation and for the repression by enhancing the CytR binding.

Transcription activation by CRP

The mechanism of transcription activation by CRP has been extensively studied as a paradigm for understanding how a single activator can act. Numerous studies indicate that the interaction between CRP and RNAP plays a pivotal role in transcription activation at CRP-dependent promoters (Busby and Ebright, 1997; Busby *et al.*, 1994; Kolb *et al.*, 1993a). In particular, recent genetic and biochemical studies of CRP and RNAP have identified particular sites or regions located at the surface of both proteins which are involved in protein-protein interaction and thereby responsible for transcription activation. Isolation and characterization of CRP mutants that bind normally to the target DNA but fail to activate transcription have established that a surface exposed loop around amino acids 156-164 of CRP constitutes an activating region essential for transcription activation at both type I and type II promoters (activating region I; AR1) (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Zhou *et al.*, 1993). An additional contact between the N-terminal domain of CRP (activating region II;AR2, corresponds to amino acid residues 19, 21, 96, and 101) and the N-terminal domain of RNAP α-subunit is involved in transcription activation at type II promoters (Niu *et al.*, 1996). CRP is reported to interact to C-terminal region of σ

subunit in certain type II promoters (Busby and Ebright, 1997). CRP is also known to induce a sharp bend in the DNA which may play a role in transcription activation (Crothers, 1992; Dethiollaz *et al.*, 1996; Wu and Crothers, 1984). The contribution of CRP-induced DNA bending to transcription activation is still unclear especially in type I and type II promoters.

It has been believed that CRP can affect different steps depending on the relative location of the CRP binding site in the promoter (Figure 1-3). Kinetic studies have claimed that CRP enhances initial binding of RNAP at the *lac* promoter (Malan *et al*, 1984), stimulates both initial binding and isomerization to the open complex at the *gal* promoter (Herbert *et al.*, 1986; Lavigne *et al.*, 1992), and accelerates the rate of promoter clearance at the *malT* promoter (Eichenberger *et al.*, 1997; Eichenberger *et al.*, 1996; Menendez *et al.*, 1987). It should be noted, however, that CRP is inferred to affect predominantly the isomerization step at the *lac* promoter in another study (Straney *et al.*, 1989). This suggests that our understanding of which step(s) of transcription initiation is affected by CRP is not yet certain.

In *lac* promoter, CRP binds to its binding site and interacts specifically with the αCTD of RNAP, apparently stabilizing its association with the DNA between the CRP-binding site and the –35 region (Kolb *et al.*, 1993b). The UP element which is the target site for αCTD located at the upstream region of the *lac* promoter increases the transcription activity without CRP (Rao *et al.*, 1994). The artificial proteins that make arbitrary contacts with RNAP were reported to sufficiently act as activators (Hochschild and Dove, 1998). These findings suggest that the role of CRP in transcription activation, at least in part, is to stabilizing the binding of RNAP to the promoter, thus facilitating closed complex formation. The recruitment model for the role of activators is simple,

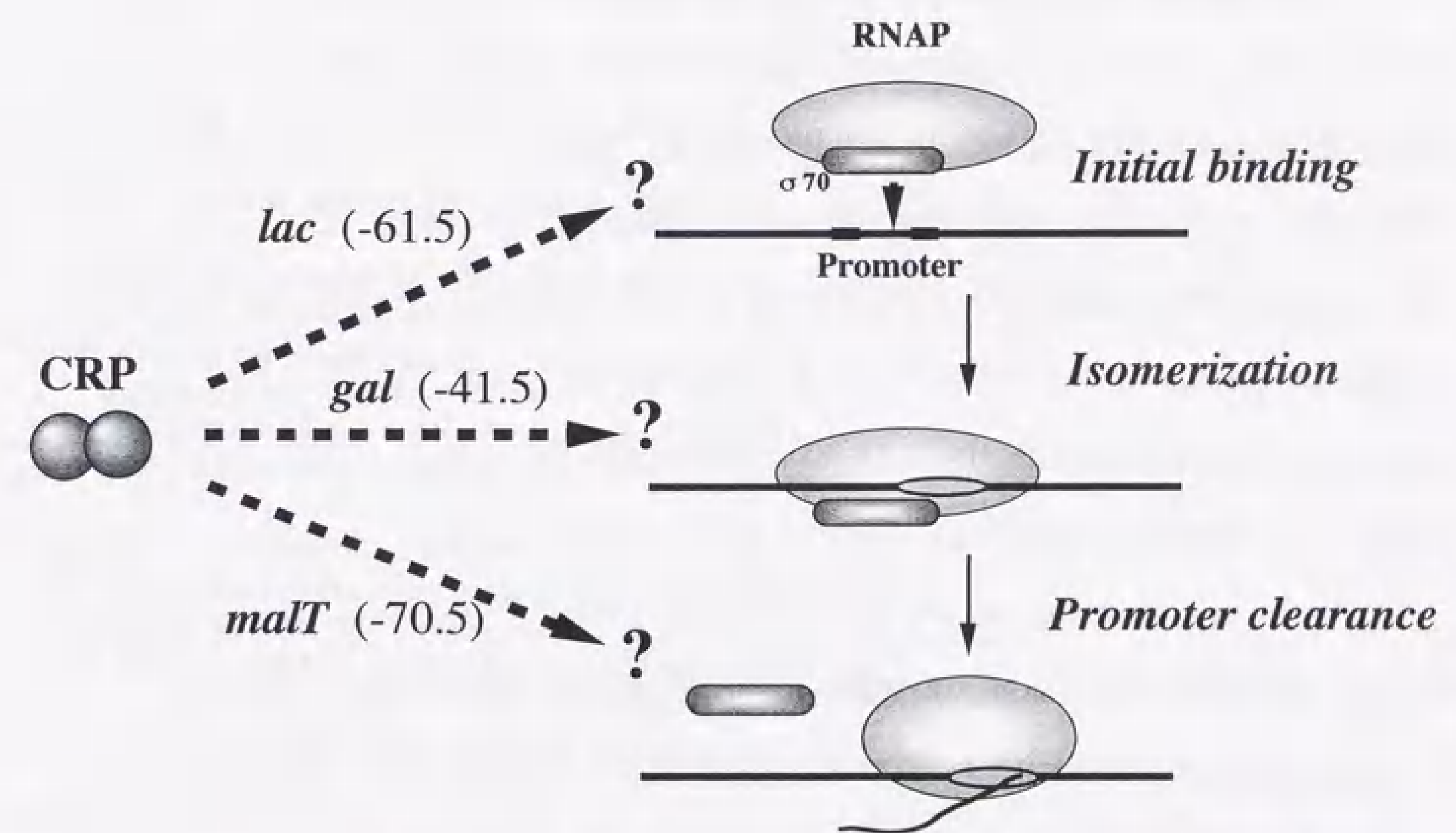

Figure 1-3. Kinetics model for CRP action in transcription initiation. CRP was reported to affect different steps in transcription initiation depending on the relative location of the CRP-binding site. The numbers in parentheses represent the center of the CRP-binding site relative to the transcription start site.

but the further experiments are required for understanding what happened at the step(s) and the effective stimulation *in vivo*.

OUTLINE OF THIS THESIS

Numerous studies using CRP system have been carried out to understand how activator stimulates transcription and then established the fine mapping of the sites in both CRP and RNAP involving the protein-DNA and/or protein-protein interactions. Structural studies have been progressing and have revealed the spatial information both of the interaction region of CRP and α subunit of RNAP. However, the important questions such as when and how the interactions lead to transcription activation have remained to be studied. One way to address this issue is to define which step of initiation is affected by CRP.

First, I investigated the role of CRP in transcription activation focusing on the steps after the formation of open complex at *lac* promoter, which is the best-characterized CRP-dependent promoter (*Chapter 2*). As CRP binds synergistically to the promoter with RNAP and stabilizes the ternary open complex, it is possible that CRP contributes to the maintenance of the open complex and functions at the promoter clearance step. I established a method that dissociates CRP from the open complex specifically by addition of a high concentration of heparin. The dissociation of CRP did not affect the interaction between RNAP and promoter. The resulting binary complex retains the characteristics of a functional open complex and is competent for transcription. The results indicate that CRP is necessary for neither the stability of the open complex nor the subsequent initiation process. I conclude that the contact between CRP and RNAP is not essential for transcription activation after the formation of the open complex at the *lac* promoter.

I performed the heparin challenge experiment on the *malT* and *gal* promoters to examine the role of CRP in transcription activation at a diverse set of CRP-dependent promoters (*Chapter 3*). At the *malT* promoter, RNAP forms a non-productive RNAP-promoter binary complex in the absence of CRP while it forms a productive CRP-RNAP-promoter ternary complex in the presence of CRP. CRP can be removed by a moderate concentration of heparin from the *malT* ternary complex. The resulting binary complex is functionally identical to the ternary complex. A very high concentration heparin can dissociate CRP from the *galP1* ternary complex without changing the properties of the complex. I conclude that the common role of CRP in transcription activation is to stimulate events leading to the formation of a productive open complex at a diverse set of CRP-dependent promoters. I suggest that the CRP-RNAP interaction is only transiently needed for transcription activation. Hence, the step(s) up to the formation of productive open complex is a key to solve the mechanisms of transcription initiation and its regulation.

RNAP forms a heparin resistant nonproductive transcription complex, while it forms a productive complex in the presence of CRP at *malT* promoter. Next, I investigate a role of UP element for the formation of the nonproductive and productive complex (*Chapter 4*). Specific interaction between the α subunit of RNAP and UP element stimulates transcription at some promoters in *Escherichia coli*. The *malT* promoter has an A/T-rich upstream sequence separated 9 bp from the -35 region. I showed that the α-UP interaction leads to the formation of an inactive open complex when UP element is situated at a certain position at *malT* and *lac* promoters. The region around the transcription start site is melted both in the productive and nonproductive complexes. I conclude that the UP element mediates the formation of both productive and nonproductive open complexes depending on its location.

I studied the functional significance of the UP element and the nonproductive complex in the regulation of *malT* expression (*Chapter 5*). I found that the

nonproductive complex is converted to the productive complex by CRP and that CRP-dependent activation is markedly reduced in the absence of the UP element. It indicates that CRP alone is not sufficient to recruit RNAP to the promoter. I suggest that CRP predominantly acts at the post-recruitment step to form the productive complex by modulating the RNAP-promoter interaction at *malT* promoter.

Chapter 2

Role of CRP in transcription activation at *Escherichia coli lac* promoter: CRP is dispensable after the formation of open complex

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SUMMARY

The role of cAMP receptor protein (CRP) in transcription activation at the *Escherichia coli lac* promoter was investigated focusing on the steps after the formation of open complex. Although CRP binding to the *lac* DNA is stabilized in the ternary open complex, a high concentration of heparin dissociates CRP from the open complex without affecting the interaction between RNA polymerase and promoter, resulting in a binary complex. The release of CRP is directly shown by Western blotting and DNase I footprinting. The binary complex exhibits a slightly increased gel mobility compared to the ternary complex. The binary complex retains the characteristics of the open complex in footprinting pattern which is essentially identical with that of the open complex of the *lac* UV5 promoter. The binary complex is competent for transcription. These results indicate that CRP is not necessary for the maintenance of active open complex. In addition, the removal of CRP does not increase the production of abortive RNAs. I conclude that the contact between CRP and RNA polymerase is not essential for transcription activation after the formation of the open complex at the *lac* promoter. In other words, the role of CRP in the *lac* promoter is restricted to the steps up to the formation of open complex.

INTRODUCTION

The cyclic AMP receptor protein (CRP or CAP) of *Escherichia coli*, the prototype of a DNA-binding regulatory protein, controls transcription of a large number of genes either positively or negatively in response to carbon nutrient conditions (Botsford and Harman, 1992; Crothers, 1992; Kolb *et al.*, 1993a). The protein is a dimer of two identical subunits composed of 209 amino acids, which is activated by cAMP to bind to a specific sequence of 22 bp located within or near target promoters. How CRP activates transcription has been a long-standing question since its discovery. Two ways in which CRP activates transcription have been proposed: one involving CRP-RNA polymerase (RNAP) interaction (Gilbert, 1976), and one involving changes in DNA structure (Dickson *et al.*, 1975).

Recent genetic and biochemical studies have revealed that both mechanisms may contribute to the action of CRP. First, CRP appears to contact directly with RNA polymerase to activate transcription (Busby and Ebright, 1994; Ebright, 1993; Ishihama, 1993). A surface exposed loop around amino acids 156 to 164 of CRP and the C-terminal of α subunit of RNAP are responsible for this protein-protein interaction (Busby and Ebright, 1994; Ebright, 1993; Ishihama, 1993), although the nature of the interaction is not known. Second, CRP induces a sharp bend in the DNA upon binding to its recognition site (Schultz *et al.*, 1991; Wu and Crothers, 1984). This bending is believed to play a role in the formation of open complex (Zinkel and Crothers, 1991). Thus, it is likely that CRP affects upon both RNA polymerase and DNA structure to activate transcription. However, the molecular mechanisms by which the CRP-RNAP interaction and CRP-induced DNA bending stimulate transcription are largely unknown.

One way to address this issue is to investigate which step of initiation is affected by CRP. Transcription initiation is composed of several steps. RNA polymerase is known to pass through at least three distinct stages prior to chain elongation

(Chamberlin, 1974; Crothers, 1992; McClure, 1980). First, the holoenzyme interacts reversibly with the promoter to form a closed complex that is transcriptionally inactive. Second, the closed complex is isomerized to an open complex in which a short region of DNA around the transcription start site is unwound. The open complex is transcriptionally competent and is able to make abortively a series of short RNAs by the addition of ribonucleotides. The final step is the promoter clearance in which RNA polymerase escapes from the promoter with a loss of σ factor to form a stable elongation complex.

Kinetic studies have suggested that CRP affects different steps depending on the relative location of the CRP site. When the CRP site is located upstream from the promoter, CRP appears to increase both the binding affinity for a closed complex and the rate of transition from closed to open complexes (Gaston *et al.*, 1990; Malan *et al.*, 1984; Straney *et al.*, 1989). When the CRP site is located within the promoter, the primary effect of CRP seems to be at the isomerization step (Gaston *et al.*, 1990). In either case, it is obvious that CRP accelerates the formation of the ternary open complex that is transcriptinally competent.

On the other hands, little is known whether CRP plays any roles after the formation of the open complex. Here, I show that CRP can be removed from the ternary open complex of the *lac* promoter with the competition by a high concentration of heparin. The resulting binary complex was stable and active to initiate transcription from the P1 promoter (Reznikoff, 1992) as the ternary complex containing CRP. No significant increase in abortive RNA products was observed after the removal of CRP. The binary open complex was also shown to have the same characteristics as those of the open complex of *lac* UV5 promoter (Reznikoff, 1978). The data indicate that the CRP-RNAP interaction and probably CRP-induced DNA bend are not required for the maintenance of open complex, initiation of transcription, and promoter clearance in the wild-type *lac* promoter.

MATERIALS AND METHODS

DNA and proteins

The 185-bp *Bam*HI-*Hin*dIII fragments carrying either the wild-type or UV5 *lac* promoters were used in this study (Figure 2-1). The fragments were prepared from plasmids pLAC11 and pLAC12, respectively. A 320-bp *Pvu*II fragment containing the wild-type *lac* promoter region was isolated from pUC19. After joining *Bam*HI linker, the fragment was digested *Bam*HI and *Hin*dIII. The resulting *lac* promoter fragment was cloned between the *Bam*HI and *Hin*dIII sites of pMS437C (Kawamukai *et al.*, 1985) to generate pLAC01. The 190-bp *Eco*RI-*Hin*dIII fragment derived from pLAC01 was recloned between the *Eco*RI and *Hin*dIII sites of pBR322 to create pLAC11. The plasmid pLAC12 containing the *lac* UV5 promoter was created from pLAC11 by PCR mutagenesis using synthetic oligonucleotides. In pLAC12, the G at position -9 and T at position -8 in the -10 region of the wild-type promoter are together changed to A. CRP was purified from cells harboring pHA7 (Aiba *et al.*, 1982) by the procedure of Eilen *et al.* (Eilen, 1978). RNA polymerase was purified from strain W3350 according to the method of Fukuda *et al.* (Fukuda and Ishihama, 1974).

Gel shift assay

The gel mobility shift assay was performed in a total volume of 20 ml of a transcription buffer (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 50 μg/mg bovine serum albumin) containing 5 mM $CaCl_2$, 50 mM cAMP, and 5 % glycerol. The *Bam*HI-*Hin*dIII fragment ^{32}P-labeled at its 5' *Hin*dIII end (1 nM) was incubated first with CRP (0-40 nM) for 5 min at 37°C and then with RNA polymerase (0 or 20 nM) for 10 min. Then the mixture was treated with 2 μl

of competitor for 3 min. After adding 3 μl of 40 % glycerol, 0.025 % bromophenol blue, 0.025 % xylene cyanol, the mixture was fractionated by electrophoresis on a native 5 % polyacrylamide gel containing 0.1 mM cAMP in 1/2 TBE at room temperature.

Western blotting

After exposing for autoradiogram, the gel mentioned above was equilibrated in a soaking buffer (375 mM Tris-HCl pH 7.5, 1 % SDS) for 1 hr as described by Zinkel and Crothers (Zinkel and Crothers, 1990). The proteins were transferred to a PVDF membrane (Immobilon-P, Millipore) with a Milliblot apparatus. The membrane was probed with polyclonal anti-CRP antibody according to the method described by Ishizuka *et al.* (Ishizuka *et al.*, 1993).

DNase I footprinting

The *Bam*HI-*Hin*dIII fragment ^{32}P-labeled at its 5' *Hin*dIII end (1 nM) in 100 μl of transcription buffer containing 5 mM $CaCl_2$, 50 μM cAMP and 5 % glycerol was first incubated with CRP (0-100 nM) for 5 min and then incubated with RNA polymerase (0 or 90 nM) for 30 min at 37°C. The mixture was treated with heparin for 3 min at 37°C and placed at 25°C for 5 min. DNase I was added at a concentration of 50 ng/100 μl and the incubation was continued for 1 min at 25°C. After addition of 25 μl of 1.5 M sodium acetate, 20 mM EDTA, 100 μg/ml tRNA, the mixture was treated with phenol and precipitated with ethanol. The pellet was dissolved in loading buffer (8 M urea, 0.025 % bromophenol blue and 0.025 % xylene cyanol in TBE) and analyzed on an 8 % polyacrylamide-8 M urea gel.

In vitro *transcription*

The transcription assay was performed in a total volume of 30 μl of transcription buffer containing 50 μM cAMP and 5 % glycerol. The *Bam*HI-*Hin*dIII fragment of a final concentration of 5 nM was first incubated with CRP (0 or 40 nM) for 5 min at 37°C and then with RNA polymerase (30 nM) for 10 min. The mixture was treated with 3 μl of heparin for 3 min. The transcription was started by adding 3 μl of a substrate solution containing 0.05 mM [α-^{32}P] UTP (5 μCi) and 0.5 mM each of ATP, GTP, and CTP. After 15 min of incubation at 37°C, the reaction was terminated by addition of 2 μl of 0.5 M EDTA. The 20 μl of the mixture was mixed with 40 μl of loading buffer and fractionated by electrophoresis on a 20 % polyacrylamide gel containing 8 M urea.

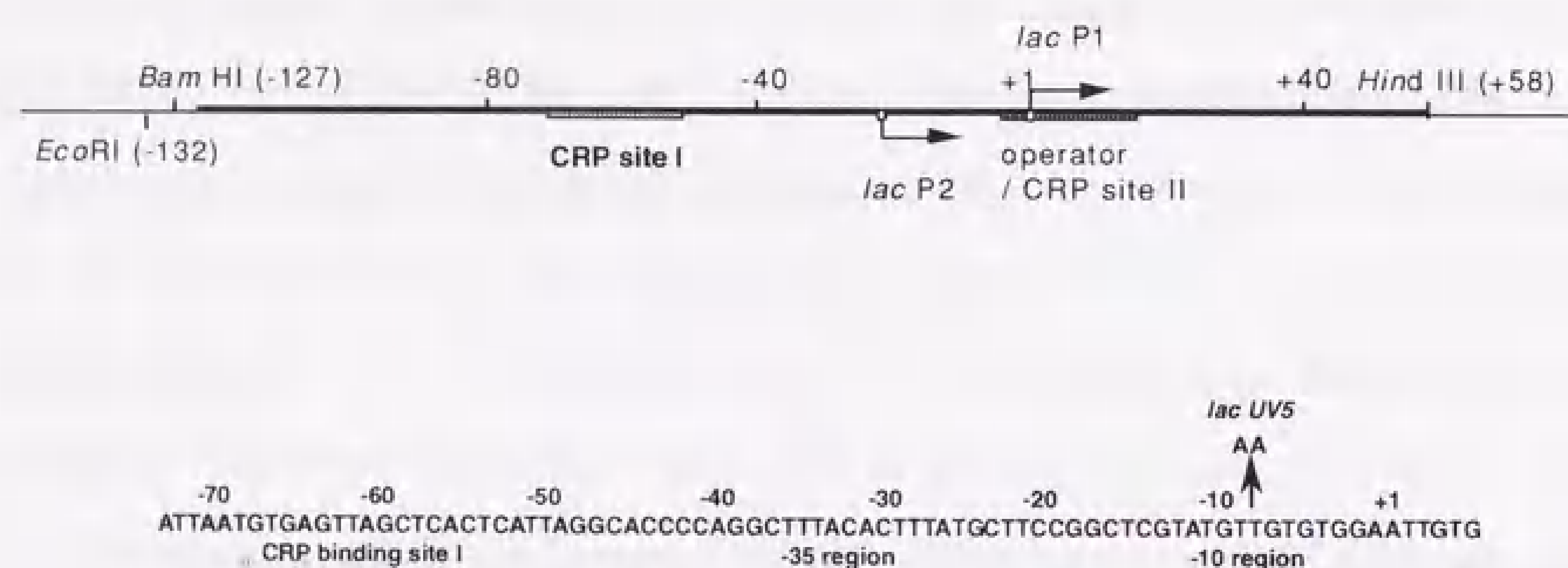

Figure 2-**1**. Diagram of the *lac* promoter region. The upper panel shows the partial map of pLAC11 that carries the wild-type *lac* promoter. The transcription start site for the *lac*P1 promoter is numbered +1. The thick line corresponds to the *lac* promoter sequence derived from pUC19. The 185-bp *Bam*HI-*Hin*dIII fragment was used in this study. The lower panel shows the nucleotide sequence of the upper strand of wild-type *lac* promoter region along with the base changes in *lac* UV5 mutation.

