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Antibodies as Probes of the Structure and Function of the Alpha and Beta' Subunits of the *E. coli* RNA Polymerase

by

Karim Ahmad Sharif

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

1996

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Date

Chair of the Examining Committee

(Joseph S. Krakow)

January 30, 1996

Date

Executive Officer

(Richard L. Chappell)

Dr. David H. Calhoun, City College

Dr. David A. Foster, Hunter College

Dr. Harvey L. Ozer, UMDNJ

Dr. Rivka Rudner, Hunter College

Supervisory Committee

The City University of New York

ABSTRACT

Antibodies as Probes for the Structure and Function of the Alpha and Beta' Subunits of the *E. coli* RNA Polymerase

by

Karim Ahmad Sharif

Advisor: Dr. Joseph S. Krakow

The epitopes have been localized for a set of monoclonal antibodies specific for the α subunit of the *E. coli* RNA polymerase. These antibodies were also used to investigate the role of α in transcription activation with CRP dependent as well as activator independent promoters. Immunogenic regions representing the surfaces that may be exposed on α have been assessed by using polyclonal antibodies specific to shorter α fragments. Subunit specific monoclonal antibodies have also been used to assess σ interaction with the individual subunits of RNA polymerase.

Anti-α monoclonal antibodies studied are classified into 3 groups based on their epitopic assignments. Group 1 mAb 123C2 maps in the N-terminus of α between amino acids 1-23; Group 2 antibodies (mAb 129C4, mAb 124D1 and mAb 121C5) map in the central region between amino acids 190-210; Group 3 antibodies

(mAb 130B1 and mAb 125C6) map in the C-terminus between amino acids 310-320. mAb 130C2 is anomalous since it maps to the N-terminus between amino acids 1-23 as well as the C-terminus between amino acids 320-329. Three antibodies (mAb 130C2, mAb 121C5, and mAb 125C6) inhibited CRP-dependent initiation with *lac* P^+ but not with *lac* UV5 or *gal* P^+. Inhibition was observed with free RNA polymerase and RP_c; the preformed RP_o was insensitive. Only *lac* P^+ was sensitive to these anti-α antibodies supporting the concept that the mode of interaction of RNA polymerase with CRP differs between *lac* P^+ and *gal* P^+. Anti-α antibodies failed to inhibit transcription from several activator independent promoters.

The immunogenic regions on α, revealed by polyclonal antibodies, mostly correspond to the regions involved in either subunit-subunit contacts or molecular interactions with *cis*-acting UP elements or *trans*-acting transcription factors. The determined regions also are in good agreement with the epitopes identified for the monoclonal antibodies.

Subunit specific monoclonal antibodies were used to assess interaction between the individual subunits and σ^{70} by the ability of another polymerase subunit to coimmunoprecipitate σ. The results indicate that free β' can bind to σ in solution in the presence or absence of heparin, tRNA or nonspecific DNA. This was also confirmed by HPLC gel filtration. This interaction was mapped to the N-terminal domain of β' including residues 201-477. None of the other free subunits or the $\alpha_2\beta$ complex coimmunoprecipatated the σ subunit.

DEDICATION

This work is dedicated to Hazarat Mirza Nasir Ahmad, the third successor to Hazrat Imam Mahdi and the Messiah

(May Allah be pleased with them both)

His devotion to science and education, and the dream for more scientists in our community inspired me to pursue a career in science.

In the name of Allah, the Gracious, the Merciful

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ABBREVIATIONS

BSA:	bovine serum albumin;
cAMP:	3', 5' cyclic adenosine monophosphate;
CRP:	cyclic AMP receptor protein;
DMEM:	Dulbecco's Modified Eagle Medium
DTT:	dithiothreitol;
EDTA:	ethylenediamine tetraacetic acid;
IPTG	isopropyl-1-thio-β-D-galactopyranoside
lac:	the lactose operon;
*lac*P$^+$:	wild-type *lac* promoter;
mAb:	monoclonal antibodies;
NFCM:	non-fat carnation milk;
PAGE:	polyacrylamide gel electrophoresis;
PBS:	phosphate buffered saline;
SDS:	sodium dodecyl sulphate;
TCA:	trichloroacetic acid;
TEMED:	N, N, N', N'-tetramethylethylenediamine;
Tris:	Tris-(hydroxymethyl) aminomethane.

INTRODUCTION

The DNA dependent RNA polymerase of *Escherichia coli* is a multi subunit enzyme which is found in two states: a catalytically competent core enzyme or a promoter selective holoenzyme. The core enzyme is composed of at least four subunits; two identical α subunits, one β and one β' subunit ($\alpha_2\beta\beta'$), with molecular masses of 36511, 150615, 155159 Daltons respectively (Burgess *et al.*, 1987). Addition of one of several molecular species of the σ subunits converts the core enzyme into a promoter selective holoenzyme. The β and β' subunits are encoded by *rpoB* and *rpoC* genes respectively which are transcribed as a polycistronic mRNA. However, α and σ subunits are encoded by two different genes, *rpoA* and *rpoD* respectively (von Hippel *et al.*, 1984). All of the subunit genes have been sequenced and cloned into expression vectors (Zalenskaya *et al.*, 1990; Gribskov and Burgess, 1983).

The enzyme engages in a complex sequence of steps for RNA synthesis including recognition of the specific sites on the DNA template, initiation of the RNA chain *de novo*, elongation of the chain and termination of the chain. The initiation of transcription is subdivided into three steps (Chamberlin, 1974; McClure, 1985) which are schematically represented as follows:

$$\begin{array}{ccccccc} & & & & & & \text{NTPs} \\ R + P & \rightleftharpoons & RP_c & \leftrightharpoons & RP_o & \rightharpoonup\rightharpoonup\rightharpoonup & RNA \\ & & K_b & & K_f & & \text{Promoter clearance} \end{array}$$

The enzyme is believed to slide along the chromosomal DNA to find specific promoter sequnces. Visual demonstration of this sliding phenomenon has been reported (Kabata *et al.*, 1993). Upon recognition of a promoter sequence, RNA polymerase reversibly binds to it to form a closed promoter complex (RP_c). This step is σ dependent and core polymerase without σ cannot recognize a promoter site. RP_c readily undergoes an almost irreversible trasformation to form an open promoter complex (RP_o). The rate of the enzyme release from this complex is extremely slow with a half-time of many hours (Hinkle and Chamberlin, 1971, 1972). This step involves significant conformational changes both in the RNA polymerase holoenzyme (Polyakov *et al.*, 1995) as well as in the promoter DNA, including melting of about 18 base pairs (54Å) at the promoter (reviewed in McClure, 1985). The enzyme reads the single stranded template DNA strand and catalyzes the synthesis of RNA complimentary to the template DNA strand using the substrate nucleoside triphosphates. The enzyme, thus moves forward sliding on the DNA template, clearing the promoter for another round of transcription. Once about 9 bases have been added to the growing RNA chain, σ falls off demarkating the end of initiation and the start of the elongation complex (McClure, 1985). Elongation is carried out by the core polymerase and termination occurs by a variety of different mechanisms (reviewed in Platt, 1986; Yager and von Hippel, 1987). Positive and negative regulatory proteins are also involved in the expression of certain genetic regions. The RNA polymerase from *E. coli* has been extensively studied in an effort to elucidate the structure and function of this multi subunit enzyme.