RESULTS

Cooperative binding of RNA polymerase and CRP-cAMP to lac promoter

It is well established that CRP-cAMP stimulates the binding of RNA polymerase to the *lac*P1 promoter (Ren *et al.*, 1988; Straney *et al.*, 1989). In addition, it is known that CRP-cAMP binding to the *lac* promoter-containing DNA is also markedly stabilized by RNA polymerase (Straney *et al.*, 1989). To investigate the nature of the *lac* open complex, I tested these previous observations by using both the wild-type and UV5 *lac* promoters. A DNA fragment containing the wild-type *lac* promoter was incubated with RNA polymerase and/or CRP-cAMP and the protein-DNA complexes were analyzed by a gel mobility shift assay (Figure 2-2). Under my experimental condition, CRP in the presence of cAMP preferentially binds to the CRP site I centered at -61.5 relative to the start point of P1 transcription, resulting in a distinctive band (Figure 2-2, lane 2). This complex is extremely sensitive to heparin while it is relatively stable to the competition of poly(dA-dT) (Figure 2-2, lanes 3 and 4), as reported by Straney *et al.* (Straney *et al.*, 1989). RNA polymerase formed a stable P1 open complex in the presence of CRP-cAMP (Figure 2-2, lane 5) while it formed a P2 open complex with a low yield in the absence of CRP-cAMP (data not shown). The ternary P1 open complex was stable to the competition with heparin (Figure 2-2, lane 6), suggesting that CRP still exists in the open complex after the treatment of heparin. The appearance of free DNA by the treatment of heparin suggests that heparin-sensitive complexes including the closed complex also exist in the reaction mixture. I show later by Western blotting and DNase I footprinting that CRP is indeed retained within the open complex after the heparin-treatment (see Figures 2-3B and 2-4). These results are consistent with those of Straney *et al.* (Straney *et al.*, 1989), indicating again that both RNA polymerase and CRP-cAMP bind cooperatively to the *lac* promoter. I also analyzed DNA-protein

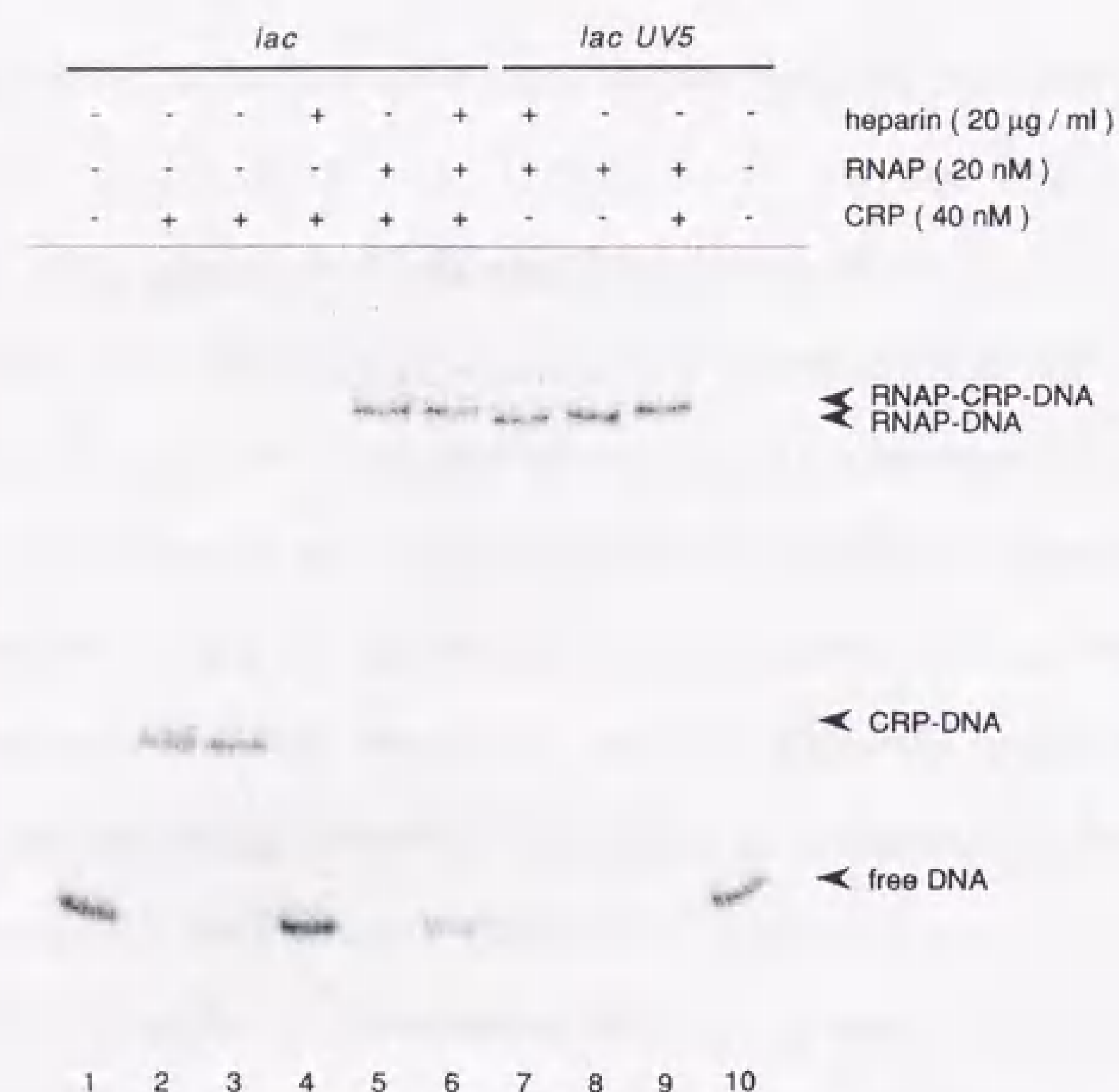

Figure 2-2. Gel shift analysis of protein-DNA complexes of the *lac* promoter fragment. The ^{32}P-labeled 185-bp *Bam* HI-*Hind* III fragment containing wild-type *lac* promoter (lanes 1-6) or *lac* UV5 promoter (lanes 7-10) was incubated with protein(s) in the presence of cAMP. The mixture was further incubated with or without heparin and separated on a native 5% polyacrylamide gel. Lanes:1 and 10, DNA alone; 2, CRP added (no competitor); 3, CRP then poly(dA-dT) (12.5 µg/ml) added; 4, CRP then heparin added; 5 and 9, CRP and RNA polymerase added (no competitor); 6, CRP and RNA polymerase then heparin added; 7, RNA polymerase then heparin added; 8, RNA polymerase added (no competitor).

complexes of the *lac* UV5 promoter fragment. As expected, RNA polymerase in the absence of CRP-cAMP formed a stable binary P1 open complex that has a slightly increased gel mobility (Figure 2-2, lanes 7 and 8). When the *lac* UV5 fragment was incubated with both RNA polymerase and CRP-cAMP, a ternary open complex was formed that has the same mobility of the complex containing the wild-type *lac* promoter fragment (Figure 2-2, lane 9).

Effect of high concentrations of heparin on the P1 open complex

Although the CRP binding to *lac* DNA is stabilized in the ternary open complex, I thought that a more drastic competition with heparin might affect the binding of CRP and RNA polymerase to the *lac* promoter. To examine the stability of the ternary complex, I have treated the ternary complex with increasing concentrations of heparin and analyzed the products by a gel shift assay (Figure 2-3A). When the complex was treated with a low concentration (20 µg/ml) of heparin, no clear change in the mobility of the complex was detected (Figure 2-3A, lane 3). However, increasing concentrations of heparin caused a slight reduction in the mobility of the complex (Fig. 3A, lanes 4-6). The mobility of the complex treated with 1000 µg/ml of heparin was the same as that of the binary open complex of the *lac* UV5 promoter (Figure 2- 3A, lanes 7 and 8). This suggests that CRP was lost from the ternary open complex with the competition of a high concentration of heparin. The resulting binary complex was apparently stable at least for 60 min at 37°C even in the presence of heparin (data not shown), although I have not determined quantitatively the stability of the complex.

A

B

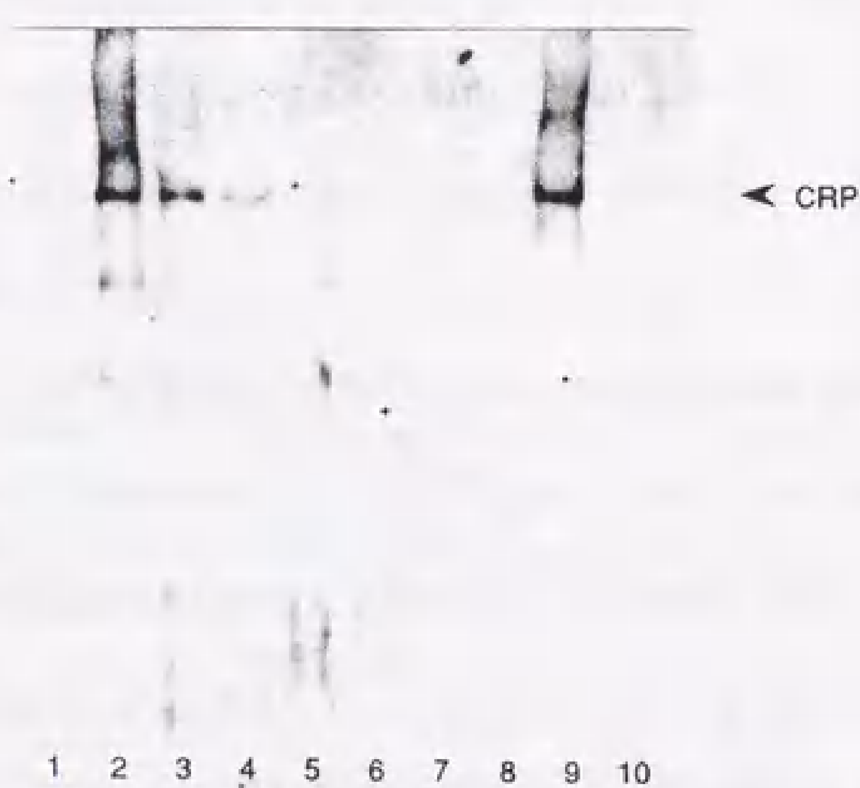

Figure 2-3. Effect of increasing concentrations of heparin on open complexes. (A) Gel mobility shift analysis of open complexes. The ^{32}P-labeled 185-bp *Bam* HI-*Hind* III fragment containing the wild-type *lac* promoter (lanes 1-6) or *lac* UV5 promoter (lanes 7-10) was incubated with protein(s) in the presence of cAMP. The mixture was treated with indicated concentrations of heparin and separated on a native 5% polyacrylamide gel.
(B) Immunoblot analysis using an anti-CRP antibody. The gel shown in (A) was directly used for Western blotting. The arrow indicates the existence of CRP in open complexes.

A direct evidence for the dissociation of CRP by Western blot analysis

In order to determine directly whether CRP is present in the complex, a Western blot analysis of the gel shown in Figure 2-3 was carried out by using anti-CRP antibody. As expected, CRP was detected before the treatment of heparin (Figure 2-3B, lanes 2 and 9), indicating the presence of CRP in the open complex. The treatment of a low concentration of heparin did not affect the content of CRP (Figure 2-3B, lane 3) while an increased concentration of heparin to 100 μg/ml significantly reduced the content of CRP (Figure 2-3B, lane 4). When the concentration of heparin was further increased, CRP was no longer detected in the complex (Figure 2-3B, lanes 5 and 6). The result indicates that CRP was selectively removed from the ternary open complex by the treatment of a high concentration of heparin. In other words, the CRP-RNAP interaction, which is a characteristic of the *lac* open complex, was disrupted under this particular condition.

Characterization of ternary and binary complexes by footprinting

To characterize further the nature of the open complex before and after heparin treatment, a DNase I footprinting experiment was performed. A DNA fragment containing either the wild-type or UV5 *lac* promoters in which the 5' end of the lower strand was labeled with ^{32}P was incubated with RNA polymerase and/or CRP-cAMP. The reaction mixture was treated with various concentrations of heparin and subjected to DNase I digestion. The products were fractionated on a denatured 8% polyacrylamide gel. When the wild-type *lac* promoter fragment was incubated with CRP in the presence of cAMP, the CRP site I was protected (Figure 2-4, lane 3). The same protection by CRP-cAMP was observed with the *lac* UV5 promoter fragment (data not shown). As expected, the CRP binding to the CRP site I was lost by a low concentration of heparin

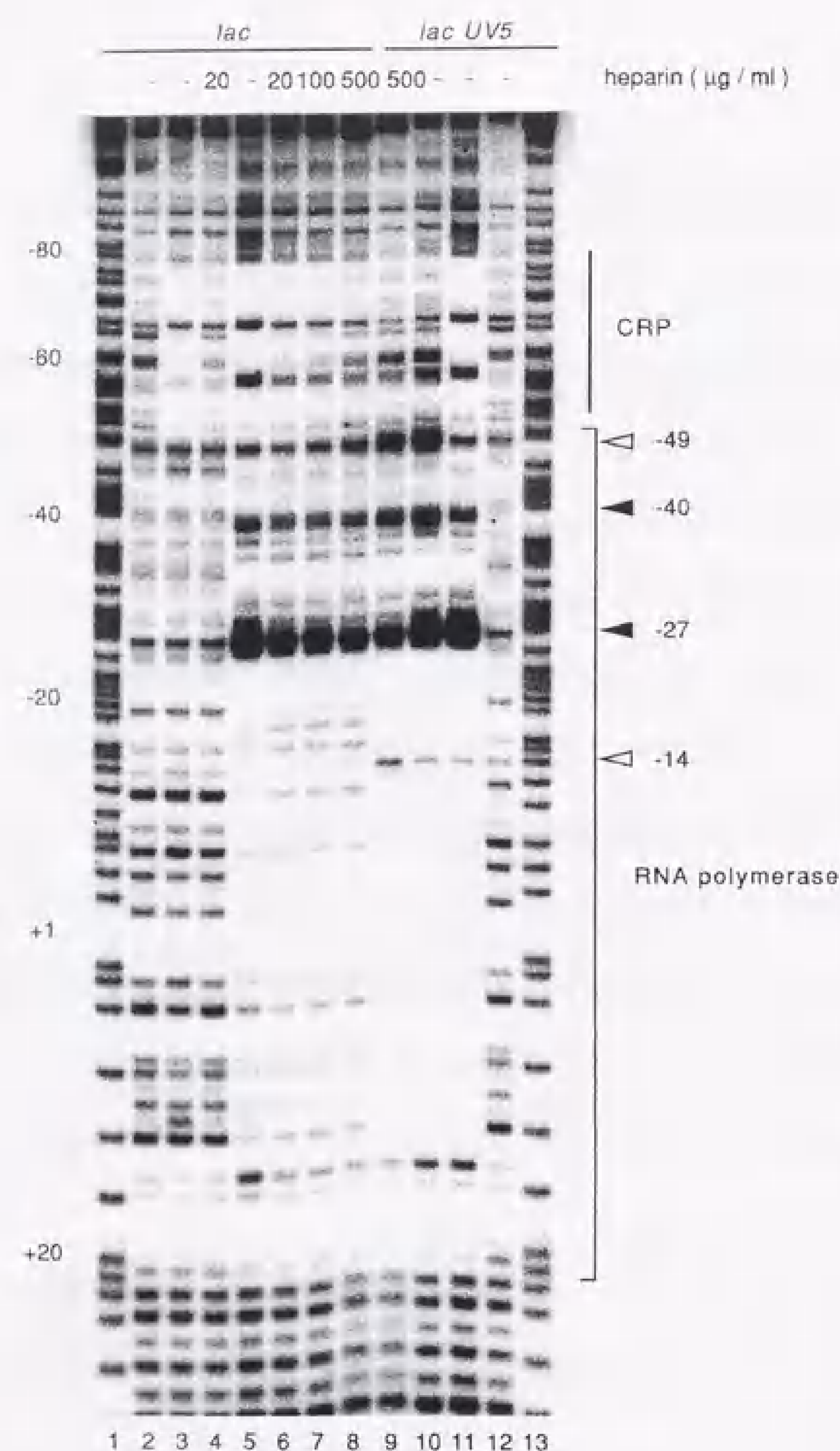

Figure 2-4. DNase I footprinting analysis of the promoter fragments. The 185-bp *Bam*HI-*Hin*dIII fragment ^{32}P-labeled at its *Hin*dIII 5' end containing wild-type *lac* promoter (lanes 1-8) or *lac* UV5 promoter (lanes 9-13) was incubated with protein(s) in the presence of cAMP. The mixture was treated with indicated concentrations of heparin and subjected to a partial digestion by DNase I. The products were fractionated on a denatured 8% polyacrylamide gel. Lanes: 2 and 12, no added protein; 3, CRP (100 nM) added; 4, CRP (100 nM) then heparin (20 μg/ml) added; 5 and 11, CRP (40 nM) and RNA polymerase (90 nM) added; 6, CRP (40 nM) and RNA polymerase (90 nM) then heparin (20 μg/ml) added; 7, CRP (40 nM) and RNA polymerase (90 nM) then heparin (100 μg/ml) added; 8, CRP (40 nM) and RNA polymerase (90 nM) then heparin (500 μg/ml) added; 9, RNA polymerase (90 nM) then heparin (500 μg/ml) added; 10, RNA polymerase (90 nM) added. Lanes 1 and 13 are the products of A + G chemical reaction. The regions protected from DNase I digestion by CRP-cAMP and/or RNA polymerase are shown by vertical lines. The filled triangles indicate enhanced bands specific for the P1 open complex of wild-type and UV5 *lac* promoters. The open triangles indicate enhanced bands specific for the open complex of *lac* UV5 promoter. Numbers on the left indicate positions relative to the transcription start site of P1.

(Figure 2-4, lane 4). When RNA polymerase alone was incubated with the wild-type *lac* DNA, no clear protection against DNase I digestion was observed presumably due to a low yield of the P2 open complex (data not shown). On the other hand, RNA polymerase alone protected strongly the *lac* UV5 promoter fragment in the region between +20 and -50 against DNase I attack. Several sites (-14, -27, -40, and -49) exhibited an enhanced cleavage (Figure 2-4, lanes 9 and 10). This protection/enhancement pattern is known to be characteristics to open complex of *lac* UV5 promoter (Carpousis and Gralla, 1985; Spassky *et al.*, 1985). In the presence of both CRP-cAMP and RNA polymerase, the protection was extended to the CRP site in the *lac* UV5 promoter (Figure 2-4, lane 11). It should be noted that the CRP binding did not change the pattern of RNA polymerase binding to the *lac* UV5 promoter except the suppression of enhanced cleavage at position -49. In the case of the wild-type *lac* DNA, CRP markedly stimulates the binding of RNA polymerase to P1, resulting in a clear protection/enhancement pattern that is essentially the same as that of the *lac* UV5 promoter (Figure 2-4, lane 5). When the ternary open complex was treated with increasing concentrations of heparin, the protection at CRP site I gradually disappeared. This protection by CRP-cAMP was almost completely lost when 500 μg/ml of heparin was used. In contrast, the protection/enhancement pattern between +20 and -50 remained unchanged after the heparin treatment. These results clearly indicate that a high concentration of heparin selectively dissociates CRP from the ternary open complex without affecting RNA polymerase binding to P1 in the wild-type *lac* promoter. This means that the stable ternary open complex has been converted to the binary open complex that is resistant to the competition with heparin.

Binary complex without CRP is transcriptinally competent

The experiments mentioned above established that the CRP can be removed from the ternary P1 open complex with little change in RNA polymerase binding resulting in a stable binary complex. It is interesting to address whether this binary complex is transcriptionally competent. For this, I have carried out *in vitro* transcription assay. First, I formed the binary open complex of *lac* UV5 promoter fragment and treated it with heparin. Then the complex was subjected to a single round transcription by adding a substrate mixture and the products were analyzed on a denatured polyacrylamide gel. As expected, a P1 transcript of 58 bases was produced without being affected by the increase of heparin concentration (Figure 2-5, lanes 5 and 6). In addition, a series of abortive short RNAs were produced which level appears to decrease with increasing concentration of heparin. The similar effect of heparin on the production of abortive RNAs was also observed at T7 A1 promoter (Metzger *et al.*, 1993). Next, the P1 open complex was formed by incubating the wild-type *lac* promoter fragment with RNA polymerase and CRP-cAMP. After treating the complex with a low concentration of heparin, a transcription reaction was carried out by adding ribonucleotides. The P1 transcript identical with that of the UV5 promoter was produced (Figure 2-5, lane 2). The competition with increased concentration of heparin did not affect the amount of P1 transcript (Figure 2-5, lanes 3-5). This is true even when 1000 μg/ml heparin was used. It should be noted that CRP is completely lost under this condition (Figures 2-3 and 2-4). In addition, the removal of CRP caused no increase in the production of abortive RNAs. In other words, the presence or absence of CRP does not affect the transcriptional activity in the single round run-off assay, suggesting that the binary open complex without CRP has the same ability for transcription as that of the ternary complex with CRP.

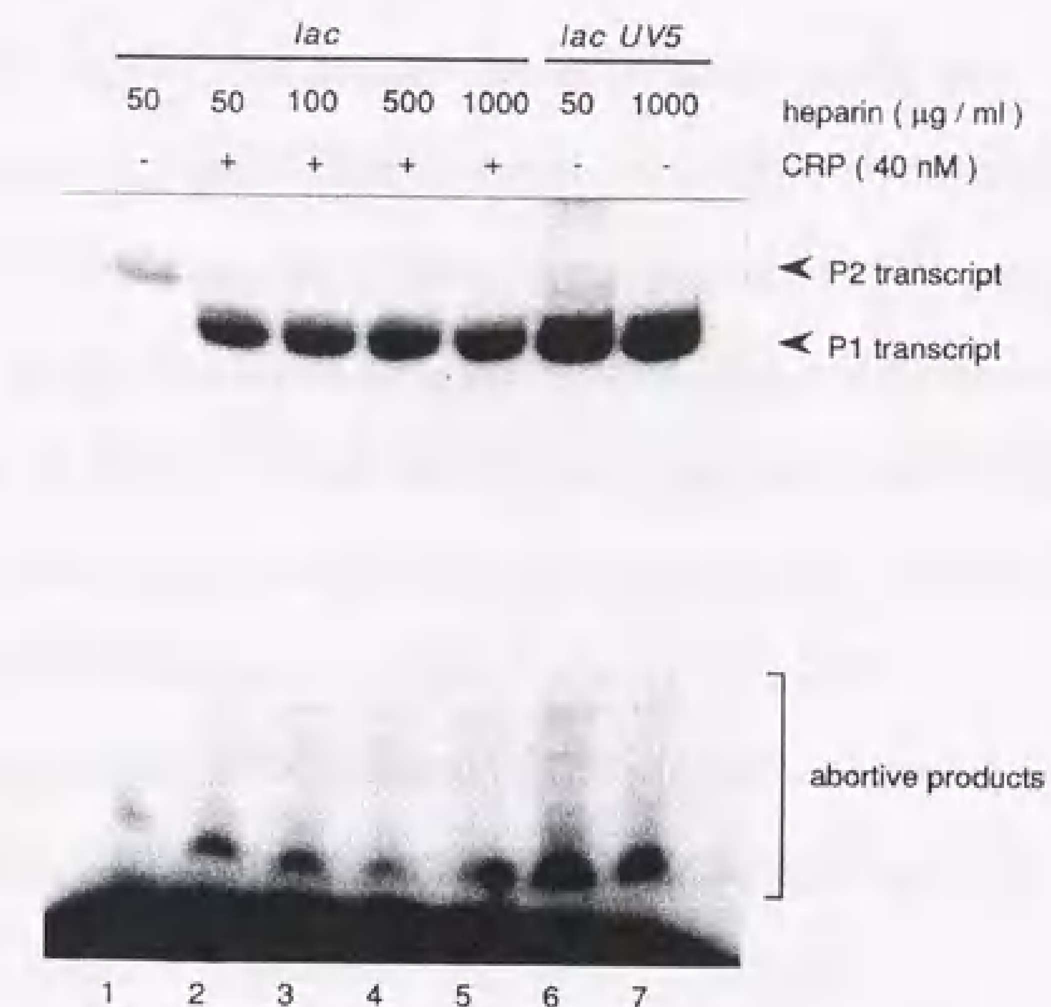

Figure 2-5. *In vitro* transcription assay of open complexes. The 185-bp *Bam*HI-*Hin*dIII fragment containing wild-type *lac* promoter (lanes 1-5) or *lac* UV5 promoter (lanes 6 and 7) was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with indicated concentrations of heparin. Transcription reaction was started by adding ribonucleotides containing [α-^{32}P]UTP. The reaction products were analyzed on a denatured 20% polyacrylamide gel. Run-off and abortive RNAs were shown by arrow heads and a bracket, respectively.

DISCUSSION

The role of CRP-cAMP in the lactose operon has been a paradigm of how a sequence specific DNA binding protein activates transcription. At the *lac* promoter, CRP-cAMP binds to a site centered at position -61.5 relative to the transcription start point and stimulates transcription either affecting the initial binding of RNA polymerase or the isomerization rate to open complex, although which step is more important is still controversial (Crothers, 1992). In any case, it is well established that CRP-cAMP facilitates the formation of the open complex that is an essential step for transcription initiation. On binding to the target site, CRP-cAMP induces a strong bend in the DNA (Schultz *et al.*, 1991; Wu and Crothers, 1984), and bindings of both RNA polymerase and CRP-cAMP to the *lac* DNA are mutually stabilized (Ren *et al.*, 1988; Straney *et al.*, 1989). Thus, DNA is believed to wrap up both CRP and RNA polymerase in the ternary open complex in which CRP is likely to be entrapped (Crothers, 1992; Zinkel and Crothers, 1991).

On the other hand, it is poorly understood whether CRP plays any roles after the formation of the open complex. In this paper, I addressed whether CRP-RNAP interaction and CRP-induced DNA bending play any roles after the formation of open complex. I specifically asked the following questions. (i) Is it possible to remove CRP from the ternary complex? (ii) If so, does it affect the binding of RNA polymerase to the promoter? (iii) What is the nature of the resulting binary complex? I found that the competition with a high concentration of heparin dissociates CRP but not RNA polymerase from the ternary open complex of the wild-type *lac* promoter. I showed that the binary complex is apparently stable and retains the activity to initiate and elongate the transcription from P1. In addition, the production of abortive RNAs was not increased by removing CRP. These results suggest that the binary open complex without CRP is functionally equivalent with the ternary open complex containing CRP

(Figure 2-6). This means that CRP-RNAP contact is not essential for the maintenance of the open complex and does not affect the promoter clearance step. In other words, CRP is no longer required for the transcription activation after the formation of the open complex.

The data presented in this study suggest an important feature of the structure of open complex. Since CRP induces the DNA bending and RNA polymerase recognizes this CRP-DNA complex to form the ternary complex, it is rather natural to assume that the removal of CRP affects the overall structure of the open complex. However, the functional integrity of the binary complex suggests that the structural characteristics of the open complex are still retained after the removal of CRP. This is strongly supported by the observation that the removal of CRP did not affect the pattern of DNase I footprint except CRP site. In addition, it is important to note that the protection/enhancement pattern of the resulting binary complex is essentially identical with that of the *lac* UV5 binary complex. This means that two open complexes are not only functionally but also structurally similar to each other.

Does the removal of CRP from the ternary complex release the DNA bending induced by CRP? The structural similarity between two binary open complexes suggests that the CRP-induced DNA bend itself is probably lost when CRP is removed. I believe that the loss of the CRP-induced DNA bend does not affect drastically the overall structure of the complex since a strong interaction between DNA and RNA polymerase is already formed to produce a stable nucleosome-like complex. A very little change in the gel mobility during the transition from the ternary to binary complex is consistent with this view. A nucleosome-like model in which the promoter is wrapped around RNA polymerase was originally proposed by Buc *et al.* (Buc, 1986) for the *lac* UV5 open complex based on the crosslinking (Chenchick *et al.*, 1981) and footprinting (Spassky *et al.*, 1985) data.

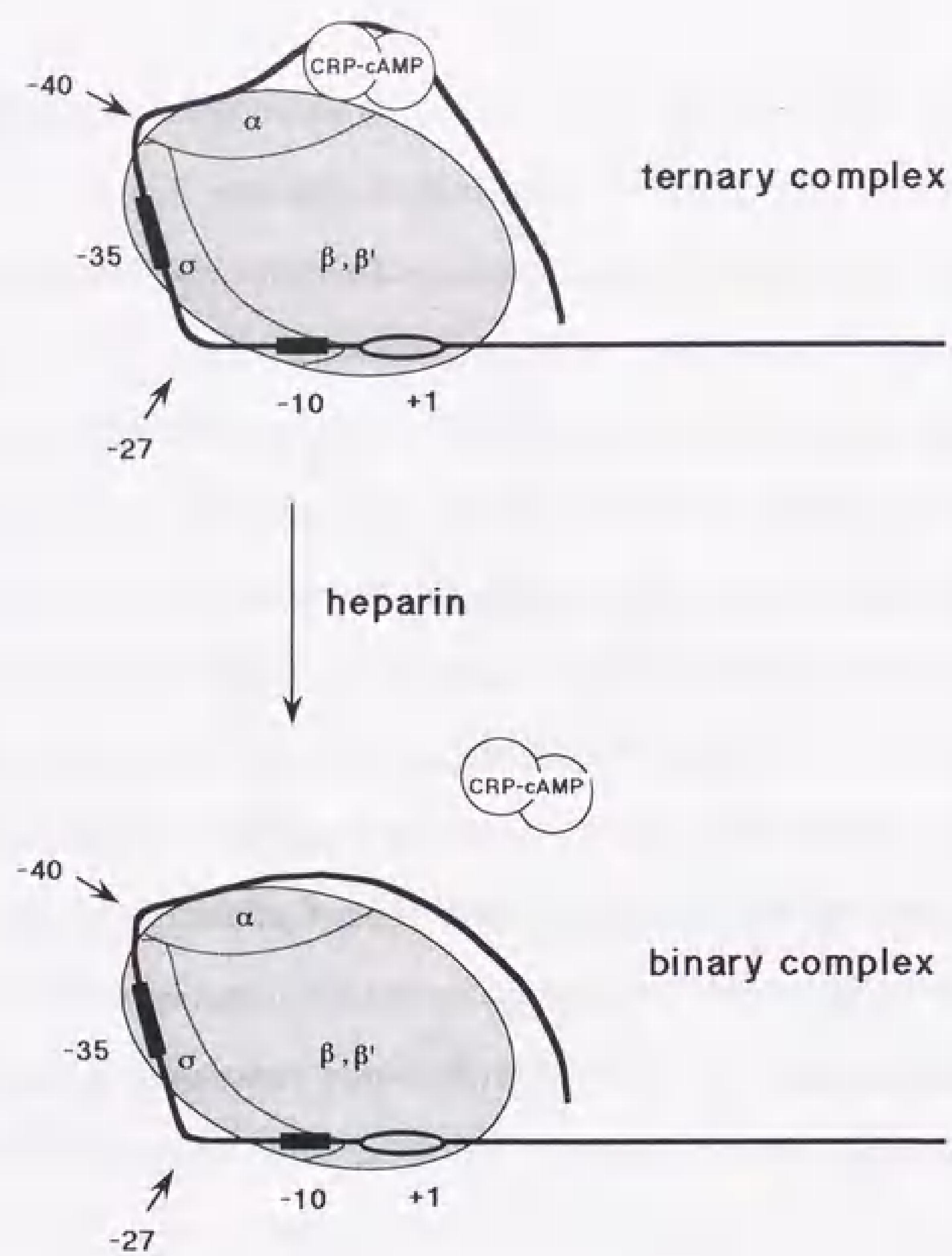

Figure 2-6. Model for the transition from ternary to binary complexes. A high concentration of heparin converts the ternary complex to the binary complex by removing CRP. A wrapping model for the complex between *lac* UV5 and RNA polymerase was originally proposed by Buc (1986). A similar model for the ternary complex of wild-type *lac* promoter in which DNA is wrapped around both CRP and RNA polymerase was described by Zinkel and Crothers (1991). We refined the previous wrapping models by incorporating the present results and recent findings concerning the role of RNA polymerase subunits. Two open complexes are essentially equivalent each other except the binding of CRP. The arrows indicate the hypersensitive sites to DNase I cleavage. Recent work has shown that the C-terminal region of α subunit binds immediately upstream of the -35 element and appears to make a direct contact with CRP (Busby and Ebright,1994). In addition, several lines of evidence suggest that s subunit is responsible for binding to -35 and -10 elements (Severinov,*et al.*, 1994).

The observations that RNA polymerase appears to induce a DNA bending in several promoters are consistent with the wrapping model (Heumann *et al.*, 1988; Kuhnke *et al.*, 1987; Meyer-Almes *et al.*, 1994; Rees *et al.*, 1993). A similar model (caging of RNA polymerase) is also described by Adhya *et al.* (Adhya *et al.*, 1993). Furthermore, the recent crosslinking study with T7 A1 promoter suggests that the spatial distance between -35 promoter region and transcription start site is closer than expected from one dimensional distance (Severinov *et al.*, 1994). Thus, it is highly possible that DNA wraps around RNA polymerase even in the wild-type binary open complex with a significant bent between -10 and -35 regions. The DNase I hypersensitive sites at -27 and -40 in the open complexes can be interpreted as the result of the DNA bending. Taken together, I propose possible structural models for the ternary and binary open complexes in which the recent findings concerning the role of α and σ subunits are also incorporated (Busby and Ebright, 1994; Gross, 1992). This model predicts that RNA polymerase may additionally contact with the upstream region of the promoter. In fact, Buckle *et al.* (Buckle *et al.*, 1992) observed a weak protection by RNA polymerase around -90 in *lac* UV5 promoter against OH radicals. However, my DNase I footprinting data do not show a clear evidence for the upstream touch, suggesting that the RNAP-upstream interaction may be rather moderate.

It should be noted that Zinkel and Crothers (Zinkel and Crothers, 1991) reported the stability of the ternary complex of a *lac* promoter derivative in which A-tract bend sequence was inserted into the region of immediately upstream of the CRP site. They found that the CRP-RNAP-DNA open complex releases easily CRP with heparin treatment. The resulting binary complex exhibited a significant increase in gel mobility, suggesting that the CRP-induced bend was lost when CRP was removed from the ternary complex. It would be interesting to test whether this binary complex still retains the nature of open complex.

The another issue to be studied is how the relative location of the CRP site affects the stability of the CRP-RNAP-DNA ternary complex. The quantitative measurement of the sensitivity of CRP binding to heparin could be a useful test to probe the architecture of the ternary open complexes in various CRP-dependent promoters. For example, it is interesting to examine the stability of CRP to heparin in the ternary complex with type II CRP dependent promoter in which the CRP site is located within the core promoter (Aiba *et al.*, 1989). In addition, it is particularly interesting to remove CRP from the ternary open complex with *malT* promoter in which CRP is reported to act by stimulating the rate of escape from the promoter (Menendez *et al.*, 1987). The characterization of both ternary and binary *malT* open complexes should give a definite answer as to the role of CRP in the promoter clearance step.

ACKNOWLEDGMENTS

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Chapter 3

A common role of CRP in transcription activation : CRP acts transiently to stimulate events leading to open complex formation at a diverse set of promoters

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SUMMARY

I showed that CRP is not required after the formation of open complex at the *lac* promoter (*Chapter 2*). Here, I have investigated the role of CRP in transcription activation at the *malT* and *galP1* promoters. At the *malT* promoter, RNA polymerase (RNAP) forms a non-productive RNAP-promoter binary complex in the absence of CRP while it forms a productive CRP-RNAP-promoter ternary complex in the presence of CRP. CRP can be removed by a moderate concentration of heparin from the *malT* ternary complex. The resulting binary complex is functionally identical to the ternary complex. At the *gal* promoter, RNAP forms predominantly a binary complex at the *P2* promoter in the absence of CRP while it forms a ternary complex at the *P1* promoter in the presence of CRP. A very high concentration heparin can dissociate CRP from the *galP1* ternary complex without changing the properties of the complex. These data indicate that CRP is not required for the maintenance of the ternary complex and plays no role in the subsequent steps, irrespective of the promoter context. I conclude that the common role of CRP in transcription activation is to stimulate events leading to the formation of a productive open complex at a diverse set of CRP-dependent promoters. I suggest that the CRP-RNAP interaction is only transiently needed for transcription activation.

INTRODUCTION

Transcription initiation is assumed to be composed of three major steps (Levin *et al.*, 1987; McClure, 1985). Firstly, RNA polymerase (RNAP) binds reversibly to promoter DNA to form a closed complex that is transcriptionally inactive. Secondly, the closed complex is converted to an open complex in which a short region of DNA around the transcription start site is unwound. The open complex is able to produce a series of short RNAs abortively in the presence of ribonucleotides. The third step is the promoter clearance in which RNAP escapes from the promoter with a loss of σ factor to form a stable elongation complex. The efficiency of each of these steps can be subject to regulation by transcriptional activator or repressor proteins.

The cyclic AMP receptor protein (CRP; also known as CAP, catabolite gene activator protein) of *Escherichia coli* is involved in the regulation of transcription of a large number of genes either positively or negatively in response to carbon nutrient conditions (Botsford and Harman, 1992; Kolb *et al.*, 1993a). The protein is a dimer of two identical subunits composed of 209 amino acids (Aiba *et al.*, 1982) When complexed with its allosteric effector cAMP, CRP undergoes a conformational transition and binds to a specific sequence of 22 bp located within or near target promoters to regulate transcription (Botsford and Harman, 1992; Kolb *et al.*, 1993a). The CRP binding sites lie at different locations relative to the transcription start site in various promoters. The promoters where CRP alone is sufficient for activation can be divided into two groups: type I (also referred to as Class I) promoter where the CRP binding site is located upstream of the -35 region; type II (Class II) promoter where the CRP binding site overlaps the -35 region (Aiba *et al.*, 1989; Ebright, 1993). The *lac* and *malT* promoters are examples of type I promoter, each of which has a CRP binding site at position -61.5 and -70.5, respectively. The prototype of type II promoter is the *galP1* promoter which has a CRP binding site at position -41.5.

The mechanism of transcription activation by CRP has been extensively studied as a paradigm for understanding how a single activator can act. Numerous studies indicate that the interaction between CRP and RNAP plays a pivotal role in transcription activation at CRP-dependent promoters (Busby and Ebright, 1994; Busby and Ebright, 1997; Kolb *et al.*, 1993a) In particular, recent genetic and biochemical studies of CRP and RNAP have identified particular sites or regions located at the surface of both proteins which are involved in protein-protein interaction and thereby responsible for transcription activation. Isolation and characterization of CRP mutants that bind normally to the target DNA but fail to activate transcription have established that a surface exposed loop around amino acids 156-164 of CRP constitutes an activating region essential for transcription activation at both type I and type II promoters (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Zhou *et al.*, 1993). Studies of RNAP α-subunit mutants have revealed that the C-terminal domain of α-subunit is involved in the interaction with the activating region of CRP (Igarashi and Ishihama, 1991). An additional contact between the N-terminal domain of CRP and the N-terminal domain of RNAP α-subunit is involved in transcription activation at type II promoters (Niu *et al.*, 1996). CRP is also known to induce a sharp bend in the DNA which may play a role in transcription activation (Crothers, 1992; Dethiollaz *et al.*, 1996; Wu and Crothers, 1984).

Despite the remarkable advances in the understanding of the action of CRP, several important questions remain to be resolved. For example, it is not well understood how the CRP-RNAP interaction can lead to transcription activation. The related question is which step(s) of transcription initiation is modulated by the CRP-RNAP interaction. It has been believed that CRP can affect different steps depending on the relative location of the CRP binding site in the promoter. Kinetic studies have claimed that CRP enhances initial binding of RNAP at the *lac* promoter (Malan *et al.*, 1984), stimulates both initial binding and isomerization to the open complex at the *gal*

promoter (Herbert *et al.*, 1986; Lavigne *et al.*, 1992), and accelerates the rate of promoter clearance at the *malT* promoter (Eichenberger *et al.*, 1997; Eichenberger *et al.*, 1996; Menendez *et al.*, 1987). It should be noted, however, that CRP is inferred to affect predominantly the isomerization step at the *lac* promoter in another study (Straney *et al.*, 1989). This suggests that our understanding of which step(s) of transcription initiation is affected by CRP is not yet certain. In particular, much less is known about the role of CRP-RNAP interaction in the steps after the formation of open complex.

I demonstrated that removal of CRP from the *lac* ternary open complex by a high concentration of heparin did not change the structural and functional properties of the complex (*Chapter 2*). This finding clearly established that CRP-RNAP interaction is no longer necessary for the maintenance of open complex and for any other steps after the formation of open complex. In this chapter, I performed the heparin challenge experiment on the *malT* and *gal* promoters to examine the role of CRP in transcription activation at a diverse set of CRP-dependent promoters. I show evidence that the ternary open complex containing CRP at two promoters can be converted to the respective binary complex although the concentration of heparin required for the removal of CRP substantially different from that at the *lac* promoter. The resulting binary complexes retained the same properties as those of the ternary complexes, indicating that CRP has no role in maintenance of the open complex and in the promoter clearance at the *malT* and *galP1* promoters. I conclude that the major target of CRP action is restricted up to the formation of productive open complex irrespective of the location of the CRP binding site. I suggest that the CRP-RNAP interaction is only transiently needed for transcription activation.

MATERIALS AND METHODS

DNA and proteins

DNA fragments used in this study are shown in Figure 3-1. The *malT* promoter region was amplified by PCR from the chromosomal DNA of wild-type *E. coli* strain PP6 (Aiba *et al.*, 1981) using two synthetic primers: 5'-TATCCAGTGTGCTCCATCTC-3' and 5'-GCTCACGAACCACGGTATGG-3'. The amplified DNA was digested with *Sau*3AI and *Hpa*II. The resulting 208 bp *Sau*3AI-*Hpa*II fragment containing the *malT* promoter region was cloned between the *Bam*HI and *Acc*I sites of pUC19 to construct pMT100. The 247 bp *Eco*RI-*Hin*dIII fragment containing the *malT* promoter region was prepared from pMT100 and used for most experiments. The 230 bp *Sac*I-*Hin*dIII *malT* fragment was used only for DNase I footprinting experiment. The 240 bp *Hpa*II fragment containing the *gal* promoter region derived from plasmid pBdC1 (DiLauro *et al.*, 1979) was cloned into the *Hinc*II site of pUC119 to construct pGAL100. The 167 bp *Hha*I-*Eco*RI fragment carrying the *gal* promoter region was prepared from pGAL100. CRP was purified from cells harboring pHA7 (Aiba *et al.*, 1982) by the procedure of Eilen *et al.* (Eilen *et al.*, 1978). RNA polymerase was purified from strain W3350 according to the method of Fukuda *et al.* (Fukuda and Ishihama, 1974).

Gel shift assay

The gel mobility shift assays were performed in a total volume of 30 μl of transcription buffer (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 50 μg/ml bovine serum albumin) containing 50 μM cAMP and 5 % glycerol. The DNA fragments of a final concentration of 5 nM were incubated first with CRP (0 or 50 nM) for 5 min at 37°C and then with RNA polymerase (30 nM) for 30 min. Then the mixture was treated with 3 μl of heparin (final concentration of 2-6400 μg/ml) for 3 min. 5 μl of the mixture was fractionated by electrophoresis on a native 5 %

polyacrylamide gel containing 0.1 mM cAMP in 1/2 TBE at room temperature. The gel was stained with 5 μg/ml ethidium bromide solution and exposed on UV lamp. The reversed photographs were used for Figures.

Western blotting

The gels used above were subjected to Western blotting as described (*Chapter 2*). Polyclonal anti-CRP antibody (Ishizuka *et al.*, 1994) was used to probe CRP on the membrane.

DNase I footprinting

The reactions were performed in a total volume of 100 μl of a transcription buffer containing 5 mM $CaCl_2$, 50 μM cAMP and 5 % glycerol. The ^{32}P-end-labeled DNA fragments of a final concentration of 1 nM were incubated with CRP (0 or 110 nM) for 5 min and then incubated with RNA polymerase (0 or 110 nM) for 30 min at 37°C. The mixtures were treated with heparin (0-50 μg/ml) for 3 min at 37°C and placed at 25°C for 5 min. DNase I was added at a concentration of 50 ng/100 ml and incubation was continued for 1 min at 25°C. After addition of 25 μl of 1.5 M sodium acetate, 20 mM EDTA, 100 μg/ml tRNA, the mixture was treated with phenol and precipitated with ethanol, dissolved in 8 M urea loading buffer (0.025 % bromophenol blue and 0.025 % xylene cyanol in TBE). The products were analyzed on an 8 % polyacrylamide-8 M urea gel.

In vitro transcription

The run-off transcription assays were performed in a total volume of 30 μl of transcription buffer containing 50 μM cAMP and 5 % glycerol. The open complexes were formed under the same condition as in the case of gel shift assays. The transcription was started by adding 3 μl of a substrate solution of 0.5 mM 4NTPs containing [α-^{32}P] UTP (5 μCi). After 15 min of incubation at 37°C, the reaction was terminated by addition of 60 μl of phenol, 30 μl of 0.6 M sodium acetate (pH 5.5), 20 mM EDTA, and 200 μg/ml tRNA. The products were precipitated with ethanol and fractionated by electrophoresis on 8 % or 20 % polyacrylamide gels containing 8 M urea. For the abortive initiation assay, the *Eco*RI-*Hin*dIII fragment containing *malT* promoter (5 nM) in 30 μl of transcription buffer containing 50 μM cAMP and 5 % glycerol was incubated with RNA polymerase (30 nM) in the absence and the presence of CRP (50 nM) as mentioned above. The reaction was started by adding 3 μl of a solution containing 2 mM ApU and 0.1 mM [α-^{32}P] UTP (10 μCi). Following the incubation at 37°C, the products were precipitated with ethanol and analyzed by electrophoresis on a 20 % polyacrylamide gel containing 8 M urea.

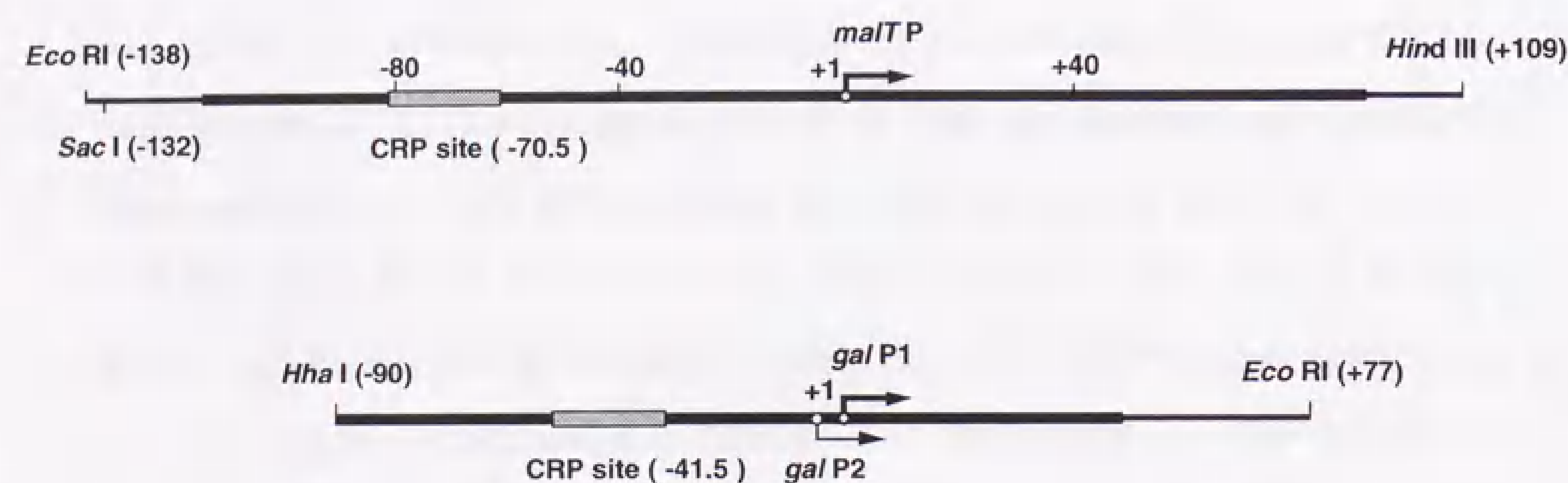

Figure 3-1. Promoter fragments used in this study. The upper and lower lines represent the *malT* and *gal* promoter regions derived from plasmid pMT100 and pGAL100, respectively. The thick lines correspond to the native promoter regions and the thin lines are derived from the vectors. The relevant restriction sites are shown. The transcription start sites for *malT* and *galP1* are numbered as +1. The numbers in the parentheses represent the distance in base pairs relative to the transcription start site. The hatched boxes represent the CRP binding site.