A diverse range of approaches has generated information on the shape and dimensions of the enzyme as well as the modes of subunit interactions. Early studies using low-angle X-ray measurements revealed that the enzyme had a somewhat elongated shape with a radius of gyration of approximately 60Å and elongation of no greater than 150Å (Pilz *et al.*, 1972). Further studies refined the shape and dimensions of this complex enzyme (Lederer *et al.*, 1991; Coggins *et al.*, 1977; Hillel and Wu, 1977). A model, based on small-angle neutron scattering suggested the core enzyme to be an extended triangle of elongated subunits. Subunits β and β' were found to be curved (Stockel *et al.*, 1980a). The σ subunit was found to be nestled up to the core in a space filling manner, making contacts with one of the two α's, as well as with β and β' (Stockel *et al.*, 1980b). Using the small angle X-ray scattering, Meisenberger and coworkers (1980b) confirmed and extended the model proposed by Stockel. Both β and β' were found to be conical in shape, lying side by side with their thick ends in the same orientation, and curved, elongated α discsinteracting with these thick ends (Meisenberger *et al.*, 1980c). Free σ was found to be a Y-shaped molecule (Meisenberger *et al.*, 1980a). A computer generated three dimentional model, derived from two dimensional X-ray crystallography, revealed and confirmed the elongated shape of the enzyme (100 Å X 100 Å 160 Å). A striking feature in this model was the presence of a thumb-like projection surrounding a channel of about 25 Å in diameter and 55 Å in length that could accomodate about 16 bases of double stranded DNA. This may relate to the melted segment of DNA that is seen in the form of a bubble at the transcription site.

Therefore, this cleft has been proposed to be the catalytic site of the RNA polymerase composed of the β and β' subunits. This is supported by the fact that this projection has a significant homology to the catalytic site of the DNA polymerase I Klenow fragment (Darst *et al.*, 1989) and amino acid sequence homologies have been found between Pol I and the β' subunit of the RNA polymerase (Allison *et al.*, 1985). More recently, refined two dimensional crystals of core RNA polymerase have been analyzed by EM of negatively stained crystals tilted at different angles at a resolution of 23 Å (Polyakov *et al.*, 1995). The structure revealed fascinating changes in the conformation as compared to the RNA polymerase holoenzyme, yet resembled the yeast RNA polymerase II in structure. All of the three structures show the thumb-like projection, however, it is closed, in the form of a ring, in core polymerase and yeast polymerase II, while it is open in the RNA polymerase holoenzyme from *E. coli.* Owing to the limits of resolution, identification of the individual subunits in the functional enzyme or the regions involved in either subunit-subunit contacts or molecular interactions with *cis*-acting UP elements or trans-acting transcription factors have not been possible. Therefore, our knowledge and understanding regarding the structure in regard to its function as well as subunit interactions is still limited to biochemical and molecular biological techniques.

The native enzyme can be dissociated into individual subunits by increasing amounts of urea, and removal of the urea under appropriate conditions will result in the reconstitution of a functional enzyme (Lill and Hartmann, 1970; Heil and Zillig,

1970; Ishihama and Ito, 1972). The purified subunits can be assembled *in vitro* to form a functional enzyme (Zalenskaya *et al.*, 1990). The *in vitro* reconstitution of the enzyme occurs in a sequential manner and in, at least, three steps: the formation of α homodimer, the addition of β to form an obligatory intermediate complex $\alpha_2\beta$, and finally the addition of β' to form a premature core. Addition of DNA, or incubating at 37° C converts it to a mature core enzyme (reviewed in Ishihama *et al.*, 1987). Addition of σ also rapidly converted the premature core enzyme into a mature holoenzyme (Fukuda and Ishihama, 1974).

The β subunit has been proposed to be involved in the catalytic activity of the enzyme (Lisitsyn *et al.*, 1988; Kashlev *et al.*, 1990) as well as in the binding of nucleotide substrates and rifampicin (Landick *et al.*, 1990). *In vitro* analysis of a purified mutant RNA polymerase containing an internally deleted β subunit revealed an altered promoter selectivity by the enzyme and thus suggested a role of β in transcriptional initiation (Glass *et al.*, 1986). Mutations conferring resistace to rifampicin, and streptolygidin have been mapped to this subunit (Raussay and Zillig, 1969; Iwakura *et al.*, 1973). Rifampicin mutations were found to be in the form of clusters mostly in the central region of β: cluster I between residues 507-533, cluster II between residues 563-572, and a distal site at residue 687 (Jin and Gross, 1988). Some N-terminal rifampicin mutants were also identified at residue 146 (Lisitsyn *et al.*, 1984). Strptolygidin mutations were found between the two rifampicin clusters, at residues 544 and 545 (Lisitsyn *et al.*, 1985). Further mutation analysis confirmed the rifampicin localization to the previously identified clusters and that of

streptolygidin to the spacer region between the two clusters (Severinov *et al.*, 1993). Earlier studies suggested that the N-terminal half of β was involved in the assembly of core enzyme and the extreme C-terminal region interacted with the σ subunit (Glass *et al.*, 1986c, 1988). Further studies by Landick *et al.*, (1990) suggested that the N-terminal region of the β subunit was very important for the proper folding and assembly of this subunit. Two dispensible regions in β of ~250 amino acids, one centered around residue 300 and the other around residue 1000 were shown to be non-conserved and dispensible (Severinov *et al.*, 1994). More recently, it has been demonstrated that β fragments encoded by segments of *rpoB* flanking the two dispensible regions could reconstitute a functional enzyme *in vitro* suggesting that the β subunit was composed of at least three distinct domains (Severinov *et al.*, 1995). Some data also suggest that β binds with σ and it also interacts with ppGpp (Ishihama, 1988;). Crosslinking studies identified a βσ complex among other complexes (McMahan and Burgess, 1994; Hillel and Wu, 1977).

The β' subunit was initially thought to be involved in transcription termination (Jin and Gross, 1989; Ito *et al.*, 1991). Further studies strongly suggested a possible role of β' in the catalytic activity of the enzyme as well as in the interaction with the σ subunit (Kumar, 1981; Lazcano *et al.*, 1988). Among five monoclonal antibodies specific for the β' subunit, mAb 311G2 strongly inhibited the initiation and elongation activity of the RNA polymerase directed by $d(A\text{-}T)_n$ or *lac* promoter while not interfering with the enzyme's promoter binding ability, further suggesting a catalytic function for this subunit (Rockwell *et al.*, 1985; Rockwell, 1986). The mAb

was also shown to inhibit abortive initiation with both *lac* P or *lac* UV5 promoters. Based on the epitope map of the mAb, the catalytic activity might be located around amino acid residues 1047 and1072 (Luo and Krakow, 1992). β' also shares conserved regions of homology with the largest subunit of eukaryotic RNA polymerase II as well as the archaebacterial largest subunit (Allison *et al.*, 1985; Biggs *et al.*, 1985; Ahearn *et al.*, 1987; Puhler *et al.*, 1989; Nawrath *et al.*, 1990; Woychik and Young, 1990). It has the ability to strongly bind to DNA and can also bind to the polyanion, heparin, which competes with DNA for a template-binding site on the RNA polymerase (Walter *et al.*, 1967). The conserved regions contain a highly basic region which is believed to be the DNA binding domain. This binding is weakened by the addition of σ, relating the binding ability of core and a reduced affinity of holoenzyme for non-specific DNA (Zillig *et al.*, 1970, 1971; Fukuda and Ishihama, 1974). Binding ability of a β'σ complex to a promoter sequence has not yet been documented. Several mutant RNA polymerases defective in DNA binding show mutations in the β' subunit (Panny *et al.*, 1974; Gross *et al.*, 1976; Sugiura *et al.*, 1977). Reconstitution experiments demonstrated the β' dependence for σ interaction with core polymerase, suggesting a β'-σ interaction (Palm *et al.*, 1975). Stable β'σ complexes have been isolated from *L. curvatus* RNA polymerase by phosphocellulose chromatography (Stetter and Zillig, 1974). Crosslinking resulted in a β'σ complex among other crosslinked complexes (McMahan and Burgess, 1994; Hillel and Wu, 1977). Several internal deletions covering all regions in the β' subunit downstream from amino acid residue 201, could still assemble with other