RESULTS

Transcription complexes at the malT promoter

In order to examine the role of CRP in transcription activation at the *malT* promoter, a DNA fragment containing the *malT* promoter was incubated with RNAP in the presence and the absence of CRP. Then, the protein-DNA complexes were treated with increasing concentrations of heparin and the products were analyzed by a gel mobility shift assay (Figure 3-2A). RNAP formed a binary complex, which is resistant to heparin, even in the absence of CRP (Figure 3-2A, lanes 3-7). When the *malT* promoter was incubated with RNAP and CRP without heparin treatment, an extremely retarded complex was formed which presumably corresponds to an aggregate (Figure 3-2A, lane 8). In the presence of a low concentration (2 μg/ml) of heparin two major complexes were observed (Figure 3-2A, lane 9). Increasing concentrations of heparin reduced the amount of the upper band and increased that of the lower one (Figure 3-2A, lanes 9-12). I assumed that the upper band corresponds to a ternary complex containing both RNAP and CRP while the lower one is a binary complex without CRP. This assumption was directly examined by Western blot analysis of the gel. As shown in Figure 3-2B, CRP was detected only in the upper complex, indicating that CRP was dissociated from the *malT* ternary complex by heparin treatment. To examine the effect of CRP on the stability of the complex, I changed the incubation time after the addition of heparin. Although increasing incubation times reduced the amount of the complexes, no significant difference in the decay rate was observed between binary and ternary complexes (Figure 3-2C). In other words, CRP did not affect the stability of the open complex. The concentration of heparin required for the complete dissociation of CRP from the *malT* ternary complex was 50 μg/ml which is about 10-fold less than that at the *lac* promoter (*Chapter 2*). It should be noted that the presence of CRP did not increase

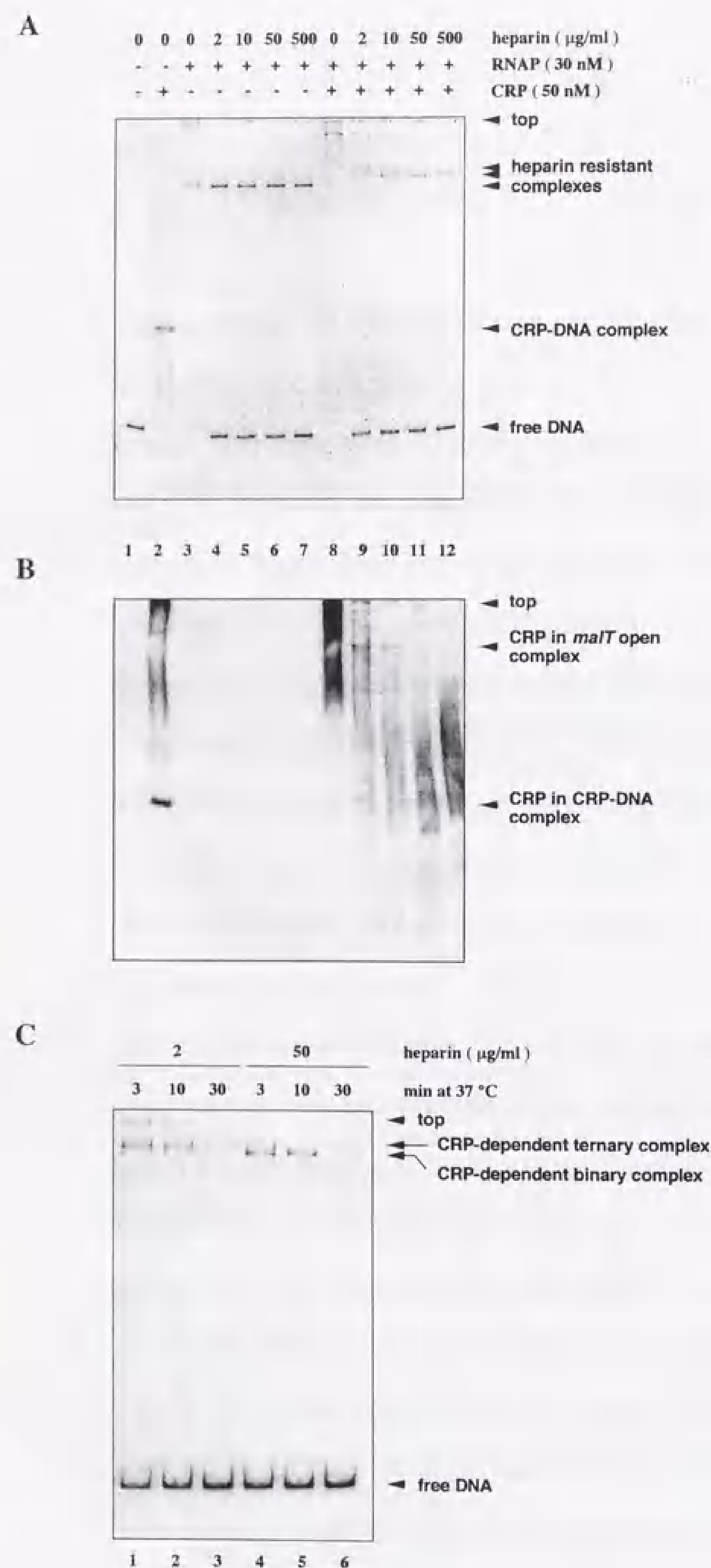

Figure 3-2. Effect of increasing concentrations of heparin on transcription complexes at malT the promoter. (A) Gel mobility shift analysis. The 247 bp EcoRI-HindIII fragment containing the malT promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with indicated concentrations of heparin for 3 min and was analyzed by electrophoresis in a 5% polyacrylamide gel containing cAMP. (B) Immunoblot analysis using an anti-CRP antibody. The gel shown in (A) was directly used for Western blotting. The arrow indicates the existence of CRP in the CRP-dependent malT open complex. (C) Stability of transcription complexes. The DNA fragment containing the malT promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with 2 μg/ml (lanes 1-3) or 50 μg/ml (lanes 1-3) of heparin for indicated times and was analyzed by electrophoresis in a 5% polyacrylamide gel containing cAMP.

the amount of the heparin resistant complex itself. An important observation is that the CRP-dependent binary complex, derived from the ternary complex by the heparin treatment, exhibited a decreased mobility compared to the CRP-independent binary complex. This indicates that two binary complexes are structurally different each other.

Protein-DNA interaction in the complexes at the malT promoter

To characterize the *malT* complexes further, I performed a DNase I footprinting experiment. Figures 3-3A and 3-3B show the footprinting patterns of the coding and template strands, respectively. CRP binds to a region between -60 and -80 in the presence of cAMP as reported by Chapon and Kolb (Chapon and Kolb, 1983). When RNAP alone was incubated with the *malT* DNA, a region between +20 and -60 was protected against DNase I digestion. Several sites both in the coding and template strands exhibited an enhanced cleavage (Figures 3-3A and 3-3B, lane 4). The presence of heparin caused little change in the protection/enhancement pattern (Figure 3-3A, lanes 5 and 6; Figure 3B, lane 5). In the presence of both CRP and RNAP, the protection was extended to the CRP site at both strands (Figure 3-3A, lane 7, and 3-3B, lane 6). The protection/enhancement pattern at the CRP binding site was almost lost in the presence of 50 μg/ml of heparin (Figure 3-3A, lane 9, and 3-3B, lane 7). On the other hand, the binding of RNAP to the *malT* promoter was essentially retained after the heparin treatment although the overall protection signal was reduced due to an increase in the amount of free DNA. It should be noted that the protection/enhancement pattern and strength of band signals in the CRP-dependent binary complex is significantly different from those of CRP-independent complex, especially in the region between -20 and -60, although RNAP appears to occupy the same DNA region in two complexes (Figure 3-3A, lanes 6 and 9; 3-3B, lanes 5 and 7). The data suggest that CRP modulates the interaction between RNAP and DNA in the left half of the *malT* promoter.

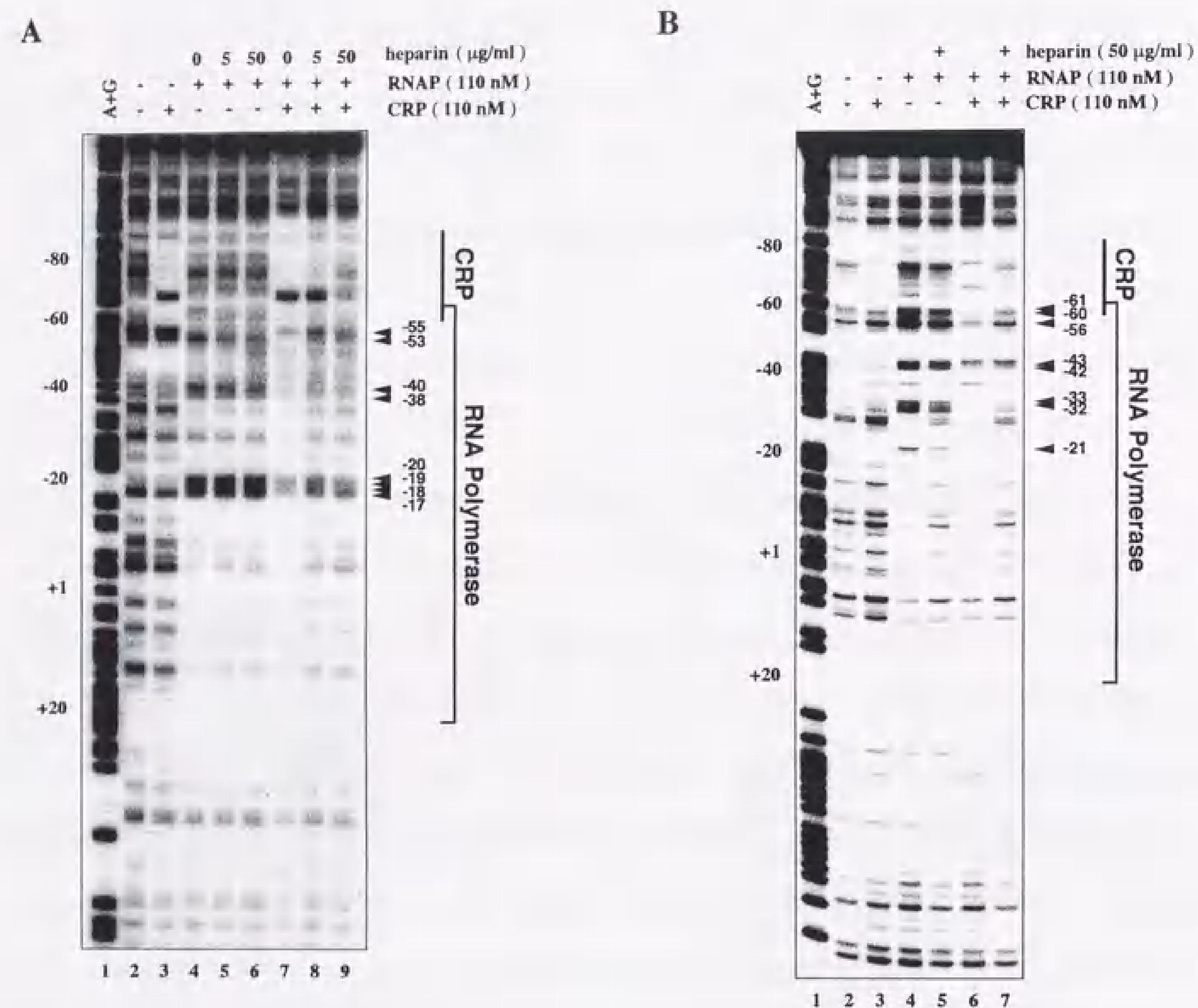

Figure 3-3. DNase I footprinting analyses of the *malT* promoter fragments. The 247 bp *Eco*RI-*Hin*dIII fragment ^{32}P-labeled at its 3' *Hin*dIII end (coding strand) (A) and 230 bp *Sac*I-*Hin*dIII fragment ^{32}P-labeled at its 5' *Hin*dIII end (template strand) (B) were uesd. Lane 1 is the products of A + G chemical reaction. The protected/enhanced regions by CRP-cAMP and/or RNA polymerase are shown by vertical lines and brackets, respectively. Asterisks mark the sites where a significant difference is observed between the CRP-independent binary complex (A, lane 6 and B, lane 5) and CRP-dependent binary complex (A, lane 9 and B, lane 7). Numbers on the left indicate positions relative to the transcription start site of the *malT* promoter.

Transcriptional activity of the complexes at the malT promoter

A major role of CRP at the *malT* promoter has been believed to facilitate the escape of RNAP from the promoter (Eichenberger *et al.*, 1997; Menendez *et al.*, 1987). If this were the case, removal of CRP from the ternary complex would reduce the amount of the run-off transcript and increase the amount of abortive transcripts. To examine this, both CRP-independent and CRP-dependent transcription complexes were formed under the same condition of the gel mobility shift assay mentioned above. After treating the complexes with increasing concentrations of heparin, a single round run-off transcription reaction was started by adding ribonucleotides. The CRP-independent complex has little ability to make the run-off transcript (Figure 3-4A, lanes 2) while the CRP-dependent complex produced a significant amount of the run-off transcript (Figure 3-4A, lane 7). When transcription was performed without heparin treatment, the transcripts increased significantly, as expected (Figure 3-4A, lanes 1 and 6). When CRP was completely removed from the ternary complex with increasing concentrations of heparin, the amount of the run-off transcript was unaffected (Figure 3-4A, lanes 6-8). I also observed that the rate of production of the run-off transcript was not affected by the presence of CRP (unpublished result). These results clearly indicate that CRP has no role in transcription activation in any steps following the formation of open complex at the *malT* promoter.

Since the complex at the *malT* promoter formed in the absence of CRP is reported to have a significant activity in abortive initiation assays (Menendez *et al.*, 1987), I also performed the abortive initiation assay with the *malT* complexes. The RNAP-promoter complexes were formed both in the presence and the absence of CRP and the abortive synthesis was started by adding a dinucleotide ApU and [α-^{32}P]UTP. The synthesis of the trinucleotide ApUpU, which corresponds to a transcript originated from the *malT* promoter, was analyzed. As shown in Figure 4-4B, the abortive product

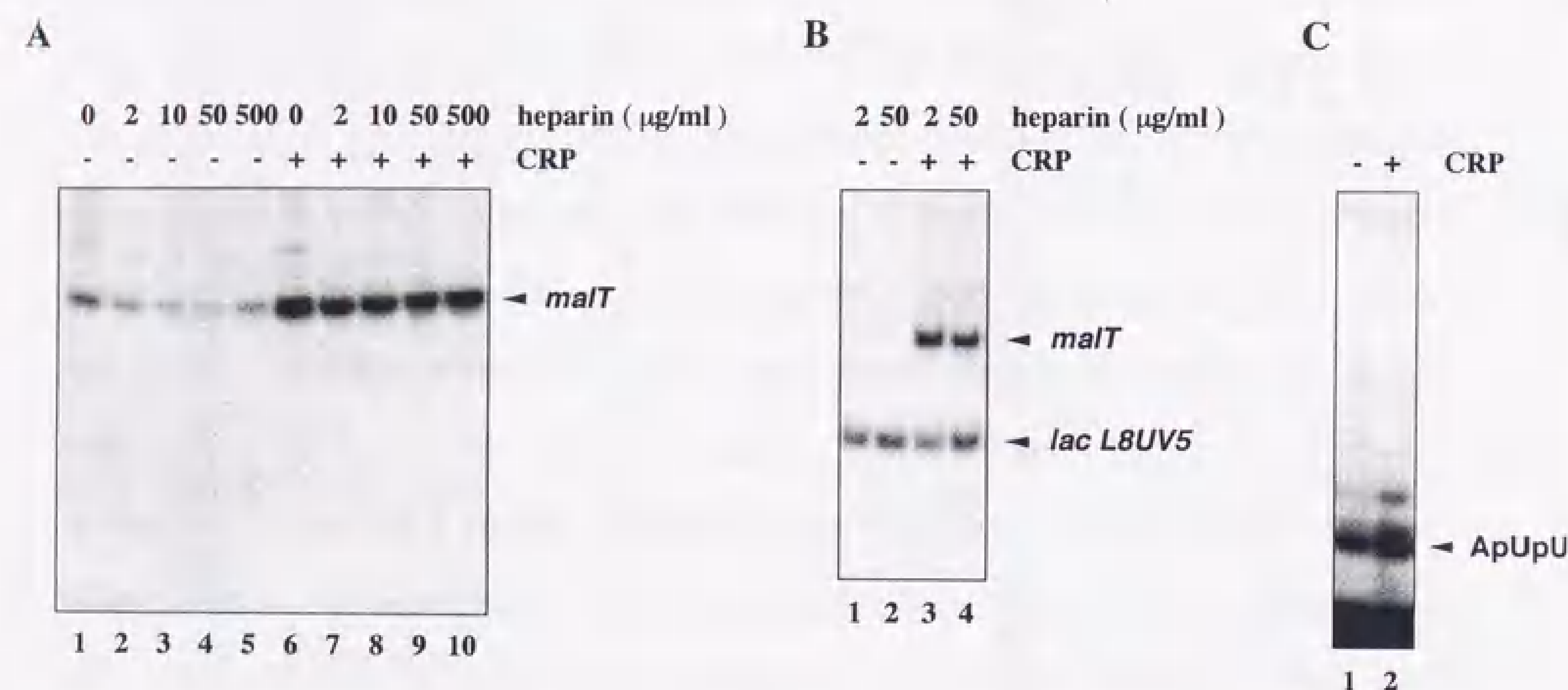

Figure 3-4. Transcriptional activities of complexes at the *malT* promoter. (A) *In vitro* run-off transcription assay. The 247 bp *Eco*RI-*Hin*dIII fragment containing the *malT* promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with indicated concentrations of heparin for 3 min. The transcription reaction was started by adding ribonucleotides containing [α-^{32}P]UTP. The products were analyzed by electrophoresis on a 8% polyacrylamide gel containing 8M urea. (B) Abortive initiation assay. The transcription complexes were formed as same condition of (A). The abortive reaction was started with the addition of ApU and [α-^{32}P]UTP to final concentrations of 0.2 mM and 0.1 mM, respectively. The reaction mixtures were incubated for 15 min and the products were analyzed by electrophoresis on a 20% polyacrylamide gel containing 8M urea.

was clearly produced both in the presence and the absence of CRP. This result is consistent with that of the previous report. Thus, the CRP-independent heparin resistant complex has an ability to produce abortive RNAs but not to make the run-off transcript. I conclude that the major role of CRP in transcription activation at the *malT* promoter is to stimulate events leading to the formation of productive transcription complex and to prevent the formation of non-productive one. Thus, the promoter clearance step *per se* can not be the major target for CRP action.

Transcription complexes at the gal promoter.

I next investigated the role of CRP at the *gal* promoter which is a prototype of the type II CRP-dependent promoter. Figure 3-5A shows a gel mobility shift assay of transcription complexes formed at the *gal* promoter with increasing concentrations of heparin. It is well known that RNAP preferentially binds to the *galP2* promoter in the absence of CRP while it binds to the *galP1* promoter in the presence of CRP (Aiba *et al.*, 1981; DiLauro *et al.*, 1979). Although RNAP formed the complex more efficiently in the presence of CRP, no difference in the mobility between the CRP-dependent (*P1*) and CRP-independent (*P2*) complexes was observed (Figure 3-5A). The increasing concentrations of heparin affected neither the mobility nor the amount of the two complexes. Western blot analysis revealed that the content of CRP in the *galP1* complex was reduced by the addition of increasing concentrations of heparin (Figure 3-5B). However, a significant amount of CRP was still detected in the complex even when 1.6 mg/ml of heparin was used. About 6 mg/ml of heparin was required to remove most of the CRP from the ternary complex. Thus, CRP can be selectively removed from the ternary complex at the *galP1* promoter. The increasing concentrations of heparin affected neither the mobility or the amount of the complex, suggesting that removal of

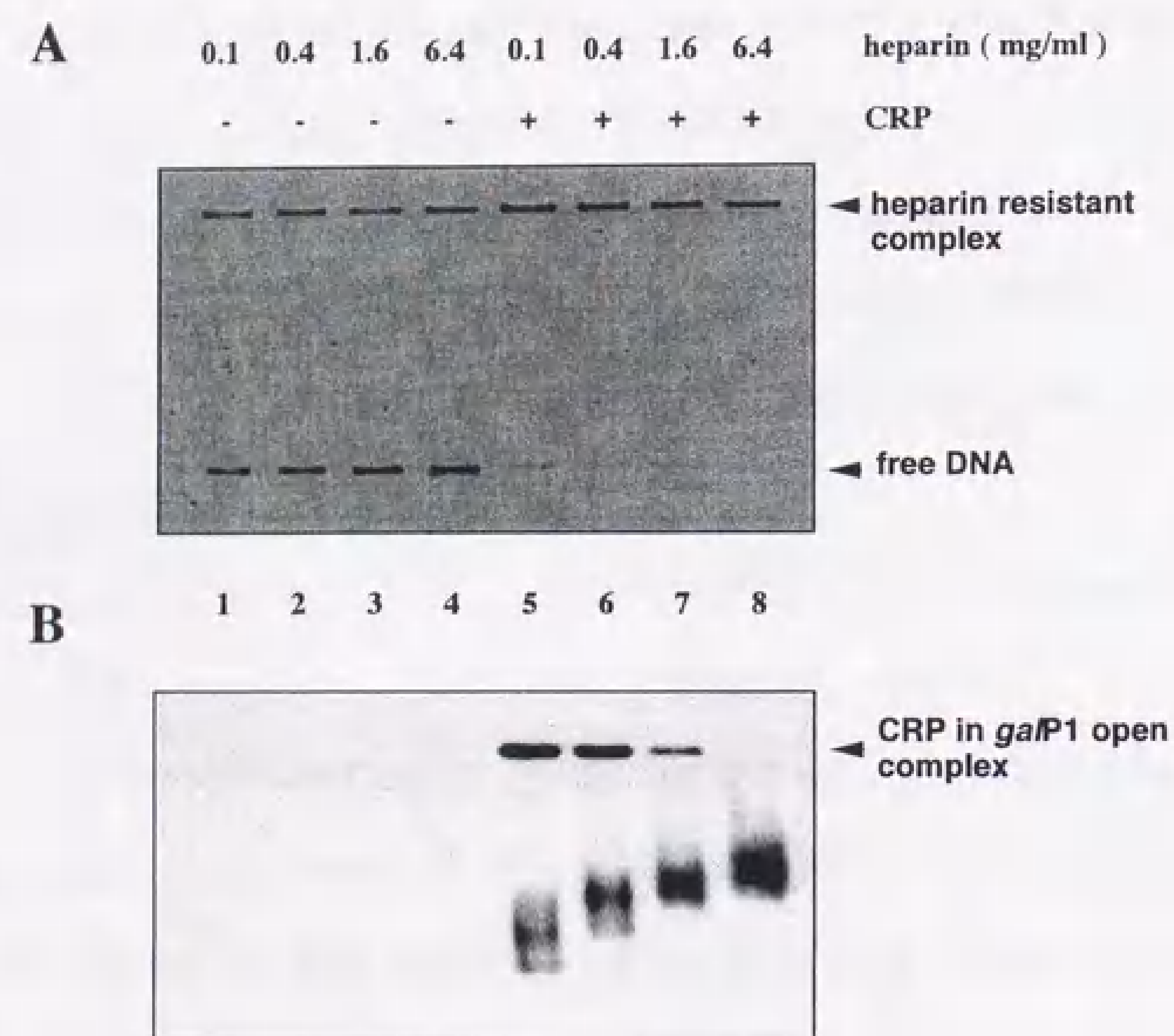

Figure 3-5. Effect of increasing concentrations of heparin on transcription complexes at the *gal* promoter. (A) Gel mobility shift analysis The 167 bp *HhaI-EcoRI* fragment containing the *gal* promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with indicated concentrations of heparin for 3 min and was analyzed by electrophoresis in a 5% polyacrylamide gel containing cAMP. (B) Immunoblot analysis using an anti-CRP antibody. The gel shown in (A) was directly used for Western blotting. The arrow indicates the existence of CRP in the *galP1* open complex.

CRP does not change drastically the overall structure and the stability of the open complex.

Transcriptional activity of the complexes at the gal promoter

To examine the transcriptional activity of the *gal* complexes, a run-off transcription assay was performed. As shown in Figure 3-6, two run-off transcripts were produced in the absence of CRP. As well-known, the upper and lower bands correspond to the *P2* and *P1* transcripts, respectively (Aiba *et al.*, 1981). In addition, a large amount of short RNAs were produced in the absence of CRP (Figure 3-6, lane 1). As expected, CRP stimulated the *P1* transcription while it inhibited the *P2* transcription. The presence of high concentrations of heparin did not affect the transcription profile although it affected nonspecifically the mobility of the transcripts when 6.4 mg/ml heparin was used (Figure 3-6, lanes 1-4). Thus, the binary complex without CRP, which was produced from the *galP1* ternary complex with the heparin treatment, retained the ability to produce the *P1* run-off transcript. In addition, neither the run-off *P2* transcript nor *P2*-specific short RNAs were produced in the CRP-dependent binary complex. In other words, the *P1* complex was not converted to the *P2* complex by the removal of CRP. This means that once formed the *P1* open complex no longer needs CRP for any subsequent steps as in the case of the *lac* and *malT* promoters.

DISCUSSION

CRP can activate transcription at different locations relative to the transcription start point when a correct helical phasing is maintained between CRP and RNAP (Gaston *et al.*, 1990; Ushida and Aiba, 1990). The positions around -41, -61, and -71 are three favorable positions for CRP action and they correspond with those at naturally

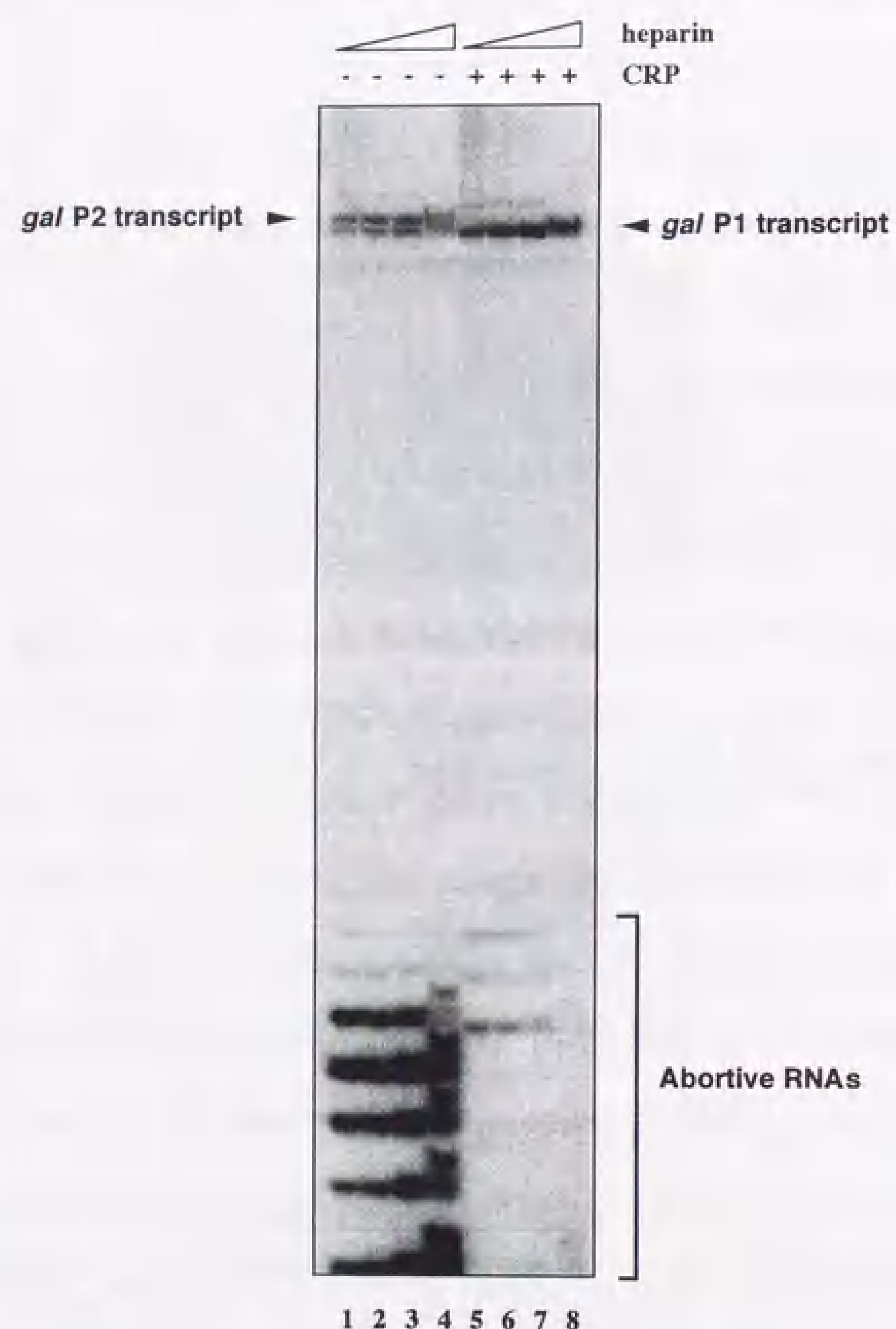

Figure 3-6. *In vitro* transcription assay of complexes at the *gal* promoter. The 167 bp *HhaI-EcoRI* fragment containing the *gal* promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with increasing concentrations of heparin (0.1, 0.4, 1.6 and 6.4 mg/ml) for 3 min. The transcription reaction was started by adding ribonucleotides containing [α-^{32}P]UTP. The products were analyzed by electrophoresis on a 20% polyacrylamide sequence gel containing 8M urea. Run-off and abortive RNAs were shown by arrow heads and a bracket, respectively.

occurring promoters represented by the *gal*, *lac*, and *malT* promoters, respectively. I showed previously based on a heparin challenge experiment that CRP is no longer required for the transcription activation after the formation of open complex (*Chapter 2*). The major advantage of this approach is that one can directly examine the role of CRP and CRP-RNAP interaction in steps after open complex formation. By using this approach, I now investigated the role of CRP and CRP-RNAP interaction in transcription activation at the *malT* and *gal* promoters in which CRP are reported to act by different mechanisms from that in the *lac* promoter.

The kinetic studies have suggested that CRP may increase the rate of escape from the promoter at the *malT* promoter (Eichenberger *et al*., 1997; Menendez *et al*., 1987). On the contrary, it has been suggested that the mechanism of transcription activation at the *malT* may be similar with that at the *lac* promoter because the same activating region of CRP and the C-terminal domain of α-subunit are involved in CRP-RNAP interaction at two promoters (Busby and Ebright, 1994; Ebright, 1993). Here I showed that CRP can be removed from the ternary *malT* open complex by heparin and that the resulting binary complex is functionally equivalent to the ternary complex (Figure 3-4). This clearly established that CRP is no longer necessary for the transcription activation after the formation of CRP-dependent open complex at the *malT* promoter. Thus, I conclude that the promoter clearance step can not be the major target for CRP action. It should be noted that a moderate concentration of heparin was sufficient to remove CRP from the ternary complex. This suggests that CRP is loosely entrapped into the ternary complex at the *malT* promoter compared to the *lac* promoter.

How is my conclusion reconciled with the previous report by Menendez *et al*. (Menendez *et al*., 1987)? They found that open complexes were formed at the *malT* promoter with an equivalent efficiency whether CRP was present or not in the abortive initiation assays while the yield of the full-length of transcript in the run-off transcription assay was markedly enhanced in the presence of CRP. I also observed that

RNAP can bind to the *malT* promoter to form a stable heparin resistant complex that has the ability to make an abortive transcript but not the run-off transcript in the absence of CRP. An important finding is that the CRP-independent complex is different structurally and functionally from the CRP-dependent binary complex. The identification of two different binary complexes can easily explain the apparent discrepancy between the two studies. My results clearly indicate that a major role of CRP at the *malT* promoter is to stimulate events leading to the formation of productive open complex and there is no further role for CRP once the productive open complex formed.

How does CRP lead to the formation of productive open complex at the *malT* promoter? Since RNAP can form a stable heparin resistant complex even in the absence of CRP and this CRP-independent complex binds to the same *malT* promoter region as the CRP-dependent complex does, the initial binding of RNAP to the promoter may not be the major target for CRP action. The *malT* promoter has an A/T-rich sequence between the -35 region and the CRP binding site that is similar to the UP-element found in the *rrnBP1* promoter (Ross *et al.*, 1993). In fact, it has been shown recently that the interaction between the C-terminal domain of α-subunit and the A/T-rich region at the *malT* promoter may play a role in the formation of the CRP-independent transcription complex (Dethiollaz *et al.*, 1996; Eichenberger *et al.*, 1997). Taken together, I suggest that CRP stimulates the formation of productive open complex by modulating the RNAP-promoter interaction at the *malT* promoter. It is likely that the role of CRP is to reposition the C-terminal domain of α-subunit from one location to another. Although I observed that the CRP-independent non-productive complex can be converted to the productive one by the addition of CRP (unpublished results), it is not clear yet whether the non-productive complex is an intermediate for the productive ternary complex or it is a product in a branched pathway in the early stage of transcription (Kubori and Shimamoto, 1996). In any case, further characterization of the CRP-independent binary complex will be needed to understand what CRP is exactly doing at the *malT* promoter.

I also investigated the role of CRP-RNAP interaction at the *gal* promoter as a representative of type II CRP-dependent promoter. The role of CRP in the transcription of *gal* operon is to activate one promoter, *P1* and to repress the other one, *P2* (Aiba *et al.*, 1981; DiLauro *et al.*, 1979). The major effect of CRP at the *galP1* appears to accelerate the rate of isomerization to the open complex (Herbert *et al.*, 1986). However, little is known whether CRP plays any other roles after the formation of open complex. CRP appears to be tightly entrapped into the open complex through the multiple interactions with RNAP (Niu *et al.*, 1996). I succeeded in removing CRP selectively from the *galP1* open complex by using a very high concentration of heparin which was 10-fold higher than that at the *lacP1* promoters. The removal of CRP from the *galP1* open complex caused no change in its mobility and amount. I conclude that the CRP-RNAP interaction does not contribute to the maintenance of the *galP1* open complex. In addition, the resulting binary complex retained the ability to initiate and elongate the *galP1* transcription, indicating that the CRP-RNAP interaction is not required for the subsequent steps after the formation of open complex at the *gal* promoter.

Figure 3-7 represents a summary of the present study. It has been suggested that the RNAP-promoter open complex forms a nucleosome-like structure in which the promoter DNA wrapped around RNAP (see references in *Chapter 2*) The architecture of ternary open complexes at the *malT*, *lac*, and *gal* promoters must be different from one another with respect the CRP-RNAP interaction. This is because the concentration of heparin needed to dissociate CRP from the ternary complex markedly decreases with increasing the distance between the CRP site and the transcription start site. This indicates that the stability of CRP-RNAP-DNA interaction in the ternary complex at the *malT*, *lac*, and *gal* promoters increases in that order. Thus, the location of the CRP binding site certainly determines the structural property of the ternary open complex. However, I would like to point out striking similarities among the transcription complexes formed at three representative CRP-dependent promoters. They are: 1) in the

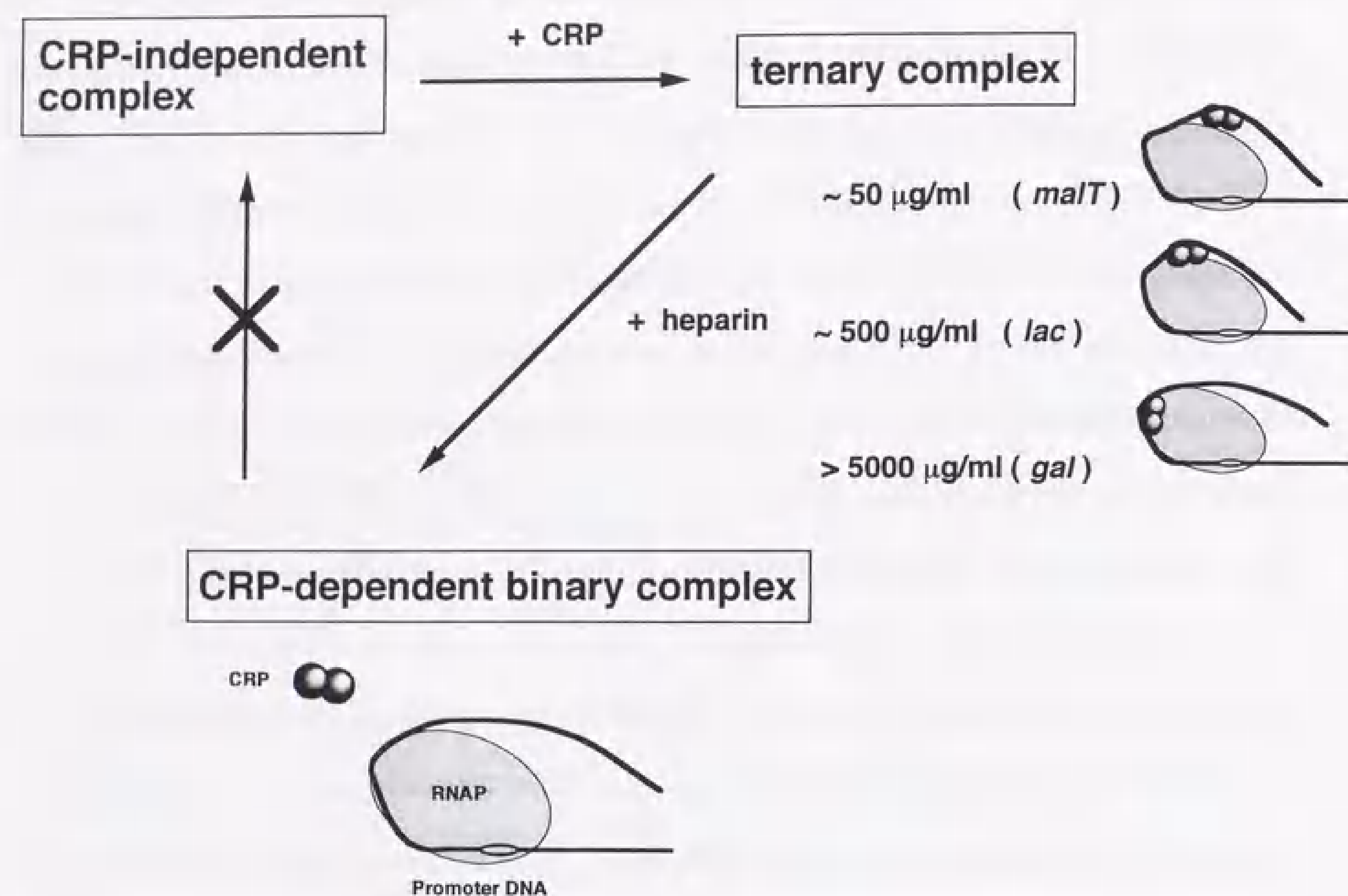

Figure 3-7. Common properties of transcription complexes at *malT*, *lac*, and *gal* promoters. RNAP forms a CRP-independent binary complex in the absence of CRP while it forms a CRP-dependent ternary complex in the presence of CRP at three representative CRP-dependent promoters. The CRP-independent binary complex is inactive (at the *malT* promoter) or less active (at the *lac* and *gal* promoters). The ternary complex is fully competent for transcription at three promoters. CRP can be removed from the ternary complex by heparin, resulting in an active binary complex that is functionally equivalent to the parent ternary complex. The concentration needed to dissociate CRP varies depending on the promoters. Models for the geometry of CRP-dependent ternary and binary complexes are depicted. The transition from the CRP-dependent binary complex to the CRP-independent binary complex never occurs. In contrary, the CRP-independent binary complex can be converted to the ternary complex. A common major role of CRP in transcription activation at three promoters is to stimulate events leading to the formation of the transcriptionally active ternary complex.

absence of CRP, RNAP forms a CRP-independent binary complex that is either non-productive (*malT* case) or less active (*lac* and *gal* cases); 2) in the presence of CRP, RNAP forms a CRP-dependent complex that is fully competent for transcription; 3) CRP can convert the CRP-independent binary complex to the ternary complex; 4) removal of CRP from the ternary complex results in a CRP-dependent binary complex that is functionally equivalent to the ternary complex; 5) the CRP-dependent binary complex does not change to the CRP-independent binary complex.

An important general conclusion of the present study is that a major common role of CRP at three representative CRP-dependent promoters is to stimulate events leading to the formation of open complex. In other words, the role of CRP-RNAP interaction is already over when the productive open complex has been formed at these promoters although CRP is present in the ternary open complex. I propose that the CRP-RNAP interaction is only transiently needed for transcription activation at CRP-dependent promoters, irrespective of the location of the CRP binding site. A recruitment model was formulated recently as a common mechanism of transcription activation in bacteria and yeast (Ptashne and Gann, 1997). My model is analogous to the recruitment model although the latter model did not address the role of activator after the formation of open complex. It is interesting to test whether my conclusion would be generally applicable to a variety of transcriptional activators both in bacteria and eukaryote. The heparin challenge experiment would be useful to answer this important question at least in bacteria. Furthermore, how the interaction between activator(s) and RNAP can facilitate the formation of productive open complex is the most challenging problem for future work.

ACKNOWLEDGMENTS

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Chapter 4

A novel inactive open complex mediated by UP element at *Escherichia coli* promoter.

Submitted (1998), in collaboration with Hiroji Aiba

SUMMARY

Specific interaction between the α subunit of RNA polymerase (RNAP) and the A/T-rich upstream sequence (UP element) stimulates transcription at some promoters in *Escherichia coli*. I report here that the α-UP interaction leads to the formation of an inactive open complex when the UP element is situated at a certain position. RNAP forms a heparin resistant nonproductive complex at the *malT* promoter. The *malT* promoter has an A/T-rich upstream sequence separated 9 bp from the -35 region. The substitution of the A/T-rich sequence to other sequences eliminated both the formation of the heparin resistant complex and the binding of α subunit to the *malT* promoter. The 5 bp deletion between the A/T-rich sequence and the -35 region caused an increase in promoter activity. The UP element derived from the *rrnB* P1 stimulated the transcription of the *malT* core promoter when fused properly. The 4 bp insertion between the UP and the -35 region eliminated the transcription activity without loosing the ability to form a heparin resistant complex. The similar effects of UP element were also observed in the UP-*lac* hybrid promoters. The region around the transcription start site is melted both in the productive and nonproductive complexes. I conclude that the UP element mediates the formation of both productive and nonproductive open complexes depending on its location.

INTRODUCTION

The recognition of promoter elements by RNA polymerase (RNAP) and the formation of open complex are central events in transcription initiation. In *Escherichia coli*, the RNAP σ subunit is primarily responsible for these processes (Gross *et al.*, 1996). The σ^{70} of RNAP holoenzyme is involved in sequence-specific contacts with two hexamer promoter elements located approximately 10 and 35 bp upstream of the transcription start site (Dombroski *et al.*, 1992). The similarity of -10 and -35 hexamers to consensus sequences, and the spacing between two hexamers are major determinants for promoter strength (McClure, 1985; Mulligan *et al.*, 1984). It was recently shown that an A/T-rich sequence located upstream of the -35 hexamer acts as the third promoter element (UP element) in certain promoters (Rao *et al.*, 1994; Ross *et al.*, 1993).

The best characterized UP element is the A/T-rich sequence of the *rrnB* P1 promoter that increases promoter activity more than 30-fold *in vivo* (Ross *et al.*, 1993). The C-terminal domain of RNAP α subunit (αCTD), which functions as the target for a number of transcription factors (Busby and Ebright, 1994; Ishihama, 1993), binds directly to the UP element and therefore is responsible for the specific interaction between the UP element and RNAP (Blatter *et al.*, 1994; Estrem *et al.*, 1998; Murakami *et al.*, 1996; Ross *et al.*, 1993). Recently, a consensus UP element sequence consisting of alternating A- and T-tracts was determined by *in vitro* selection (Estrem *et al.*, 1998). In addition, it was shown that the effect of the UP elements on the promoter activity correlated generally with their degree of similarity to the consensus sequence in several promoters (Ross *et al.*, 1998). The A/T-rich upstream sequences are found at many other promoters (Galas *et al.*, 1985; Helmann, 1995; Ozoline *et al.*, 1997). It has been estimated that approximately 3% of promoters for mRNAs and 19% of promoters for stable RNAs among the entire *E. coli* genome have sequences upstream of the -35

hexamer similar to the consensus (Estrem *et al.*, 1998). However, the functional significance of most of UP element-like sequences remains to be studied.

I found that RNAP forms a stable nonproductive complex at the *malT* promoter in the absence of added factors (*Chapter 3*). The nonproductive complex is resistant to heparin and has an ability to produce the trinucleotide ApUpU from ApU and UTP, but not the run-off transcript. The relative location of the *malT* A/T-rich sequence to the -35 hexamer is 5 bp longer compared to the *rrnB* P1 UP element. Here, I investigate how the location of UP element affects the nature of RNAP-promoter complexes. I show that the formation of the heparin resistant nonproductive complex is dependent upon the A/T-rich sequence that is recognized by the α subunit. A shortening the distance between the A/T-rich region and the -35 hexamer increases the formation of the productive complex. The analogous nonproductive and productive heparin-resistant complexes are formed when the *malT* A/T-rich region is substituted to the *rrnB* P1 UP element and also in the *rrnB* P1 UP-*lac* hybrid promoters. The region around the transcription start site is melted in both the nonproductive and productive complexes. I conclude that the UP element mediates the formation of both productive and nonproductive open complexes depending on its location with respect to the core promoter.

MATERIALS AND METHODS

DNAs and Proteins.

The promoters used in this study are shown in Figure 4-1. The *Hin*dIII end of the 247 bp *Eco*RI-*Hin*dIII fragment containing the wild-type *malT* promoter of pMT100 (see *Chapter 3*) was filled-in and cloned between the *Eco*RI and *Sma*I sites of pUC19. The 261 bp *Eco*RI-*Xba*I fragment from the resulting plasmid was cloned into pLA100

to construct pMT201. The pMT202 (Δ5bp), pMT203 (-A/T) and pMT220 (*malT* H) were constructed from pMT201 by PCR mutagenesis. The 180 bp *Pvu*II-*Hin*dIII fragment containing the *lac* promoter derived from pUC19 was cloned between the *Sma*I and *Hin*dIII sites of pMS437C (Kawamukai *et al.*, 1985). The *Hin*dIII site of the resulting plasmid was filled-in and a *Xba*I linker was inserted to construct pLA02. The *Bam*HI (-78), *Kpn*I (-45) and *Hin*dIII (-37) sites were introduced into the *lac* promoter region of pLA02 by PCR mutagenesis to construct pLA100. The *Bam*HI and *Hin*dIII region of pLA100 was replaced by a 30-bp synthetic oligonucleotide containing the *rrnB* P1 UP element to construct pLA200. The *Eco*RI-*Hin*dIII region of pMT220 was replaced by the *Eco*RI-*Hin*dIII fragment derived from pLA200 to construct pMT221 (UP4-*malT*) . The *Bam*HI and *Hin*dIII region of pLA100 was replaced by a 29-bp synthetic oligonucleotide containing the *rrnB* P1 UP element to construct pLA201 (UP4-*lac*). To construct pMT222 (UP8-*malT*) and pLA211 (UP8-*lac*), pMT221 and pLA201 were digested with *Hin*dIII and filled-in, respectively.

CRP and RNA polymerase were purified previously (*Chapter 3*). The α subunit of RNAP was purified from cells harboring pGEMAX185 according to the method described (Igarashi and Ishihama, 1991).

Electrophoretic Mobility-Shift Assay (EMSA).

The EMSA assay for RNAP was carried out in a total volume of 30 μl of a transcription buffer (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and 50 μg/ml bovine serum albumin) containing 50 μM cAMP and 5 % glycerol as described (*Chapter 3*) by using a 5 nM DNA fragment and 30 nM RNA polymerase except that the heparin challenge was performed by adding 3 μl of 50 μg/ml heparin for 3 min. When used, 3 μl of NTPs solutions (1 mM ATP, UTP or 1 mM 4NTPs) was added to the mixture prior to the heparin treatment. The assay for the α

subunit was performed in a total volume of 20 μl of a buffer (50 mM Tris-HCl pH 7.9, 50 mM NaCl, 3 mM DTT and 5 % glycerol). A DNA fragment of a final concentration of 5 nM was incubated with the α subunit (0 - 0.3 μM) for 40 min at 22°C. The mixture was analyzed by electrophoresis on a native 6 % polyacrylamide gel containing 7.5 % glycerol in 1/2 TBE containing 2 % glycerol at room temperature.