subunits into core. However, the core enzymes containing mutant β' subunits that had lost sequences in the N-terminal region (residues 201-477) failed to bind σ (Luo, 1992; Luo *et al.*, in preparration) suggesting that the N-terminal extreme domain might be involved in core assembly while regions around 210-477 are involved in the σ binding. RNA polymerases with mutant β' subunits have been shown to be defective in recognizing termination sequences suggesting its role in transcription termination (Coppo *et al.*, 1975). A mutation at residue 402 was reported to restore rho-dependent termination, and inhibited λ Q-mediated antitermination without affecting N antitermination (Ito and Nakamura, 1993). A zinc-binding region in the β' subunit of RNA polymerase, involved in antitermination of early transcription of phage HK022, has also been identified (Clerget *et al.*, 1995).

The σ subunit directs the enzyme to recognize specific promoters (Reznikoff *et al.*, 1985). *E. coli* and other eubacteria have several species of σ factors besides the predominant σ^{70}, which alter the recognition specificity of RNA polymerase for different promoters (Lesley *et al.*, 1987; Fujita and Ishihama, 1987; Binnie *et al.*, 1986). Different σ factors recognize different sets of promoters and hence allow selective transcription. All σ factors, by definition, are involved in at least two of the four distinct biochemical activities i.e. core binding, activation of promoter recognition, DNA melting, and inhibition of non-specific transcription (Helmann and Chamberlin, 1988). Sigma factors have been classified into two families based on the homologies in structure as well as mode of transcription initiation. Most sigmas belong to a single family of proteins related to the major *E. coli* σ^{70} family, while σ^{54}

(σ^N), encoded by *rpoN*, belongs to a second family of sigmas that are distinct both structurally as well as functionally from the σ^{70} family and their mode of transcription initiation resembles more closely with that of the eukaryotes (reviewed in Merrick, 1993). Within the σ^{70} family, four regions of significant homology have been identified among different σ factors suggesting a common role of the conserved sequences in their general activity (reviewed in Helmann and Chamberlin, 1988; Lonetto *et al.*, 1992). Region 2 (amino acid 435-443) has been proposed to interact with the -10 hexamer, while part of the region 4 (amino acid 584-588) has been shown to recognize the -35 hexamer of a promoter (Siegele *et al.*, 1989; Waldburger, *et al.*, 1990; Gardella, *et al.*, 1989). The predominant form of the σ subunit is a 70.2 kDa protein, σ^{70}, which is present during the regular growth phase of *E. coli.* The σ subunit is essential for promoter recognition and thus for initiation of transcription in procaryotes, however, it is released from the core enzyme after the transition from initiation to elongation complex with a transcript of about 10 nucleotides long (Krakow and von der Helm, 1971; Gerard *et al.*, 1972). Intact σ does not bind to DNA, however, it has been domonstrated that C-terminal fragments of sigma, bearing the putative DNA binding domains, are able to bind DNA indicating that N-terminal region masks the DNA binding determinants (Dombroski *et al.*, 1992). This also suggests that σ undergoes conformational changes upon binding to core enzyme such that the DNA binding determinants are now exposed. Low angle X-ray scattering reveal that it is a Y-shaped molecule, interacting with β, β' and one of the two α subunits in the core polymerase (Meisenberger *et al.*,

1980a;Meisenberger *et al.*, 1980c). Biochemical studies demonstrated that σ^{70} reduced the affinity of the core subunits for non-promoter DNA by about 10^4 (Helmann and Chamberlin, 1988). Physical measurements indicated that σ induces conformational changes in core polymerase upon binding (Wu *et al.*, 1976). Comparison of three-dimensional structures of the core and holoenzyme confirmed that binding of σ causes significant conformational changes in the enzyme, the most striking of which is the opening of the channel which has been proposed to be the catalytic site in the RNA polymerase (Polyakov *et al.*, 1995; Darst *et al.*, 1989). This open channel configuration of the holoenzyme may allow it to scan for the promoter sequences while sliding along the DNA. Cross-linking of ribnucleotide analogs bound in the initiation site of the open promoter complexes to σ and subsequent proteolytic and chemical degradation suggested that the C-terminal part of conserved region 3 was part of the active center of RNA polymerase (Severinov *et al.*, 1994a). The σ subunit has been shown to cross-link to all the subunits of the core enzyme indicating its close proximity (McMahan and Burgess, 1994; Hillel and Wu, 1977).

The α subunit of *Escherichia coli* RNA polymerase is composed of 329 amino acid residues, and is encoded by the *rpoA* gene. It has significant homology with the α subunit of *Bacillus subtilis* and it is identical to the α of *Salmonella typhimurium* (Boylan *et al.*, 1989; Lombardo *et al.*, 1991). The α subunit is considered to be the center of enzyme assembly since it forms a dimer in solution upon which ß and ß' subunits are sequentially assembled. Hence, the core RNA

polymerase assembles in the following sequence: $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (reviewed in Ishihama *et al.*, 1987). Analysis of mutations in the *rpoA* gene affecting RNA polymerase assembly (Ishihama *et al.*, 1980; Kawakami and Ishihama, 1980; Igarashi *et al.*, 1990) indicated that the N-terminal region of α plays an important role in core enzyme assembly. For example, a mutant α protein that has lost as much as 94 amino acids from its C-terminus, can still be reconstituted into a functional core RNA polymerase (Igarashi *et al.*, 1991a). An anti-α monoclonal antibody that was shown to block the assembly of the enzyme (Riftina *et al.*, 1989), maps its epitope between residues 190 and 210 (Sharif *et al.*, 1994). Kimura *et al.* (1994) demonstrated that a fragment of α, that has lost 20 amino acids from its N-terminus and 94 from the C-terminus, can still assemble with other subunits to form a functional enzyme. Further deletion of the N-terminus resulted in a defect in α dimerization. Previously, it was shown that two temperature sensitive mutant alleles of α for the assembly of the enzyme, *rpoA*101 and *rpoA*112 (Kawakami and Ishihama, 1980), have undergone single amino acid substitutions at positions 191 and 45, respectively (Igarashi *et al.*, 1990). More recently, it was demonstrated that mutations at Arg45 make α inactive in binding β (Kimura and Ishihama, 1995b even though the mutant α forms dimers (Ishihama *et al.*, 1980; Kawakami and Ishihama, 1980; Igarashi *et al.*, 1990; Kimura and Ishihama, 1995b). Thus the major contact site on α for the β subunit is proposed to be located in the N-terminal region near residue 45 and for the β' subunit is located between residues 180 to 200 (Kimura and Ishihama, 1995a; 1995b).

Initial *in vitro* studies of the C-terminally truncated mutants of α suggested that the C-terminal domain of α was involved in transcription activation with *Class I* promoters, where activators bound upstream of the promoter -35 region (Igarashi and Ishihama, 1991; Igarashi *et al.*, 1991b). Activation of transcription remained unaffected with *Class II* promoters, where activators bound to a region overlapping the promoter -35 region. This was confirmed by a systematic analysis of various kinds of C-terminal deletion derivatives of α (Igarashi *et al.*, 1991a; Hayward *et al.* 1991). Several activators have now been indicated to require the C-terminus of the RNA polymerase α subunit (contact site I) and most of them bind upstream of the promoter -35 region (Giladi *et al.*, 1992; Gussin *et al.*, 1992; Tao *et al.*, 1993;). However, the studies revealed that the location of the factor DNA binding site might not determine the region of RNA polymerase with which the activating factor interacts (Gussin *et al.*, 1992). Therefore, factors are classified into those that involve contact site I or contact site II (reviewed in Ishihama, 1993). On the other hand, several point mutations have been isolated which are defective in positive control by different transcriptional activators (Sunshine and Sauer, 1975; Dale *et al.*, 1986; Garrett and Silhavy, 1987; Matsuyama and Mizushima, 1987; Giffard and Booth, 1988; Thomas and Glass, 1991; reviewed in Russo and Silhavy, 1992; Sharif and Igo, 1993). These data support the model proposed by Matsuyama and Muzishima, (1987) that the polymerase associated α subunits face away from the transcription start point. This orientation enables α to interact with regulatory proteins bound upstream of the promoter. Zou *et al.*, (1992) screened a C-terminal

random mutant library of α for mutants defective in *lac* P1 transcription. DNA sequencing of the *rpoA* gene for ten randomly picked mutants revealed that all of the mutations cluster in a narrow region between amino acids 265-270. Another PCR random mutagenesis study generated *rpoA* mutants defective in CRP-dependent but not CRP-independent transcription. All mutations were mapped to a narrow region on α between amino acids 258 and 265. Alanine scanning, using site directed mutagenesis to replace one amino acid at a time, from residue 255 to 270 revealed that Glu-261 was the most important andmutations at Asp-258 or Asp-259 were also defective in CRP-dependent transcription (Tang *et al.*, 1994). Slauch *et al.* (1991) have shown that both positive and negative regulation of porin gene transcription involves interaction between the ompR protein and RNA polymerase through the α subunit. Several of the *rpoA* mutants affecting regulation of gene expression cluster in the C-terminal portion of the α protein (Slauch *et al.*, 1991; Matsuyama and Mizushima, 1987; Thomas and Glass, 1991; Lombardo *et al.*, 1991). A third recognition element in bacterial promoters has been identified in the *Escherichia coli* ribsomal RNA promoter *rrnB* P1in addition to the -10 and -35 hexamers. It is an AT rich DNA sequence found upstream of the -35 hexamer, called UP element, and has been found to be recognized by the α subunit both in the free form or in the RNA polymerase holoenzyme assembled form indicating a direct role of α in promoter recognition (Ross *et al.*, 1993). RNA polymerases containing particular point or deletion mutations in the C-terminal region of α failed to be activated by the UP element. These mutants are also defective in some of the

factor dependent initiation reaction suggesting a common mechanism between the two processes. NMR studies of a C-terminal α fragment (residues 233-329) reveal that helix 1 (residues 264-273) recognizes the sequense of the UP element though Asn^{268} on the same side of the α-helical array may also be involved (Jeon *et al.*, 1995). Limited proteolytic studies reveal that α consists of two distinct structural domains, an N-terminal domain comprised of amino acids 8-241 which is involved in subunit assembly, a C-terminal domain comprised of amino acids 249-329 which is involved in *trans*-acting factor or *cis*-acting DNA binding, and an interdomain region of ≥13 amino acid residues which forms a flexible linker between these two domains (Blatter *et al.*, 1994). Another proteolytic analysis done using V8 protease and trypsin defines a similar structural domain organization in α, with slightly different bounderies: the amino-terminal domain upstream of residue Arg^{235}, the carboxy-terminal domain downstream of residue Glu^{244} and the linker interdomain of residues 235-244 which is highly accessible to endoproteases (Negishi *et al.*, 1995). This structural organization (Figure 1) has lead to a better understanding of the transcription activation mediated through α from various factors involved with architecturally different promoters (Busby and Ebright, 1994).

Subunit specific polyclonal as well as monoclonal antibodies have proven to be a useful tool for probing the function and topological arrangements of the RNA polymerase subunits (Stender, 1980,1981; Nikiforov, *et al.*, 1983; Rockwell *et al.*, 1985; Rockwell and Krakow, 1988; Riftina *et al.*, 1989, 1990; Dalla Venezia and Krakow, 1990; Luo and Krakow, 1992). Some of the anti-α monoclonal antibodies

have been shown to specifically inhibit abortive initiation with the *lac* P1 promoter (Dalla Venezia and Krakow, 1990). This gene is regulated by the cAMP receptor protein (CRP), a positive regulatory protein, which binds to a region centered at -61.5 on the *lac* promoter. This suggests that the mAbs, upon binding to α, interfere with the function of CRP.

The present study utilizes subunit specific monoclonal or polyclonal antibodies to probe the structure and the function of the α and β' subunits of the *Escherichia coli* RNA polymerase. In the first part of the thesis, epitopes have been mapped for a set of monoclonal antibodies specific for the α subunit and are further characterized for their effects on the initiation of transcription with CRP-dependent or independent promoters. In the second part, anti-α peptide specific polyclonal antibodies have been used to identify immunogenic and thus surface exposed regions of the α subunit. In the third part, interaction of σ with individual RNA polymerase subunits and subassembly forms have been investigated using subunit specific monoclonal antibodies and a β' domain has been identified that interacts with the σ subunit.

MATERIALS AND METHODS

Sources of Materials:

Reagents were obtained as follows: CsCl and USBioclean, United States Biochemical; ethidium bromide, aprotinin and Protein A-Sepharose, Sigma; restriction endonucleases and *In vitro* Rabbit Reticulocyte Lysate Translation Kit (Type II), T7 RNA polymerase, Boehringer Mannheim; DMEM[1], fetal bovine serum, Gibco; *In vitro* Transcription Kit, Stratagene; Tran^{35}S-label and ^{3}H-leucine, ICN Biochemicals; goat anti-mouse IgG, Sigma and Cappel; acrylamide, Serva Fine Chemicals Inc.; S-30 Lysate System and RNasin, Promega; DNA amplification kit, Perkin Elmer Cetus; goat-anti-mouse IgG-phosphatase, Kirkegaard & Perry Laboratories Inc; non-fat powdered milk, Carnation Company; WesternLight protein detection kit, Tropix.

Bacterial Strains and Plasmids:

E. coli strains used were:HMS174 F$^-$ *hsdRr*$_K$ *m*^+_K *rifR* (DE3)[pT7α] (obtained from Dr. A. Goldfarb, New York Public Health Institute, Zalenskaya *et al.*, 1990); BL21(F$^-$ *ompTr*$_B^-$ *m*$_B^-$)[λDE3] (Studier and Moffatt, 1986), from Dr. A. Ishihama, National Institue of Genetics, Japan, Plasmids used were: pT7α (obtained from Dr. A. Goldfarb; Zalenskaya *et al.*, 1990); pTAX185, pTAD235, pTAD176, and pTAD150 (Igarashi *et al.*, 1991); pGEMAX185, pGEMAD296, and pGEMAD256 (Igarashi and Ishihama, 1991)(obtained from Dr. A. Ishihama). pHW104, a plasmid that has four

copies of *lac* P^+ cloned between *Eco* RI sites, was obtained from Dr. D. M. Crothers (Yale University). pKB252 carrying the *lac* UV5 promoter (obtained from Dr. A. Ishihama; Kajitani and Ishihama, 1983); pBdC1 containing a 240 bp *gal* promoter between *Hpa* II (obtained from Dr. A. Ishihama; Taniguchi *et al.*, 1989); pBR322 was purchased from Bohringer Mannheim.