In Vitro Transcription.

The run-off transcription assay was carried out as described (*Chapter 3*) by using a 5 nM DNA fragment and 30 nM RNA polymerase except that the heparin challenge was performed by adding 3 μl of 50 μg/ml heparin for 3 min.

Permanganate Footprinting.

The reaction was performed in a total volume of 50 μl of a transcription buffer containing 5 % glycerol. The *Eco*RI-*Xba*I fragments (1 nM) ^{32}P-labeled at their 5' *Eco*RI ends were incubated with RNA polymerase (0 or 110 nM) for 30 min at 37°C. When used, 100 nM CRP and 50 μM cAMP were added before RNAP. The mixture was treated with 5 μl of 50 μg/ml heparin for 3 min at 37°C. $KMnO_4$ was added at a concentration of 2 mM and incubation was continued for 1 min at 25°C. The reaction was quenched by adding 2 μl of 2-mercaptoethanol. After addition of 20 μl of 1.5 M sodium acetate, 20 mM EDTA, 100 μg/ml tRNA, the mixture was precipitated with ethanol. The DNA was cleaved by incubating the samples in 1 M piperidine at 90°C for 30 min. The products were analyzed on an 6.5 % polyacrylamide sequencing gel containing 8 M urea.

β-Galactosidase Assay.

The promoter-*lacZ* fusion plasmids were introduced in KI70 (Δ*lac*) and OK6201 (Δ*lac crp*⁻) (Morita *et al.*, 1988). Cells were grown in LB medium containing 50 μg/ml ampicilin to an OD600 of 0.8 and the β-galactosidase activity was determined according to the method of Miller (Miller, 1972).

S1 Nuclease Assay.

An S1 assay was performed as described previously (Aiba *et al.*, 1981). KI70 (Δ*lac*) and OK6201 (Δ*lac crp*⁻) cells harboring the promoter-*lacZ* fusion plasmids were grown in LB medium containing 50 μg/ml ampicilin to an OD600 of 0.5. The 261 bp *Eco*RI-*Xba*I fragment of pMT201 ^{32}P-labeled at its *Xba*I 5' end was used as a DNA probe for the *malT* transcript. The DNA probe and total RNAs were hybridized, and treated with 100 units of S1 nuclease at 37°C for 15 min. The reaction products were analyzed on an 8% polyacrylamide gel containing 8 M urea.

RESULTS

The A/T-Rich Sequence Is Necessary for the Formation of Nonproductive Complex at the malT Promoter.

RNAP alone could form a unique heparin-resistant binary complex that has no ability to make the run-off transcript at the *malT* promoter (*Chapter 3*). The *malT* promoter has an A/T-rich sequence upstream of the -35 hexamer (Chapon and Kolb, 1983; Dethiollaz *et al.*, 1996). To examine the possible role of the A/T-rich sequence in the formation of the heparin resistant nonproductive complex, I have constructed several variants of the *malT* promoter (Figure 4-1). RNAP-promoter complexes were analyzed by an EMSA and by an *in vitro* transcription assay. The nonproductive complex exhibits

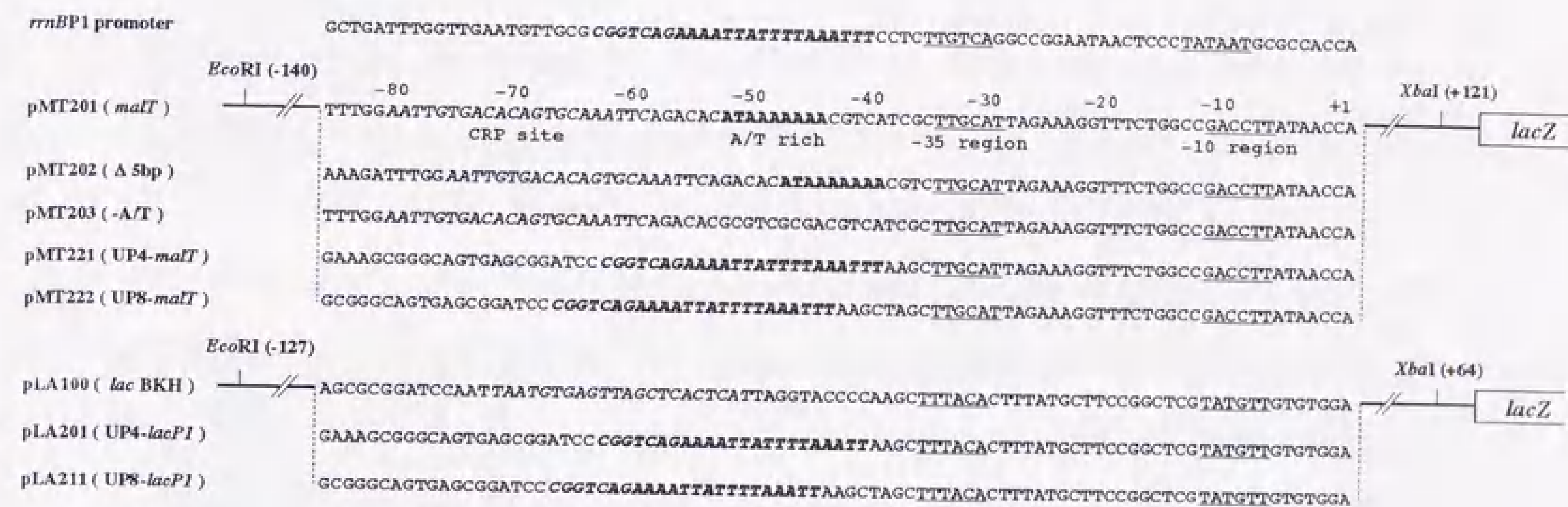

Figure 4-1. Organizations and sequences of promoters used in this study. The -35 and -10 regions are underlined. The CRP sites are italicized. The transcription start site is numbered as +1. The upstream A/T rich sequence of the *malT* promoter is shown in boldface. The UP element derived from the *rrnB* P1 is shown in bold-and italic-face.

a slightly increased mobility compared to the productive binary complex that is produced from the ternary complex containing both RNAP and CRP by heparin challenge at the *malT* promoter (*Chapter 3*). When the A/T-rich sequence was substituted to a G/C-rich sequence, the formation of the nonproductive complex was markedly reduced (Figure 4-2*A*, lane 4). A little nonproductive complex was also formed at the *lac-malT* hybrid promoter where the *malT* A/T-rich sequence was substituted to the upstream region of the *lac* promoter (data not shown). I conclude that the A/T-rich sequence is required for the formation of nonproductive heparin-resistant complex. A small amount of the productive complex formed in the absence of CRP-cAMP was not affected by the elimination of the A/T-rich sequence. In agreement with this observation, the basal transcription activity that is presumably due to the small amount of the productive complex was not affected by the lack of A/T-rich sequence (Figure 4-2*B*).

The α Binds to the A/T-Rich Sequence in the malT Promoter.

The α subunit interacts directly with the *rrnB* P1 UP element (Blatter *et al.*, 1994; Gaal *et al.*, 1996; Ross *et al.*, 1993). The *malT* A/T-rich sequence exhibits a moderate similarity to the *rrnB* P1 (Ross *et al.*, 1993) and consensus UP elements (Estrem *et al.*, 1998). To examine whether the *malT* A/T-rich sequence interacts with the α subunit, I performed an EMSA using the purified α subunit and DNA fragments containing the wild-type or -A/T *malT* promoter. The α subunit bound specifically to the wild-type *malT* promoter resulting in an α-DNA complex under my experimental conditions while little interaction between the -A/T *malT* promoter and α was observed (Figure 4-3). I conclude that the *malT* A/T-rich region functions as an UP element recognized by the α subunit and the α-UP interaction is responsible for the formation of the nonproductive complex at the *malT* promoter.

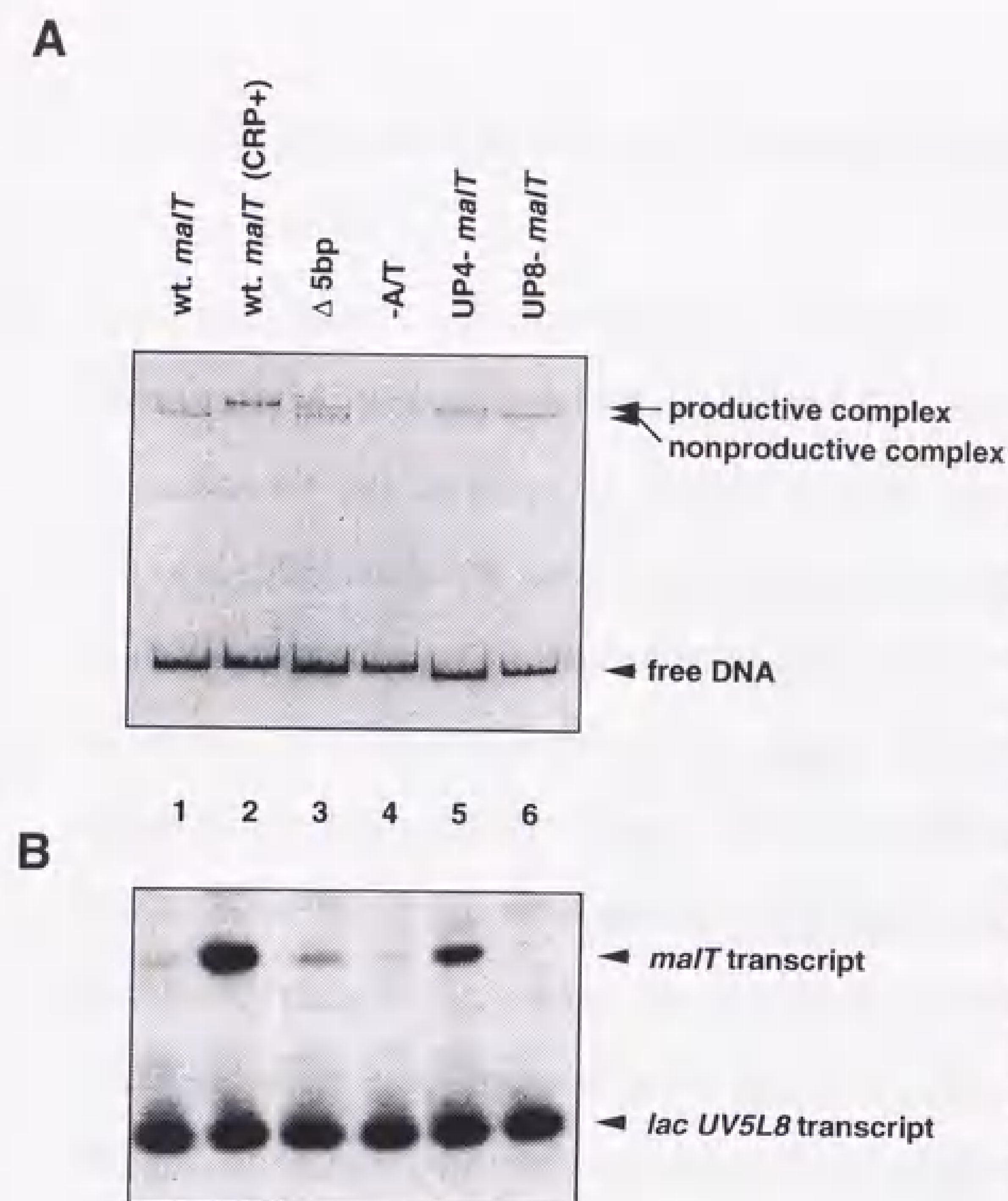

Figure 4-2. Effect of A/T-rich sequences on the *malT* core promoter *in vitro*. (*A*) EMSA. The *Eco*RI-*Xba*I fragments containing the *malT* promoter derivatives were incubated with RNAP, treated with heparin, and analyzed by electrophoresis in a 5% polyacrylamide gel. In lane 2, CRP (50 nM) and cAMP (50 μM) were added prior to the addition of RNAP. The productive complex (PC) and nonproductive complex (NC) are shown by arrows. (*B*) *In vitro* run-off transcription assay. The *Eco*RI-*Xba*I fragments containing the *malT* promoter derivatives were used as templates along with the 205 bp *Eco*RI fragment containing the *lacL8UV5* promoter as an internal control.

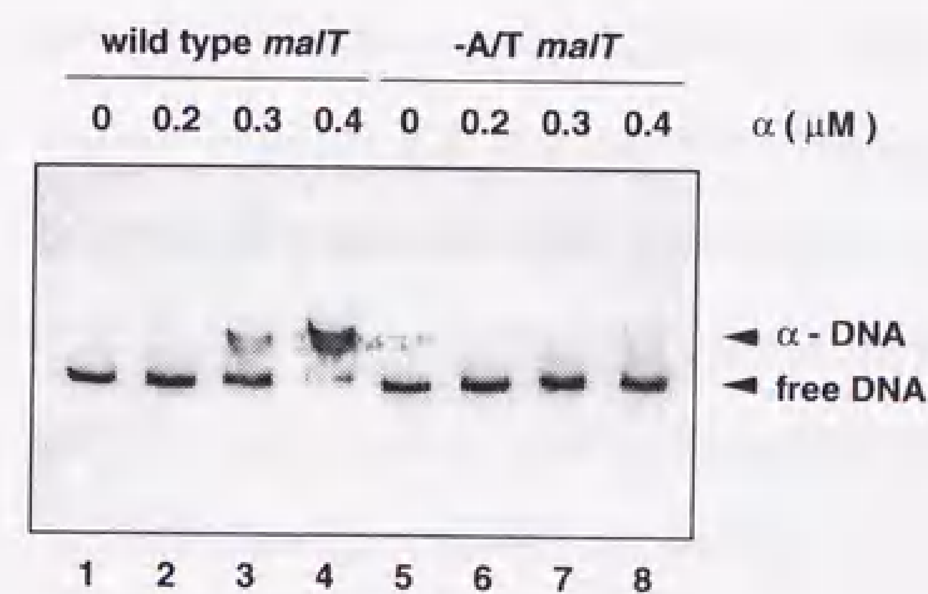

Figure 4-3. Binding of α subunit to the *malT* promoter. The 261 bp *Eco*RI-*Xba*I fragment derived from pMT201 (lanes 1-4) or pMT203 (lanes 5-8) was incubated with indicated concentrations of α subunit.

Effect of the Location of UP Element on the RNAP-malT Promoter Interaction.

The UP element has been identified as the third promoter element because it stimulates the promoter activity (Ross *et al.*, 1993). In other words, the functional significance of UP elements characterized to date is to facilitate the formation of productive open complexes. An interesting question is why the UP element leads to the nonproductive complex formation at the *malT* promoter. It should be noted that the right most end of the *malT* UP element with respect to the -35 hexamer is located 5 bp upstream compared to the *rrnB* P1 UP element (see Figure 4-1). In order to examine the effect of the location of UP element on the RNAP binding at the *malT* promoter, I have deleted 5 bp between the A/T-rich sequence and the -35 region of *malT* promoter. Interestingly, a moderate but significant increase in the formation of the upper band corresponding to the productive complex was observed at the Δ5 bp mutant (pMT202) in the EMSA (Figure 4-2*A*, lanes 1 and 3). In fact, an increased promoter activity was observed in the Δ5 bp mutant (Figure 4-2*B*, lane 3). I also constructed two *rrnB* P1 UP-*malT* hybrid promoters: the UP4-*malT* (pMT221) and UP8-*malT*(pMT222). The positions of UP element in the UP4-*malT* and UP8-*malT* approximately correspond to those of the *malT* A/T-rich sequence in the Δ5 bp and wild-type *malT* promoters, respectively. The two *rrnB* P1 UP-*malT* hybrid promoters were also analyzed by the EMSA. RNAP preferentially formed a complex of a lower mobility at the UP4-*malT* promoter while it formed predominantly a complex of a higher mobility at the UP8-*malT* promoter (Figure 4-2*A*, lanes 5 and 6). The UP4-*malT* promoter exhibited a marked transcription activity promoter *in vitro* while little activity was detected at the UP8-*malT* promoter (Figure 4-2*B*, lanes 5 and 6). These experiments clearly indicate that UP elements stimulate the formation of the productive complex therefore activates the transcription when it was suitably placed at the *malT* promoter. On the other hands, UP elements leads to the formation of the nonproductive complex when it was moved a

half-turn upstream. Thus, the location of UP elements determines the nature of heparin resistant complexes.

Transcription Start Region is Melted in the Nonproductive Complexes.

I showed that the nonproductive heparin-resistant complex at the wild-type *malT* promoter has an ability to produce the trinucleotide ApUpU which corresponds to a transcript originated from the *malT* promoter by the addition of ApU and [α-^{32}P]UTP (*Chapter 3*). This suggests that the region around the transcription start site would be melted. To examine this, I performed a permanganate footprinting of the complexes (Borowiec and Gralla, 1987). The ^{32}P-labeled DNA fragment carrying the wild-type *malT* promoter was incubated with or without CRP-cAMP and then with RNAP to form a productive or nonproductive complexes. Following the addition of heparin, KMnO4 modification was carried out. The permanganate preferentially modifies T residues in single stranded region of DNA (Borowiec and Gralla, 1987; Sasse-Dwight and Gralla, 1989). As shown in Figure 4-4*A*, a significant DNA melting around the transcription start site was detected in both the productive and nonproductive complexes. It should be noted that two functionally different complexes reveal the essentially the same melting pattern although a slight difference in the band intensity was observed between two complexes. I also performed the permanganate footprinting experiment using DNA fragments containing the UP4-*malT* (Figure 4-4*B*) and UP8-*malT* promoters (Figure 4-4*C*). The same melting pattern as in the case of the wild-type *malT* promoter was observed with both the productive and nonproductive complexes. Thus, the UP element mediates the formation of two similar but functionally different open complexes at the *malT* promoter depending on its location.

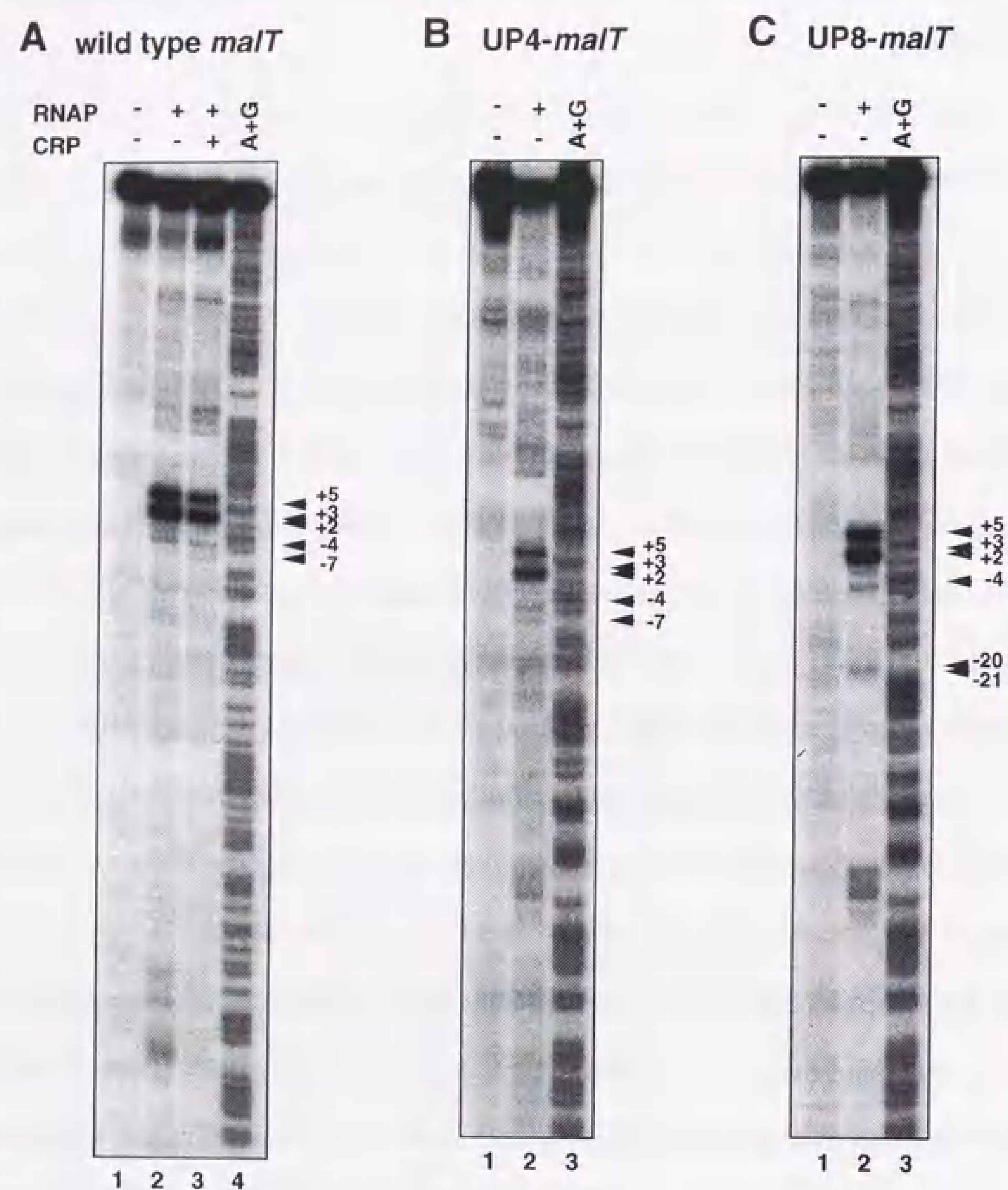

Figure 4-4. Potassium permanganate footprinting of the *malT* promoter derivatives. The 261 bp *Eco*RI-*Xba*I fragments derived from pMT201 (*A*), the 236 bp *Eco*RI-*Xba*I fragment derived from pMT221 (*B*), and the 240 bp *Eco*RI-*Xba*I fragment derived from pMT222 (*C*) were used for $KMnO_4$ footprinting. The arrowheads indicate the permanganate sensitive sites. The numbers represent the positions relative to the transcription start site.

Effect of Ribonucleotides on Transcription Complexes.

To further characterize the properties of the promoter-RNAP complexes, the complexes were incubated with ribonucleotides prior to loading the gel and the reaction mixtures were subjected to the EMSA. The addition of the initiating nucleotides (ATP and UTP) to the productive complex would allow RNAP proceed to +8. As expected, most of the productive complex was converted to an initiated complex of an increased mobility (Figure 4-5, lane 5). This is consistent with those observed with the transcription complexes at the *lacUV5* and *lac* P1 promoters (Straney and Crothers, 1985; Straney *et al.*, 1989). When 4NTPs were added to the productive complex, heparin resistant complexes were no longer detected (Figure 4-5, lane 6) indicating that the productive complex almost completely has run off. On the other hands, the addition of NTPs had essentially no effect on the nonproductive complex (Figure 4-5, lanes 2 and 3).

Effect of Location of UP Element on malT Transcription in Vivo.