Preparation of Proteins:

RNA polymerase was prepared from *E. coli* K12 by the methods indicated in Fujita *et al.* (1987) and Hager *et al.*, (1990). Protein concentrations were determined by the following extinction coefficients; core polymerase, $E^{1\%}_{280nm}$ = 5.8; holoenzyme, $E^{1\%}_{280nm}$ = 6.7 (Levine *et al.*, 1980).

The α subunit was purified with modifications to the procedure of Zalenskaya *et al.*, (1990). *E. coli* cells (HMS174(DE3)[pT7α]) were grown to log phase and induced with 0.4 mM IPTG for 3 hours at 37°C in a Magnaferm fermentor (New Burnswick Scientific). The cells were harvested by centrifugation at 3000 X g for 10 minutes at 5° C in Beckman J2-21 centrifuge. All the purification steps were carried out at 5° C, unless otherwise mentioned. After lysing the cells in lysis buffer (50 mM Tris-HCl (pH 8.0), 4 mM EDTA, 0.1 mM DTT, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1 M NaCl, 5% glycerol, and 130 µg/ml lysozyme) and sonication for six times in 20 second pulses, S-100 fractions were obtained by ultracentrifugation in 70Ti rotor (Beckman) and loaded onto a Q-Sepharose (2.5 X 10 cm) column

equilibrated with 50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM DTT and 5% glycerol (TGED) + 50 mM NaCl. After washing with the equilibration buffer, proteins were eluted with a linear gradient of 50 to 700 mM Nacl in TGED. The fractions were analyzed on SDS-PAGE and the peak fractions, containing α, were pooled, concentrated using ammonium sulfate and loaded onto a Sephacryl S-100 (2.5 X 100 cm) column equilibrated with TGED + 0.1 M NaCl. The peak fractions were pooled and subjected to another round of Q-Sepharose chromatography (0.5 X 3 cm column) followed by Sephacryl S-100 size exclusion chromatography. The peak fractions were pooled and loaded onto a hydroxyapatite column (0.5 X 5 cm) equilibrated with 50 mM Tris-HCl (pH7.9), 5% glycerol (TG) and with TG + 0.1 mM DTT (TGD) just prior to loading. The proteins were eluted with a linear gradient of 5 to 200 mM sodium phosphate (NaH_2PO_4). α eluted between 50 to 100 mM sodium phosphate and was considered to be 99% pure. Peak fractions were pooled, concentrated by ammonium sulfate precipitation and dialyzed against storage buffer (50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM DTT, 0.1 M NaCl, and 50% glycerol). The α protein concentration was determined by using the extinction coefficient $E^{1\%}_{280nm} = 3.7$ (Levine, *et al.*, 1980).

β, β' and σ^{70} and mutant α subunits were prepared in the laboratory of Dr. Ishihama and given as a generous gift. Internally deleted β' mutants were prepared by Dr. Luo in this laboratory as follows: BL21 (lDE3) pLysS or K38/pGp1-2 were transformed with plasmids containing the mutant β' constructs shown in Figure 23.

Transformants were grown in 200 ml LB medium containing 200 μg/ml of ampicillin at 37°C (for BL21 cells) or at 30°C (for K38/pGp1-2 cells). Expression of mutant β' proteins was induced at Klett 30 by adding IPTG to 1 mM (for BL21 cells) or by shifting the temperature from 30°C to 42°C (for K38/pGp1-2 cells). After induction for 3 hr, cells were harvested and stored at -80°C. For purification, frozen cells collected from a 200 ml culture were resuspended in 1.5 ml Lysis Buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 0.1 mM NaCl). After adding phenylmethanesulfonyl fluoride to 0.27 mM, lysozyme to 0.3 mg/ml and sodium deoxycholate to 0.1%, the mixture was incubated on ice for 20 min with occasional mixing. The cell lysate was sonicated 5 sec for 4 times, and then centrifuged at 15,000 rpm for 10 min. The pellet was resuspended in Lysis Buffer. After centrifugation at 15,000 rpm for 10 min, the pellet was washed with Lysis Buffer containing 0.2 mM NaCl, and extracted twice with Extraction Buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM DTT, 0.2 M KCl, 10 mM MgCl2, 20% glycerol, and 6 M guanidine-HCl. The combined extract was centrifuged at 40,000 rpm for 90 min at 4oC in a Beckman TLS55 rotor. The supernatant was stored at -20°C, and used for reconstitution of RNA polymerase.

CRP was purified by a modification of the procedure of Eilen and Krakow (1977) using *E. coli* cells transformed with pHA7 (Aiba, *et al.*, (1982).

Anti-α, anti-β, and anti-β' monoclonal antibodies were prepared according to Rockwell *et al.* (1985). Anti-σ monoclonal antibodies were prepared in the laboratory of Dr. Burgess and given to us as a generous gift. Polyclonal anti-α antibody was

raised in rabbits as indicated in Iwakura *et al.* (1974). The antisera were prepared by the laboratory of Dr. Ishihama and given as a generous gift. Anti-α peptide polyclonal antibodies were made in the laboratory of Dr. R. Glass (Edinburgh, UK) and given as a generous gift.

***Partial Digestion of the α Subunit*:**

150 µg of purified α subunit was partially digested in 100 µl of 10 mM Tris-HCl (pH 8.0), 40 mM NaCl, 1 mM EDTA and 0.1 mM DTT and 4.5 µg chymotrypsin for 15 minutes at 37°C. The reaction was stopped by adding 20 µl of 20 mM PMSF.

***Reverse-Phase HPLC*:**

150 µg of α protein partially digested with chymotrypsin was loaded onto a C-8 column equilibrated with 10% acetonitrile in HPLC grade H_2O using a Gilson HPLC model 704. The column was washed for 10 minutes with 10% acetonitrile in 0.1% trifluoroacetic acid (TFA) and the fragments were eluted using a linear gradient from 10% to 60% acetonitrile in 0.1% TFA. The peak fractions were collected using Gilson fraction collector. Samples were dried in a Speed-Vac and taken up in Laemmli buffer for SDS-PAGE.

Gel-filtration HPLC:

64 pmoles of wildtype β' and 128 pmoles of wildtype σ subunit were incubated in 100 µl of reconstitution buffer at 37°C for 30 min. The mixture was then

loaded onto an HPLC gel-filtration column (TSK-GEL G4000SW, (0.5 X 30 cm) from Tosoh) equilibrated in a buffer containing 10 mM Tris-HCl (pH 6.8), 0.1 mM EDTA, 200 mM NaCl, and 5% glycerol at a constant flow rate of 0.5 ml/min. After 8 minutes, 250 µl fractions were collected. 150 µl of each fraction was mixed with 15 µl of 100% TCA + 1% Na deoxycholate, incubated on ice for 10 min, and centrifuged at 4°C for 10 min. The protein pellets were resuspended in 60 µl 1 x SDS sample buffer and adjusted to pH 8 with 2M Trizma base. The samples were heated in a boiling water bath for 2 min and 20 µl of each from fractions 1 through 20 was loaded onto a SDS-7.5% polyacrylamide gel. The protein bands were stained with Coomassie brilliant blue.

***DNA Preparations*:**

Plasmids pTAX185, pTAD235, pTAD176, and pTAD150 (Igarashi *et al.*, 1990) and pGEMAX185, pGEMAD296, and pGEMAD256 (Igarashi and Ishihama, 1991) were purified by CsCl density gradient centrifugation as described by Davis *et al.* (1986). The *lac* P^+, *lac* UV5 and *gal* P^+ used for the transcription assays were prepared as described by Igarashi and Ishihama (1991) and Igarashi *et al.* (1991). Purified promoter fragments, *pBR* P4, *Trp* P^+, *Nus* A, *rps* A, *Leu* X and *rpl* J which produced run off RNA transcripts of 241, 141+186, 99, 90, 81, and 69 nucleotides respectively (Ishihama, personal communication), were provided by Dr. Ishihama. For PCR, pTAX185 was digested with *Xba* I to excise the *rpo*A gene and resolved by electrophoresis on a 0.8% agarose gel. The 1.2 Kb fragment bearing the *rpo*A

gene was cut out and purified using USBioclean. Following phenol/chloroform extraction and ethanol precipitation the DNA fragment was dissolved in TE buffer (5 mM Tris-HCl (pH 8.0), 0.5 mM EDTA).

***In Vitro Synthesis of Truncated α Fragments*:**

Synthesis of Capped mRNA: *In vitro* transcription and capping was carried out as using T7 RNA polymerase to synthesize capped mRNA (Nielsen and Shapiro, 1986). 40 units of T7 RNA polymerase were incubated with 2 µg of the indicated circular or linearized DNA template in the transcription buffer (40 mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$; 10 mM dithiothreitol; 4 mM spermidine; 10 mM NaCl) in the presence of 0.5 mM each of ATP, CTP, and UTP; 0.05 mM GTP; 5 mM $m^7G(5')ppp(5')G$; and 50 units of RNasin ribonuclease inhibitor in a total reaction volume of 50 µl. The reagents were made with double distilled or DEPC treated water. After incubation of the reaction with 40 units of T7 RNA polymerase for 60 minutes at 37°C, another 40 units of the enzyme were added for an additional 60 minutes to increase the yield of the transcripts. 2 µg of circular plasmid DNA: pGEMAX185, pGEMAD296, pGEMAD256, or *Nhe*I-linearized pTAD235, pTAD176, pTAD150 in the presence of 7-methyl GpppG. This produced mRNAs coding for α-wt, α-296, α-256, α-235, α-176 and α-150, respectively. Digestion of pTAX185 with *Sty* I, *Nci* I, *Cfr*13I or *Mlu* I generated templates for the preparation of mRNAs coding for α-23, α-51, α-107, and α-136 respectively. mRNA corresponding to α210-310 was produced by transcribing the PCR generated DNA template with T7

RNA polymerase.

Synthesis of Radiolabeled Proteins in Rabbit Reticulocyte Lysates: The mRNAs were translated using a rabbit reticulocyte lysate system (250 µl reaction mixture) supplemented with 15 µl of radioactive amino acid mixture (0.2 mCi Tran^{35}S-label containing^{35} S-methionine + ^{35} S-cysteine) under the conditions specified in the protocol for the Reticulocyte Translation Kit. Since the amino acid mixture contains cysteine, the protein products are primarily labeled with ^{35}S-methionine. After 120 min incubation at 30°C, aliquots were directly subjected to SDS polyacrylamide gel electrophoresis (Laemmli, 1970) and autoradiography to verify synthesis of the respective α polypeptides.

***PCR Methods*:** Primers for PCR were synthesized using an Applied Biosystems Model 380B DNA Synthesizer (Table I). The upstream primers contain a T7 promoter and a ribosomal binding site attached as a 5' extension. The presence of these sequences are designated as "T7RBS". The numbers represent the amino acid residues in the α protein. The primers designated "R" represent reverse complements of 3' terminal fragments of the α gene supplemented with tandem stop codons. PCR was carried out essentially using the protocol of Lesley *et al.* (1991) using the *Xba* I fragment containing the *rpoA* gene as template.

***S-30 Coupled Transcription-Translation Reactions*:** The DNA templates

generated by PCR were translated in an *E. coli* S-30 fraction (Promega) by a modification of the procedure of Lesley *et al.* (1991) in the presence of 5 µl (0.07 mCi) Tran^{35}S-label in a final volume of 50 µl.

Immunoprecipitation of α Protein and Fragments:

^{35}S- or ^{3}H-labeled α polypeptides in 4 to 8 µl of the reticulocyte lysate or S-30 mix were added to 0.5 ml of DMEM containing 5% fetal bovine serum and 0.02% SDS, and incubated overnight at 5°C with 2 µg of one of the monoclonal antibodies. After addition of 5 µg of goat anti-mouse IgG, the mixture was incubated for 2 hr at 5°C. Immune complexes formed were recovered after addition of 50 µl of 1 mg/ml Protein A-Sepharose in PBS (10 mM potassium phosphate (pH 7.2) and 150 mM NaCl) followed by incubation for 10 min at 5°C and centrifugation for 2 min in a microcentrifuge. The pellet was washed five times with RIPA buffer (20 mM Tris-HCl (pH 8.0), 0.15 mM NaCl, 0.5% Na deoxycholate, 1% Triton X100, 5 mM $MgCl_2$), and then resuspended in Laemmli Buffer. After SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), the gel was stained with Coomassie Blue in 10% acetic acid-50% methanol. After soaking the gel in 1 M sodium salicylate for 60 min (Din *et al.*, 1990), the gel was dried for autoradiography.

For analysis of α-51 and α-23, the amounts of labeled peptide and monoclonal antibody were increased three fold, 15 µg of aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) were added, and the time of incubation for formation of the immune complex was increased to 36 hours at 5°C.

After electrophoresis the gel was microwaved for 40 seconds in Coomassie stain to enhance the retention of these small fragments.

Immunoprecipitation of Subassembled Forms of RNA Polymerase:

20 µl of the reconstituted mixtures of the indicated free subunits was diluted in 1 ml of DMEM+5% FBS to reduce DTT concentration, and incubated with the indicated subunit specific monoclonal antibody at 5° C overnight with constant rotation. The samples were microcentrifuged for 10 minutes at 5° C. 50 µl of 10% protein A-Sepharose in PBS was added to the suppernatants and incubated for another 10 minutes. The immune complexes were recovered by microcentrifugation and washed five times with PBS. The samples were transfered to a new microfuge tubes since free sigma protein nonspecifically bound to the microfuge tubes (data not shown). The pellet was resuspended in Laemlli buffer and boiled for 5 minutes before electrophoresis.

Western Blotting using Monoclonal Antibodies:

In case of *in vivo* expressed C-terminally truncated α proteins, *E. coli* transformed with pGEMAD296 were grown to an absorbance of 0.3 at 600nm and induced with 0.4 mM IPTG for 2 hrs at 37°C. Cells from 0.5 ml of culture medium were spun down in a microcentrifuge, washed with 50 mM Tris-HCl (pH 8.0) and suspended in Laemmli buffer. After boiling for 5 minutes, the supernatant was loaded onto a SDS polyacrylamide gel. In the case of the purified proteins, they

were directly taken up in Laemmli buffer and boiled for 2 minutes. Following electrophoresis, the proteins were transferred onto an appropriate pore size nitrocellulose membrane using a MiniBlot-SDE (Millipore). The nitrocellulose membrane was treated with 5 mg/ml heparin and then blocked in 5% nonfat dry milk in TBS (Luo and Krakow, 1992). The membrane was incubated with one of the anti-α monoclonal antibodies overnight at 4°C.