To study how the UP element affects the *malT* promoter *in vivo*, the β-galactosidase activities were determined in *Δlac crp⁻* cells carrying the promoter-*lacZ* fusion plasmids. The elimination of the UP element caused little change in the basal transcription activity (Figure 4-6*A*, lanes 2 and 3). On the other hand, the 5 bp deletion between the UP element and the -35 region of *malT* promoter caused 2.5 fold increase in the basal transcription activity compared to the wild-type *malT* promoter (Figure 4-6*A*, lanes 2 and 4). Furthermore, the UP element derived from the *rrnB* P1 markedly stimulated the activity at the UP4-*malT* promoter (Figure 4-6*A*, lane 5). The activity of the UP8-*malT* promoter was reduced to the level of the wild-type *malT* promoter (Figure 4-6*A*, lane 6). The β-galactosidase activity of *Δlac crp*$^+$ cells carrying pMT201

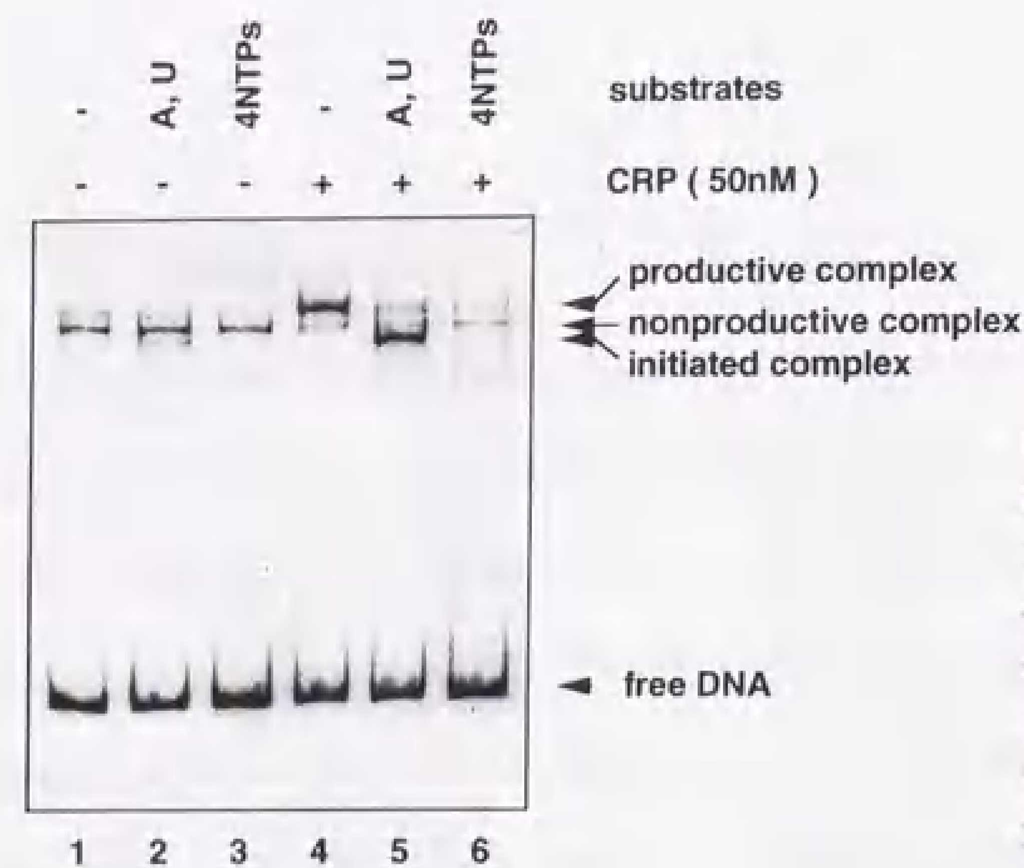

Figure 4-5. Effect of ribonucleotides on the transcription complexes at the *malT* promoter. The 261 bp *Eco*RI-*Xba*I fragment derived from pMT201 containing the *malT* promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was incubated with the indicated NTPs for 15 min prior to the treatment with heparin.

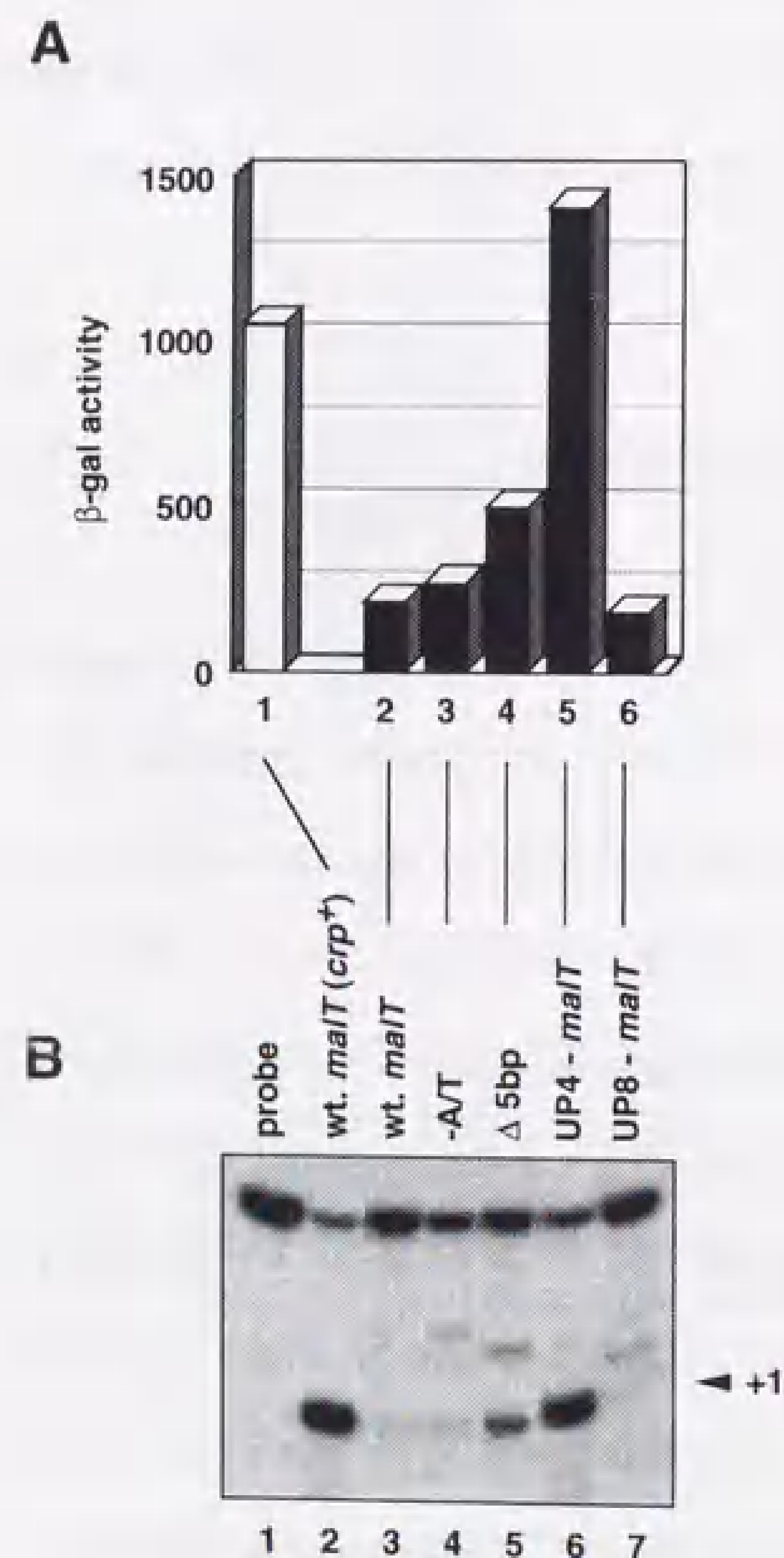

Figure 4-6. Effect of the location of UP element on the promoter activity *in vivo*. (*A*) β-Galactosidase activity of the *malT-lacZ* fusions. The β-galactosidase activity of KI70 (Δ*lac*) (lane 1) and OK6201 (Δ*lac crp-*) (lanes 2-6) cells harboring the *malT-lacZ* fusion plasmids were measured. Each bar is expressed as Miller units. The value is an average obtained from three independent experiments. (*B*) Analysis of mRNAs by S1 nuclease assay. Total RNAs (100 μg) prepared from KI70 (Δ*lac*) (lane 2) and OK6201 (Δ*lac crp-*) (lanes 3-7) cells harboring indicated plasmids were subjected to S1 assay. Lane 1 is a DNA probe without S1 treatment.

is about 5-fold higher than that of *crp*⁻ cells due to the action of CRP-cAMP (Figure 4-6*A*, lanes 1 and 2). A quantitative S1 nuclease assay was also performed to evaluate the effect of UP element on the *malT* promoter *in vivo* (Figure 4-6*B*). The results were essentially the same as those of β-galactosidase assay. Thus, UP element stimulates the transcription depending on its location relative to the core promoter *in vivo* as well as *in vitro*.

The Influence of the Location of UP Element at the lac Core Promoter.

The UP element could function in an independent manner of the promoter context (Estrem *et al.*, 1998; Rao *et al.*, 1994; Ross *et al.*, 1993). For example, the *lac* core promoter is activated by the *rrnB* P1 UP element in an *rrnB* P1-*lac* hybrid. I constructed the *rrnB* UP-*lac* hybrid promoters and the effect of UP element on the *lac* core promoter was determined by measuring β-galactosidase activity in Δ*lac crp*⁻ cells carrying the promoter-*lacZ* fusion plasmids. When the UP element is placed correctly (UP4-*lac*), the transcription activity was dramatically increased as reported (Figure 4-7*A*, lane 2). An S1 mapping experiment revealed that the transcription from the *lac* P1 is stimulated by UP element (data not shown). On the other hands, little promoter activity was detected at UP8-*lac* promoter (Figure 4-7*A*, lane 3). The EMSA revealed that the formation of heparin-resistant complex was increased in both the UP4-*lac* and the UP8-*lac* promoters compared to the wild-type *lac* promoter (Figure 4-7*B*, lanes 1-3). A significant amount of the complex at the wild-type *lac* promoter in the absence of CRP-cAMP is due to the *lac* P2 promoter (Malan *et al.*, 1984). No difference in gel mobility was observed among three different complexes (P2 complex at wild-type promoter, productive P1 complex at UP4-*lac* promoter and nonproductive P1 complex at UP8-*lac* promoter) in the *lac* system. Thus, the UP element could increase the formation of both productive and nonproductive complexes at the *lac* promoter depending on its

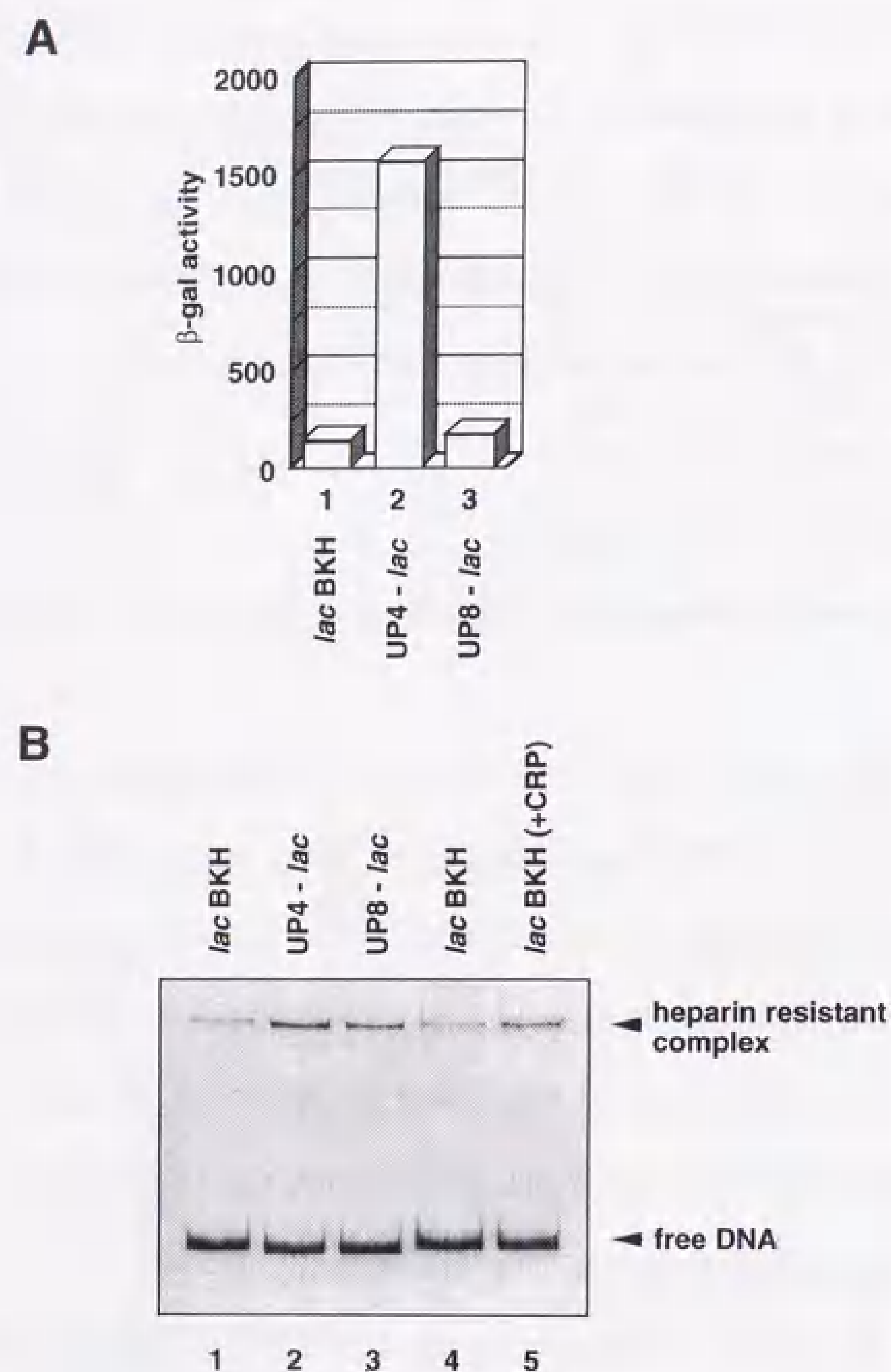

Figure 4-7. Effect of the location of UP element on the *lac* core promoter. (*A*) β-Galactosidase activity. The b-galactosidase activity of OK6201 cells harboring the indicated promoter-*lacZ* fusion plasmids were determined. Each bar is expressed as Miller units. The value is an average obtained from two independent experiments. (*B*) EMSA of transcription complexes at the UP-*lac* hybrid promoters. The *Eco*RI-*Xba*I fragments of the *lac* promoter derivatives were incubated with RNA polymerase. Lane 5 shows the CRP-dependent P1 complex formed in the presence of CRP-cAMP.

location as in the case of the *malT* promoter.

DISCUSSION

The UP element, a DNA sequence rich in (A + T) located upstream of the -35 hexamer in certain bacterial promoters, has the following properties: (1) it increases promoter strength both *in vivo* and *in vitro*; (2) it activates transcription when fused to other core promoters; (3) it interacts with αCTD; (4) the consensus sequence consists of alternating A- and T-tracts containing 15 highly conserved positions in two DNA regions (Estrem *et al.*, 1998; Rao *et al.*, 1994; Ross *et al.*, 1993). The functional properties of UP element have led to the proposal that it is a third promoter element in addition to the -10 and -35 hexamers (Ross *et al.*, 1993).

Previous studies indicated that correct alignment of UP element with respect to the core promoter seems to be necessary for function(Josaitis *et al.*, 1990; Newlands *et al.*, 1992). Here, I found that the UP element could mediate the formation of both nonproductive and productive open complexes depending on its relative location to the -35 hexamer. The distance between the -35 hexamer and the UP element is 3 to 5 bp in promoters where the UP element was shown to increase transcription (Ross *et al.*, 1998; Ross *et al.*, 1993). In contrast, the UP element leads to the formation of transcriptionally inactive open complex when the distance between two elements is around 9 bp. This suggests that the specific rotational orientation between core promoter and UP element is an important determinant for the nature of transcription complex.

The αCTD is connected to the N-terminal domain with a flexible linker (Blatter *et al.*, 1994). Concerning the α-UP interaction, it is proposed that the UP element contains two separable sections which can function independently and may be recognized by two flexible αCTD monomers (Estrem *et al.*, 1998; Newlands *et al.*, 1991; Rao *et al.*, 1994). Flexible positioning of two αCTD monomers was also detected

in the CRP-α interaction (Murakami *et al.*, 1997). I observed that the *rrnB* P1 UP element could stimulate the *malT* transcription when separated 14 bp from the -35 hexamer though the level of activation is reduced compared to the UP4-*malT* promoter (data not shown). In addition, it is known that insertion of 5 bp at -46 or deletion of 3 bp from -38 to -40 results in loss of UP element function at the *rrnB* P1 promoter (Josaitis *et al.*, 1990). It would be interesting to test whether RNAP could form a nonproductive complex at these mutated *rrnB* P1 promoters. The helical phase dependent effect of UP element on promoter function is analogous to those of upstream A-tracts (Bracco *et al.*, 1989; Gartenberg and Crothers, 1991; McAllister and Achberger, 1989) and CRP (Gaston *et al.*, 1990; Ushida and Aiba, 1990). Recent studies have suggested that A-tracts may act at least in part as UP elements because they could stimulate transcription by binding to the αCTD when fused to the *rrnB* P1 or *lac* core promoters (Estrem *et al.*, 1998).

I have shown that RNAP forms a nonproductive open complex at the *malT* and *lac* promoters when the UP element is placed 8-9 bp upstream of the -35 hexamer. Why are these complexes transcriptionally inactive? One explanation would be that the α-UP interaction at this particular position may lead to a tighter binding between RNAP and promoter to inhibit promoter escape. In fact, it is known that overstabilization of RNAP binding at the promoter by upstream A-tracts or a transcription factor would inhibit promoter clearance in some promoters (Ellinger *et al.*, 1994; Monsalve *et al.*, 1997). However, the RNAP-promoter interaction appears to be not enhanced in the nonproductive complexes compared to the productive complexes (unpublished data). I prefer a model that the α-UP interaction at a particular position relative to the core promoter presumably lead to an inactive conformation of transcription complex. A similar model has been described in the repression of the *gal* P1 promoter by GalR or LacI binding to an upstream operator in the absence of DNA looping (Choy *et al.*, 1995). In addition, it is interesting to note that RNAP carrying a specific mutation in the β

subunit is known to form a stable inactive open complex at the normal promoter (Kashlev *et al.*, 1990). All transcriptionally inactive complexes described previously are formed at constitutive promoters through interactions of RNAP either with upstream A-tracts (Ellinger *et al.*, 1994) or transcription factors (Choy *et al.*, 1995; Monsalve *et al.*, 1997). In contrast, the nonproductive complexes reported in this study are formed at weak promoters such as *malT* and *lac* through the α-UP interaction.

What is the functional significance of UP element and the nonproductive complex in the regulation of *malT* expression? I showed previously that the role of CRP in *malT* transcription is to lead to the formation of productive open complex (*Chapter 3*). I found that the nonproductive complex is converted to the productive complex by CRP and that CRP-dependent activation is markedly reduced in the absence of the UP element (*Chapter 5*). In other words, CRP alone is not sufficient to recruit RNAP to the promoter. The simplest interpretation of these results would be that the *malT* UP element is acting to recruit RNAP to the promoter and CRP predominantly acts at the post-recruitment step to form the productive complex although I do not exclude a possibility that CRP is also involved in recruitment of RNAP along with the UP element. The nonproductive complex could be a simple intermediate for the productive complex or it could be a product in a branched pathway in the early stage of transcription (Kubori and Shimamoto, 1996). One of the major roles of CRP is to increase the initial binding of RNAP through the CRP-α interaction at promoters where the CRP binding site at -61.5 (Busby and Ebright, 1994; Hochschild and Dove, 1998). I speculate that CRP action to recruit RNAP may be insufficient to fully activate promoter without the UP element when CRP site is located at -70.5. Further studies will be required to understand how CRP and the UP element cooperate to activate transcription.

ACKNOWLEDGMENTS

I thank Dr. Akira Ishihama and Dr. Katsuhiko Murakami (National Institute of Genetics) for gift of pGEMAX185 and technical comments on the purification of α subunit. This work was supported by Grants-in-Aid from Ministry of Education, Science, Sports and Culture of Japan.

Chapter 5

The complex mechanisms of CRP-dependent transcription activation at *malT* promoter: Partnerships of UP element and CRP

SUMMARY

RNA polymerase (RNAP) forms a nonproductive open complex *at Escherichia coli malT* promoter in the absence of CRP. I have shown that the interaction between the αCTD and the incorrectly positioned UP element leads to the formation of the complex (*Chapter 4*). The αCTD is also responsible for the interaction with CRP at *malT* promoter. I investigated the role of the nonproductive complex formation for CRP action. I found that CRP was able to convert from the nonproductive complex to productive one. The kinetics of the productive complex formation indicated the branched pathway in initiation of the *malT* transcription. The positive control mutant CRP (H159L) inhibited the formation of the nonproductive complex as well as the productive one. This indicates that CRP binds dominantly to the DNA and inhibits the αCTD-DNA interaction. When the UP element was substituted to a G/C-rich sequence, CRP-dependent activation was markedly reduced both *in vivo* and *in vitro* without affecting the basal transcription activity. CRP inhibited the *malT* transcription when its binding site was positioned out of phase even if the UP element was correctly positioned. Taken together, I conclude that CRP acts at the post-recruitment step to stimulate the formation of productive open complex. I suggest that UP element functions as a "concentrator" of RNAP to ready for transcription factor(s) in certain promoters.

INTRODUCTION

Transcription initiation is a multistep process (McClure, 1985). RNAP first recognizes and bind to promoter DNA, forming a closed complex. This must then isomerize to form a transcriptionally active open complex in which the DNA strands are locally melted. Then, RNAP must escape from the promoter to elongate the transcription.Promoter elements are critical determinants that dictate which step(s) is rate limit and should be targeted by transcription factors. Though activator and repressor, in theory, can affect any initiation steps,I established that a typical activator, cAMP receptor protein (CRP or CAP, catabolite gene activator protein) acts transiently to stimulate events leading to productive open complex formation (*Chapter 3*). Many prokaryotic activators and repressors contact directly the transcription machinery, RNAP, to regulate the transcription initiation. How activator stimulates the formation of open complex by protein-protein contact is one of the major issue to understand the mechanisms of transcription regulation.

A typical σ70-dependent promoter in *Escherichia coli* consists of a –10 and a –35 hexamer, both of which are directly contacted by σ in the holoenzyme. In addition, certain strong promoters comprise a third promoter element known as the UP element, which is an A/T-rich sequence located just upstream of the –35 hexamer and is bound by the C-terminal domain of RNAP α subunit (αCTD) (Ross *et al.*, 1993). The critical amino acid residues on the αCTD for UP element are positions 262, 265, 268, 296, 298, and 299 (Gaal *et al.*, 1996).

The αCTD is also known as a target domain of various transcription factors to regulate transcription (Ishihama, 1993). The best-characterized transcription factor which contacts αCTD is CRP. Recent genetic and biochemical studies of CRP and αCTD have identified particular sites or regions located at the surface of both proteins which are involved in protein-protein interaction and thereby responsible for transcription activation. Isolation and characterization of CRP mutants that bind normally to the target DNA but fail to activate transcription have established that a surface exposed loop around amino acids 156-164 of CRP constitutes an activating region essential for transcription activation (called activating region I; AR1) (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Zhou *et al.*, 1993). The most critical amino

acid residues on the αCTD for CRP-dependent activation is either position 261 (Tang *et al.*, 1994) or position 265 (Murakami *et al.*, 1996). The NMR solution structure indicates that position 261 is adjacent to helix1 and position 265 lies within helix 1 that, in combination with helix 4, is critical to the DNA binding property of the αCTD (see Figure 1-1) (Jeon *et al.*, 1995).

In *lac* promoter, CRP binds to its binding site and interacts specifically with the αCTD of RNAP, apparently stabilizing its association with the DNA between the CRP-binding site and the –35 region (Kolb *et al.*, 1993b). When the UP element derived from *rrnB*P1 located at the upstream region of the *lac* promoter, the transcription activity was increased in the absence of CRP (Rao *et al.*, 1994). These findings suggest that the role of CRP in transcription activation, at least in part, is to "recruit" αCTDand stabilizing the binding of RNAP to the promoter, thus facilitating closed complex formation. The artificial proteins that make arbitrary contacts with RNAP were reported to sufficiently act as activators (Hochschild and Dove, 1998). The recruitment model for the role of activators is simple and appears to suitable, but the further experiments are required for understanding what happened at the step(s) and the effective stimulation *in vivo*. CRP was reported to increase the binding of αCTD and synergistically stimulates in the presence of UP elementusing the semi-syntheticpromoters carrying both a consensus CRP-binding site and an UP element (Lloyd *et al.*, 1998; Savery *et al.*, 1995). However, the lack of additive effect of CRP and UP elements on the *lac* P1 promoter was reported in other studies (Czarniecki *et al.*, 1997; Noel and Reznikoff, 1998). This suggests that our understanding of the nature of CRP-αCTD interaction and the mechanisms of CRP-dependent activation is yet unclear.