Immunodetection of the antigens:

A. *Anti-mouse IgG-phosphatase:* After washing 3 times with washing buffer (0.5% nonfat dry milk in TBS), the nitrocellulose strips were incubated with goat-anti-mouse IgG-phosphatase for 2 hrs at room temperature. The strips were washed 3 times with washing buffer, and developed in Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution to visualize the bands.

B. *Enhanced Chemiluminescence*: The individual strips were incubated with goat-anti-mouse IgG-phosphatase, as provided in the kit, for 2 hr at room temperature. After separately washing them in washing buffer for three times for 10 min each, the substrate solution, provided in the WesternLight protein detection kit, was added on to the strips for 1 minute. The strips were drained of the excess solution and quickly fixed on a Whatman 3MM paper using a Scotch Double Stick Tape. They were then wrapped in Saran Wrap and exposed to an X-ray film for detection of the signal.

Single round In Vitro Transcription Assay:

The reaction mix (final volume 50 µl) contained: 50 mM Tris-HCl (pH 7.8), 3.0 mM Mg acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl, 12.5 µg bovine serum albumin, 0.1 pmol each of the indicated promoter fragments and 3 pmol RNA polymerase. For cAMP-CRP dependent promoters 2.5 pmol CRP and 100 µM cAMP was also added in the reaction mixture. After incubating for 30 min at 37°C to form the open promoter complex, 15 µl of a mix containing 0.5 mM each of ATP, GTP, CTP and 0.16 mM UTP + 2 µCi [α^{32}P]UTP and 10 µg heparin was added. Where indicated 12 pmol of one of the monoclonal antibodies was added. The closed promoter complex was formed by keeping the reaction temperature at 4°C. Therefore, antigen-antibody complex formation, in case of preformed RNA polymerase:Ab or RP_c:Ab, was done at 4°C for overnight and at 37 C for 30 minutes in case of RP_o:Ab. Single round transcription was carried out for 5 min at 37°C and stopped by adding 60 µl of a mix containing 40 mM EDTA, 18 µg of *E. coli* tRNA and 0.5 M Na acetate (pH 5.2). Following ethanol precipitation, the transcripts were resolved using 8 M urea-polyacrylamide gel electrophoresis followed by autoradiography.

Western Blotting using Polyclonal Antibodies:

Purified α fragments or core RNA polymerase reconstituted with a C-terminally truncated α fragment were subjected to SDS-PAGE. The resolved bands were transferred to a nitrocellulose membrane using a semidry protein

transfer apparatus (Bio-Rad). The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 hour at 37° C. The membrane was incubated with the indicated anti-α peptide polyclonal antibody for 2 hours at 37° C and then with goat anti-rabbit IgG-peroxidase for 2 hours at 37° C. After washing the membrane three times with the washing buffer, the antibodies bound were visualized using the immunostaining kit (Wako).

Formation of Subassemblies from Free Subunits:

64 pmoles of β or β' (wild type or mutant) or 120 pmoles of α proteins were mixed as indicated in urea buffer and dialyzed against urea buffer (7M urea, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 200 mM KCl, 0.1 mM EDTA, 10 mM DTT and 10% glycerol) and then against the reconstitution buffer (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.3 M KCl, 0.1 mM PMSF and 20% glycerol) at 4°C overnight, mixed with 128 pmoles of σ subunit in 100 µl of reconstitution buffer, and incubated at 37°C for 30 min. Samples were analyzed either by size-exclusion HPLC separation or by immunoprecipitation with monoclonal anti-β' or anti-α antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE and subjected to silver staining for protein detection. In case of β'σ complexes, coimmunoprecipitatible σ subunit was detected by Western blotting using 2D1 anti-σ monoclonal antibody (Dr. Burgess's lab) using the Western-Light protein detection kit (Tropix).

RESULTS

***Partial Chymotryptic Digestion of α Protein and Western Blotting*:**

The α subunit was partially digested as described in Materials and Methods and the resulting proteolytic fragments were resolved by SDS-20% polyacrylamide gel electrophoresis. The proteins were subjected to Western blotting by transferring them to a 0.01 μM pore size nitrocellulose membrane. All the anti-α antibodies bound to intact α, while five mAbs (125C6, 130B1, 130C2, 126C6, 128D5) crossreacted with an α fragment of approximately 130 residues (Fig 2A). The results indicated that the epitopes of these antibodies resided on a region of α spanning 130 amino acids.

***Isolation of the Chymotryptic Fragments by Reverse-Phase HPLC and Western Blotting with the Anti-α Antibodies*:**

The partially digested chymotryptic fragments were resolved by reverse-phase HPLC and subjected to Western blot analysis. Two closely migrating fragments of approximately 130 residues were resolved in fractions 35 and 36. The individual fractions were subjected to Western blot analysis with the anti-α mAbs. Five mAbs (125C6, 130B1, 130C2, 126C6, 128D5) crossreacted with the fragment recovered in fraction 36, none of the mAbs recognized the peptide contained in the fraction 35 (Fig. 2B). The results indicated that these epitopes might be clustered rather than randomly distributed on the α protein.

***Synthesis of Truncated* α:**

^{35}S- or ^{3}H-labeled C-terminal truncated fragments of RNA polymerase α subunit were synthesized *in vitro* in a rabbit reticulocyte lysate using run-off transcripts of 3'-deleted *rpoA* DNA. Internally deleted α fragments coded for by PCR generated DNA templates were synthesized in the S-30 coupled transcription-translation system as described in Materials and Methods, or in the rabbit reticulocyte lysate in the presence of ^{3}H-leucine. Products were resolved by SDS polyacrylamide gel electrophoresis. Autoradiography was performed to verify the synthesis of the desired truncated α fragments (Fig. 3 A, B and C).

α *Aggregates during Immunoprecipitation:*

Immunoprecipitation of the *in vitro* synthesized α subunit revealed that it formed aggregates during extended incubation resulting in an artifactual signal. The aggregation was too strong to be dissociated even with 0.1% SDS. However, prior addition of SDS in the antigen-antibody mixture prevented this aggregation but disrupted the antigen-antibody complex (data not shown). In order to circumvent this problem, decreasing amounts of SDS were added in the antigen-antibody solution prior to the overnight incubation (Figure 4). The amount of SDS to be added in order to prevent aggregation appears to be optimal at 0.02%. The antigen-antibody complex is dissociated as the concentration of detergent approaches 0.1%SDS.

Epitope Mapping by Immunoprecipitation:

Each of the seven indicated anti-α monoclonal antibodies were tested for their ability to bind to the ^{35}S-labeled C-terminal truncated α fragments. All of the α fragments were precipitated by the anti-α polyclonal antibody (Fig. 5). Two of the monoclonal antibodies, mAb 130B1 and mAb 125C6, bound only to intact α. Three antibodies, mAb 121C5, mAb 124D1 and mAb 129C4, crossreacted with α-235 and longer fragments. Only mAb 123C2 (data not shown) and mAb 130C2 bound to all of the fragments including α-150. The results show that the epitopes reside in three distinct regions of the α-subunit; the epitopes for mAb 125C6 and mAb 130B1 in the extreme C-terminus between amino acids 296-329 (Group 3 antibodies); those for mAb 121C5, mAb 124D1 and mAb 129C4 in the central region between amino acids 176-235 (Group 2 antibodies); and those for mAb 130C2 and mAb 123C2 in the N-terminal region between amino acids 1-150 (Group 1 antibodies).