I have shown that the incorrectly positioned UP element leads to the formation of a novel inactive open complex (*Chapter 4*). The native *malT* promoter has an incorrectly positioned UP element and therefore RNAP forms the nonproductive complex in the absence of CRP. I found that CRP stimulates the formation of the productive open complex (*Chapter 3*). Here, I investigated the roles of the UP element

and the nonproductive complex for the regulation by CRP and the functional significance. I showed that the nonproductive complex is converted to the productive one by CRP and that the UP element is required for the CRP-dependent activation at *malT* promoter. In other ward, CRP alone is not sufficient to recruit RNAP to the promoter. A positive control mutant CRP (H159L), which has an ability of DNA binding but fail to contact αCTD, inhibited both of the formation of complexes. Taken together, I conclude that CRP acts at the post-recruitment step to stimulate the formation of productive open complex. UP element may be served as a "concentrator" of RNAP to ready for transcription factor(s) in certain promoters.

MATERIALS AND METHODS

DNAs and Proteins.

The promoters used in this study are shown in Chapter 4.
CRP and RNA polymerase were purified previously (*Chapter 3*).

In Vitro Transcription.

The run-off transcription assay was carried out as described (*Chapter 4*) by using a 5 nM DNA fragment and 30 nM RNA polymerase with or without CRP (50 nM).

Electrophoretic Mobility-Shift Assay (EMSA).

The EMSA assay was carried out in a total volume of 30 μl of a transcription

buffer (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and 50 μg/ml bovine serum albumin) containing 50 μM cAMP and 5 % glycerol as described (*Chapter 4*) by using a 5 nM DNA fragment and 30 nM RNA polymerase with or without CRP (50 nM).

β-Galactosidase Assay.

The promoter-*lacZ* fusion plasmids were introduced in KI70 (*Δlac*) and OK6201 (*Δlac crp*$^-$) (Morita *et al.*, 1988). Cells were grown in LB medium containing 50 μg/ml ampicilin to an OD600 of 0.8 and the β-galactosidase activity was determined according to the method of Miller (Miller, 1972).

RESULTS and DISCUSSION

CRP is able to convert the nonproductive complex to the productive one.

I showed that RNAP forms a nonproductive complex through α-UP interaction at the *malT* promoter in the absence of CRP (*Chapter 4*). On the other hand, CRP stimulates the formation of a productive complex. In order to examine whether CRP is able to convert the nonproductive complex to the productive one, I performed an *in vitro* transcription assay in which CRP was added before and after RNAP. When CRP was first incubated with a DNA fragment containing the *malT* promoter and then added RNAP, a significant amount of the run-off transcript was observed compared to the case in the absence of CRP (Figure 5-1, lanes 1 and 3). When RNAP was incubated with the *malT* promoter for 30 min in the absence of CRP, RNAP formed a nonproductive complex (see Figure 5-2). CRP was added and incubated with this complex for 30 min. After the addition of heparin, a single round run-off transcription was started by adding ribonucleotides. The amount of the run-off transcript was little affected either if CRP was added before or after RNAP (Figure 5-1, lane 2). This result indicates that the

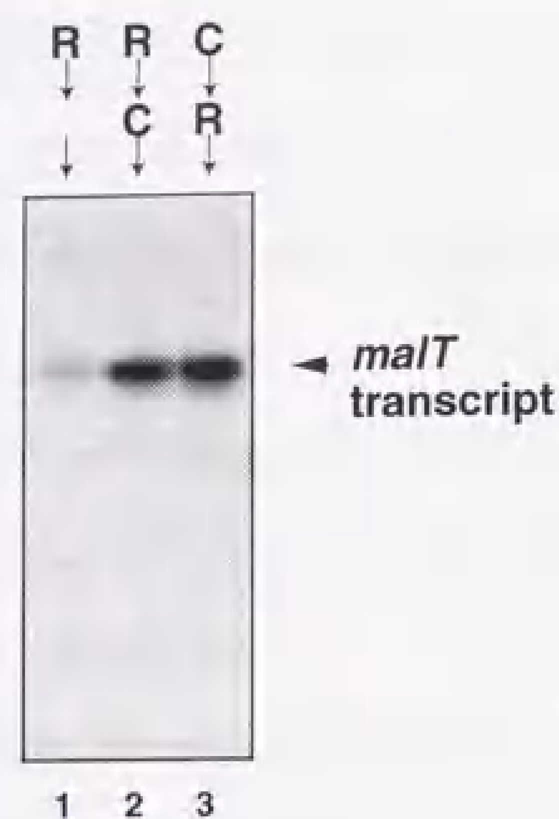

Figure 5-1. Effect of binding order for the *malT* transcription. The 5 nM of *Eco*RI-*Xba*I fragment containing the *malT* promoter of pMT201 was used as template. CRP (C) was added either 30 min before (lane 3) or after (lane 2) RNAP (R).A single round transcription was carried out by adding ribonucleotide after the heparin treatment.

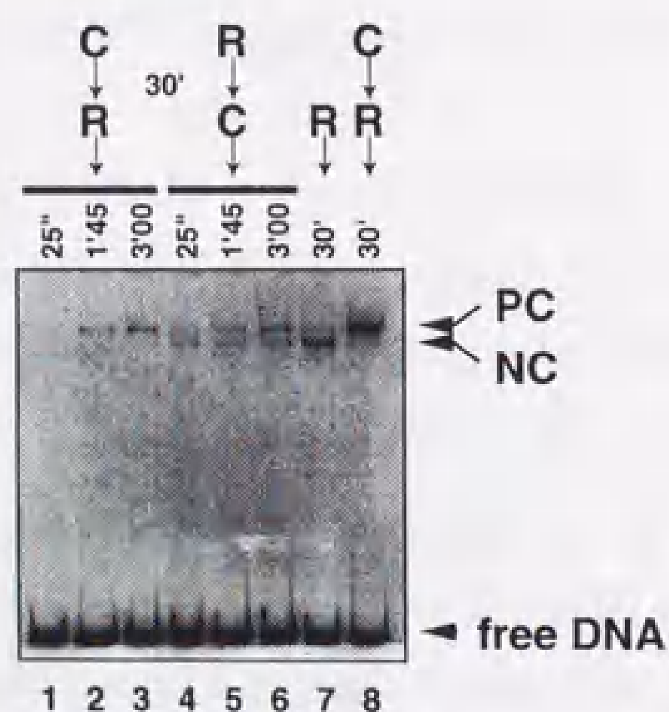

Figure 5-2. Kinetics of productive open complex formation. The 5 nM of *Eco*RI-*Xba*I fragment containing the *malT* promoter of pMT201 was used as template. CRP (C) was incubated for 30 min with DNA, then RNAP(R) was added (lanes 1-3). RNAP was incubated for 30 min with DNA, then CRP was added (lanes 4-6). At each time point, 50 µg/ml heparin was addad and then load onto the native 5 % polyacrylamide gel. Lenes 7 and 8 represents the contrl experiments. The productive complex (PC) and nonproductive complex (NC) are shown by arrows.

nonproductive complex can be converted to the productive complex by CRP.

Kinetic analysis of the formation of the productive complex.

To address how CRP converts the nonproductive complex to the productive one, I carried out a kinetic experiment of the productive complex formation by an electrophoretic mobility-shift Assay (EMSA). When CRP was incubated first with the *malT* promoter, heparin was added at each time point (25", 1'45", and 3') after the addition of RNAP to quench the complex formation. Then the mixtures were fractionated by a native polyacrylamide gel. The time-course dependent increase of the productive complex was observed (Figure 5-2, lanes 1-3). It should be noted that little of the nonproductive complex was observed in this condition. If the nonproductive complex is a simple intermediate for the productive complex formation, CRP should convert the complex by degrees. I added CRP after the formation of the nonproductive complex, then heparin was added at each time point. Surprisingly, most of the nonproductive complex was disrupted by CRP within only after 25 sec (figure 5-2, lanes 4 and 7). Nevertheless, the increase rate of the productive complex was similar the case when CRP was added before RNAP (Figure 5-2, lanes 4-6). This result indicates that the inhibition for the nonproductive complex formation and the stimulation for the formation of the productive one by CRP are independent. I suggest that the nonproductive complex is a kind of products in a branched pathway in the early stage of transcription (Kubori and Shimamoto, 1996). The complex turned to a dead-end complex may be observed as a small amount of the nonproductive complex remained after the addition of CRP.

The binding of CRP is sufficient for the inhibition of the nonproductive complex.

An activating region I (AR1) deficient H159L CRP was reported to deficient for the activation of *malT* promoter *in vivo* (Williams *et al.*, 1991) as well as other CRP-dependent promoters. I investigated the effect of the positive control mutant of CRP for the formation of the productive complex. First, I carried out an *in vitro* transcription assay. As expected, the H159L CRP-dependent activation is about 10% of that by wild type CRP (Figure 5-3A).

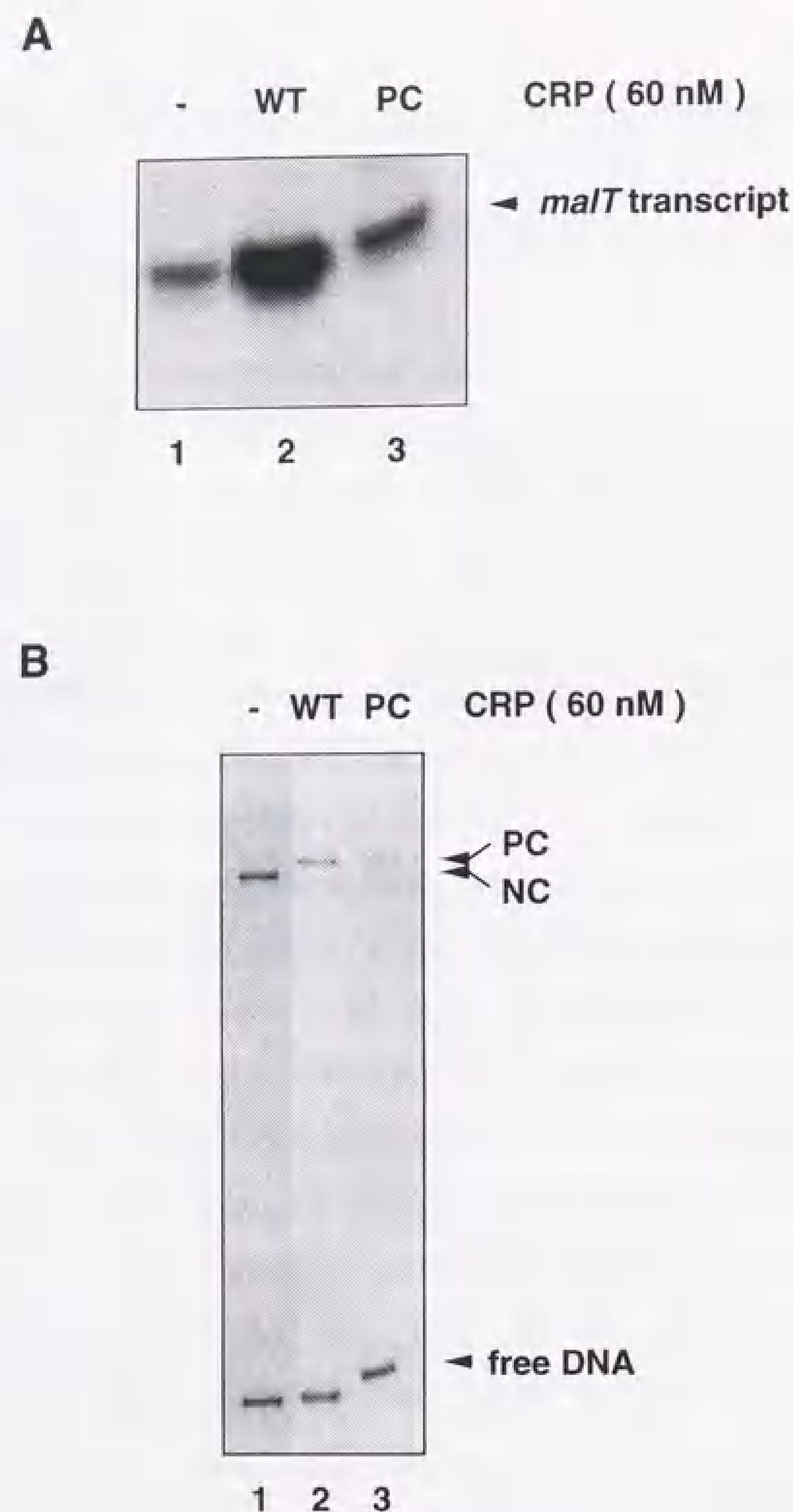

Figure 5-3. Effect of an AR1 defiecient H159L CRP for the *malT* transcription *in vitro.* (A) *In vitro* transcription assay. The 5 nM of *Eco*RI-*Xba*I fragment containing the *malT* promoter of pMT201 was used as template. (B) EMSA. The 5 nM of *Eco*RI-*Xba*I fragment containing the *malT* promoter of pMT201 was used as template. 60 nM of wild type CRP or H159L CRP were incubated for 5 min with DNA, then incubated with RNAP(R) for 30 min. After the addition of 50 μg/ml heparin, load onto the native 5 % polyacrylamide gel. The productive complex (PC) and nonproductive complex (NC) are shown by arrows.

To determine the effect for the transcription complex formation, an EMSA was performed under the same condition of the transcription assay. The H159L CRP inhibited both of nonproductive and productive complex formation (Figure 5-3B). This indicates that CRP blocks the formation of the nonproductive complex by its ability for DNA binding and that the interaction between CRP and αCTD is required for the formation of the productive complex. I suggest that CRP binds dominantly to the DNA and inhibits the αCTD-DNA interaction for the nonproductive complex formation. Actually, I obtained the data that the wild type CRP inhibited the α subunit binding to the *malT* promoter (data not shown).

Effect of the UP element of malT promoter for transcription activation by CRP

The αCTD of RNAP is responsible for both the interactions for UP element and for CRP. It is interesting to investigate the effect of the UP element for CRP action at the *malT* promoter. I have shown that the substitution of the A/T-rich sequence to the G/C-rich one inhibited the α subunit-DNA interaction and caused a significant reduction of the nonproductive complex formation (*Chapter 4*). The Δ5 bp mutation between the –35 and the UP element increased the basal transcription (*Chapter 4*). In this promoter, the CRP-binding site is positioned out of phase though the UP element is correctly positioned (see Figure 4-1). To study the effect of these mutants of the UP element for CRP-dependent activation, the β-garactosidase activities were determined both in *Δlac crp*$^-$ and *Δlac crp*$^+$ cells carrying the promoter-*lacZ* fusion plasmids. Though the elimination of the UP element caused little change in the basal transcription activity, the CRP-dependent stimulation is markedly reduced (Figure 5-4A, lane 3 and 4). The transcription activity of the 5 bp deletion mutant promoter was inhibited in the presence of CRP (Figure 5-4A, lane 5 and 6). A similar result was obtained from an *in vitro* transcription assay (Figure 5-4B). These results clearly indicate that the UP element is required for CRP-dependent activation at *malT* promoter and that CRP inhibits the transcription when its binding site is positioned out of phase even if the UP element is correctly positioned. As the UP element is required for the α subunit binding and for the formation of the nonproductive complex, CRP alone is not sufficient for to recruit RNAP to the promoter. The interaction between the CRP and RNAP is dominant for that between the RNAP and promoter through UP element. Taken together, I conclude that

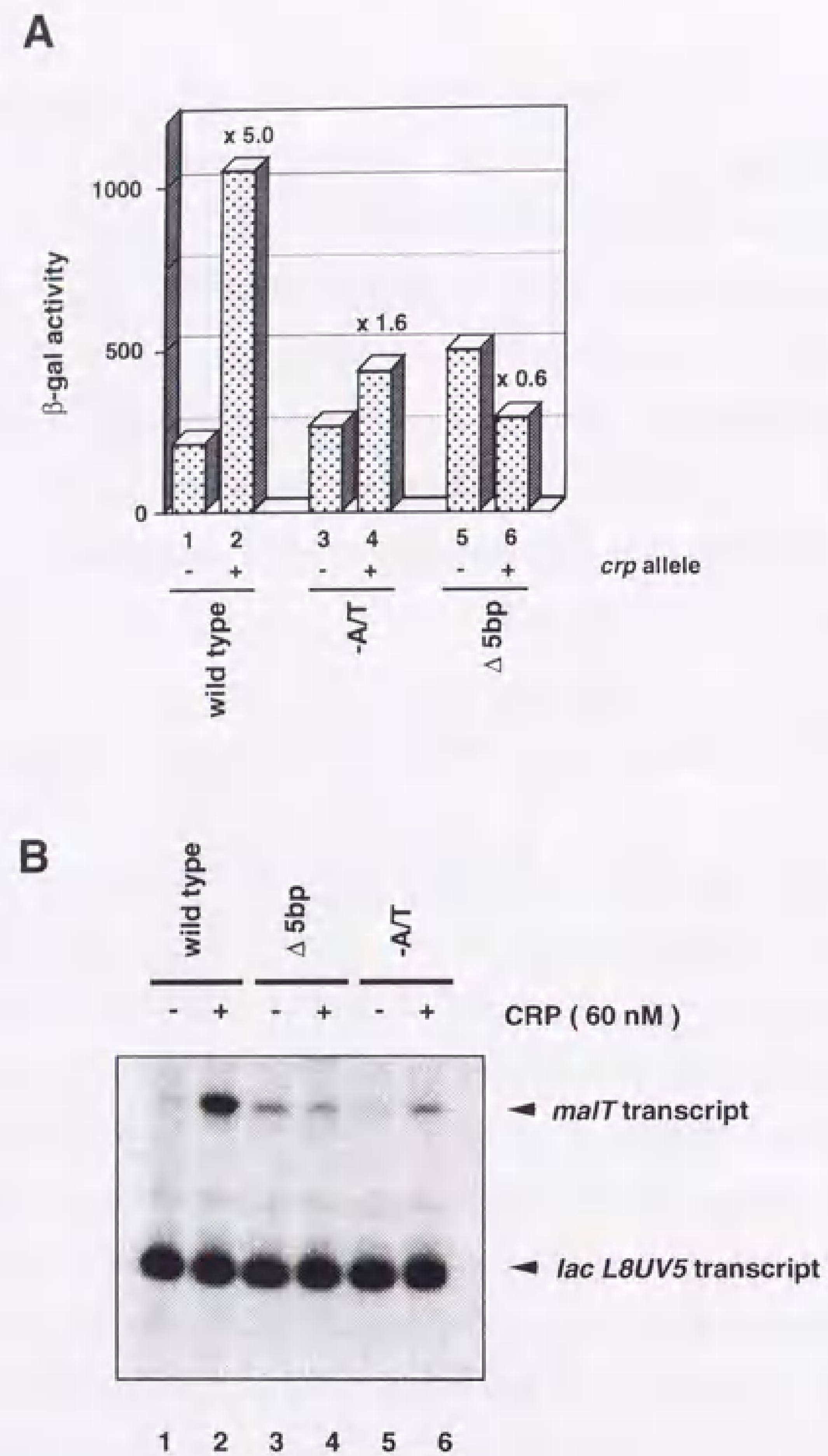

Figure 5-4. Effect of the UP element of the malT promoter for transcription activation by CRP (*A*) β-Galactosidase activity of the *malT-lacZ* fusions. The β-galactosidase activity of OK6201 (Δ*lac crp-*) (odd number of lanes) and KI70 (Δ*lac*) (even number of lanes) cells harboring the *malT-lacZ* fusion plasmids were measured. Each bar is expressed as Miller units. The value is an average obtained from three independent experiments. (*B*) *In vitro* run-off transcription assay. The *Eco*RI-*Xba*I fragments containing the *malT* promoter derivatives were used as templates along with the 205 bp *Eco*RI fragment containing the *lacL8UV5* promoter as an internal control.

CRP modulates the RNAP-promoter interaction to stimulate the formation of the productive open complex at *malT* promoter.

The requirement of UP element is likely to contradict the results shown in Figure 5-2 and 5-3, which indicate the independence of the nonproductive and productive complex formation and CRP action. I suggest a following scenario. i) RNAP is recruited by the UP element to the *malT* promoter. ii) The incorrectly positioned UP element leads to the formation of the nonproductive complex. iii) When CRP binds to its site, CRP spatially disrupts the αCTD-UP interaction therefore the nonproductive complex formation. iv) CRP interacts to αCTD to stimulate the formation of the productive complex. In other words, UP element functions as a "recruiter" or a "concentrator" of RNAP to form a nonfunctional complex and that CRP predominantly acts at the post-recruitment step in transcription initiation.

The *malT* promoter has a CRP-binding site at –70.5 that is further upstream by a DNA helical turn than that of typical CRP-dependent promoters (e.g. *lac* promoter). Some CRP-dependent promoters which have a CRP-binding site at around –70 (*glnAP1*, *gntT*, and *gntK*) has an A/T-rich sequence between –35 region and the CRP-binding site. I speculate that the UP element or other systems are required for the effective activation by CRP when the CRP site is located around –70, because the weakness of the protein-protein interaction for the RNAP recruitment by CRP alone. The partnership between UP element and CRP might be a common strategy to activate transcription effectively. My results might lead to the new aspect of the relationships of interactions between activator, RNAP, and promoter element.

CONCLUSION AND PERSPECTIVES

I described in this thesis the approaches to understand the nature of the interactions between activator, transcription machinery and promoter DNA in transcription initiation. I established a method that CRP can be dissociated specifically from the ternary open complex by adding a high concentration of heparin. I conclude that the interaction between CRP and RNAP is transiently required for the step(s) leading to the formation of productive open complex irrespective of promoter context. The conclusion was based on the results obtained that the resulting binary complex is stable and fully functional for transcription. The concentration of heparin required for the dissociation of CRP form the complexes correlated with the distance between the CRP-binding site and the core promoter, indicating the strength of the interaction between CRP and RNAP may depend on their spatial distance. The heparin challenge experiment will provide us a good probe to measure the cooperativity between protein-protein interaction. The method also can be applied for other transcription systems both of prokaryote and eukaryote to separate the role(s) of transcription factor for the steps before and after the formation of open complex.I show that CRP is dispensable after the formation of the open complex, indicating the synergistic protein-protein interaction and the architecture of ternary complex are not required for the functions on single-round transcription. Why dose CRP lie at its binding site after the formation of the open complex? I speculate that CRP may ready for the next coming RNAP for effective transcription in a living cell.

UP element, which is reported as an enhancer element, functions as a "concentrator" of RNAP and it leads a formation of nonproductive open complex if it locates certain position. It is clear that activation and repression are "two faces of the same coin". CRP and UP element act co-operatively for the transcription regulation at the *malT* promoter, though their functions may be different. My results suggest that CRP acts predominantly at the post-recruitment step to stimulate the formation of the productive open complex at the *malT* promoter. The recruitment model for the action of most of activators is proposed. This model mentions that the protein-protein interaction lead to stimulate the binding of RNAP to the promoter. One of the major role of CRP is reported to increase the initial binding of RNAP through CRP-αCTD interaction at

promoters where the CRP binding site at –61.5. I speculate that the recruitment of RNAP by CRP may be insufficient to fully activate promoter when the CRP site is located at –70.5. I believe that the studies of the partnerships of the interactions between CRP-RNAP-Promoter lead a new aspect of the complex mechanisms of transcription initiation and its regulation.

I represent the mechanistic studies of transcription using a system of *Escherichia coli* CRP in this thesis. It is also important to address the global regulation of gene expression. CRP regulates more than a hundred genes positively or negatively in response to the environmental conditions. The protein is also useful for the approach. The hole genome of *Escherichia coli* is now revealed by the Japan project team in which I also involved and the group of University of Wisconsin. The information must supply us a new strategy to solve the issue concerning the global network of gene expression. *Escherichia coli* system is a powerful tool to understanding the regulation from both the mechanistic and physiologic approach. I hope this model system provide us a knowledge how organisms live in future.

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