To further localize the epitopes for the Group 1 antibodies (mAb 123C2 and mAb 130C2), shorter N-terminal α fragments were used for immunoprecipitation. As expected mAb 129C4 did not bind to any of the N-terminal α fragments used (Fig. 6) since its epitope is not located between amino acids 1-150. Both mAb 130C2 and mAb 123C2 were able to crossreact with an α fragment as short as 23 amino acid residues. The results indicate that the epitopes for these two antibodies are located in the extreme N-terminal region between amino acids 1-23.

The results using truncated α indicate that the epitopes for the Group 2 antibodies, mAb 124D1, mAb 129C4 and mAb 121C5, are located between amino

acids 176-235, and for the Group 3 antibodies, mAb 130B1 and mAb 125C6, are present within the C-terminal region between amino acids 296-329. To ensure that the removal of these segments was directly responsible for the loss of immunoreactivity and to further define the positions of the epitopes, a set of internal α fragments was synthesized from PCR amplified DNA templates coding for smaller α fragments. A schematic representation of these α fragments is shown in Figure 7. These fragments were examined by immunoprecipitation with the indicated antibodies. As shown in Figure 8, Group 2 antibodies, mAb 124D1, mAb 129C4, and mAb 121C5 bound to α100-210; none of these antibodies crossreacted with α100-190 or α210-329. On the other hand, as shown in Fig. 9, Group 3 antibodies, mAb 130B1 and mAb 125C6 bound to both α210-329 and α210-320, while neither antibody bound to α210-310. The results indicate that the epitopes for Group 2 antibodies are located between amino acids 190-210. The epitopes for both of the Group 3 antibodies reside within the 10 amino acid C-terminal segment spanning amino acids 310-320.

Epitope Mapping by Western Blotting:

Results obtained by Western blotting of the chymotryptic fragments of α indicated that mAbs 125C6, 130B1, and 130C2 had their epitopic sequences localized within 100 to 150 residues. However, the results obtained by immunoprecipitation showed that mAb 130C2 bound to N-terminus while mAbs 125C6 and 130B1 bound to the C-terminus of α. To confirm the epitope map

derived by immunoprecipitation, we carried out Western blot analysis on the *in vivo* expressed, C-terminally truncated α proteins. Fig. 10 shows the results using α-wt and α-296. As expected the Group 1 and 2 antibodies bound to both full-length and truncated α-296 while Group 3 antibodies bound only to full-length α. The more rapidly migrating protein is probably a degraded form of the α-296. The results presented in Figure 6 derived from immunoprecipitation indicated that mAb 130C2 could bind to N-terminal fragments as short as 23 amino acids. It was surprising to find that this antibody was unable to bind to α-296 (Fig. 10) although this truncated protein retains the N-terminal sequence of α. Western blotting is carried out using SDS-denatured proteins and thus the native conformation of the N-terminal epitopic domain essential for binding of mAb 130C2 may be disrupted. It is known that some monoclonal antibodies discriminate between the native and denatured forms of an epitope. Apparently mAb 130C2 cannot bind to the N-terminal epitope on α-296 after denaturation but is still able to bind to the full-sized α as well as a C-terminal proteolytic fragment bearing epitopes for group 3 mAbs. The results indicated that mAb 130C2 recognizes another epitope located in the C-terminal region of α. To confirm this prediction, immunoprecipitation with mAb 130C2 was performed using the C-terminal α fragments, α210-320 and α210-329. mAb 130C2 bound only to α210-329 (Fig. 11). We concluded that mAb 130C2 recognizes a sequence between amino acids 320-329 as well as a conformational epitope involving amino acids 1-23 (Fig. 12).

Functional Mapping of the Monoclonal Antibodies:

A. Activator independent promoters: Single round *in vitro* mixed transcription assays were carried out in a reaction mixture containing activator independent promoter templates (*Trp* P^+, *Nus* A, *rps* A, *Leu* X and *rpl* J) as described in Materials and Methods. The antibodies were incubated with RNA polymerase at 37° C for 30 minutes prior to their addition in the reaction mixture. The substrates were then added to the reaction for RNA synthesis. All of these activator independent promoters remained insensitive to the effects of these anti-α monoclonal antibodies indicating that the antibodies did not interfere with the catalytic function of the RNA polymerase (data not shown).

B. Activator dependent promoters: Single round *in vitro* mixed transcription assays were carried out in a reaction mixture containing *lac* P^+, *lac* UV5 and *gal* P^+ DNA templates as described in Materials and Methods. The antibodies were incubated with RNA polymerase, RP_c or RP_o complexes prior to the addition of the substrates for RNA synthesis. Of the antibodies examined, mAb 130C2, mAb 121C5, mAb 125C6 and mAb 130B1 had previously been shown to strongly inhibit initiation from *lac* P^+ and partially with *lac* UV5 when RNA polymerase was preincubated with these antibodies (Dalla Venezia and Krakow, 1990). The results presented in Figure 13 show that none of the five monoclonal antibodies tested inhibited transcription initiation from the *lac* UV5 promoter. Further suggesting that these monoclonal antibodies do not interfere with the catalytic function of RNA polymerase. In contrast, mAb 130C2, mAb 121C5 and mAb 125C6 strongly and

mAb 130B1 partially inhibited cAMP/CRP-dependent initiation from *lac* P1 (Type I promoter) when these antibodies were preincubated with either holoenzyme or preformed RP_c. After the open complexes were formed, these monoclonal antibodies were without apparent effect on transcription initiation from *lac* P1. Transcription from the cAMP/CRP-dependent *gal* P1 (Type II promoter) was insensitive to all the monoclonal antibodies tested, supporting the concept that CRP makes contact with site I on α for *lac* transcription (Igarashi and Ishihama, 1991), but with site II on σ for *gal* transcription (Kumar *et al.*, 1994; Jin *et al.*, 1995). One of the Group 1 antibodies, mAb 123C2, which recognizes the N-terminal epitope, did not inhibit any of the promoter-dependent reactions. The slowest moving band seen in Figure 13 is only present when the *lac* UV5 template is usedand probably is the end to end transcript produced from this template. The antibodies were also tested for their effects on another promoter, *pBR* P4 whose activity is enhanced by cAMP-CRP. Since this is a very strong promoter, only 0.025 pmole of this promoter DNA was used as compared to 0.1 pmole of *lac* DNA. The antibodies interfered with the activity of this promoter in a similar manner aswith *lac*, though very weakly (Fig. 14) suggesting that the mechanism of CRP interaction with RNA polymerase might be similar on the two promoters.

Epitope Mapping of Anti-α Peptide Polyclonal Antibodies:

Anti-α peptide polyclonal antibodies were raised against smaller α fragments by immunizing rabbits. A schematic representation of these α fragments is shown

in Figure 15. For the determination of the major epitopic regions recognized by the anti-α peptide polyclonal antibodies, the deletion mutant α subunits (Igarashi and Ishihama, 1991; Kimura *et al.*, 1994) were subjected to Western blot analysis against the indicated peptide-specific polyclonal antibody as described in Materials and Methods.

N-terminal and internal deletion mutants lacking sequences of 10 to 20 amino acids in the region from amino acid 1 to 113 were used to assess the presence of immunogenic regions for Antibody 2 (pAb2). Figure 16 shows a Coomassie-stained gel pattern of the purified α deletion mutants used [B] and the immunostained gel pattern on nitrocellulose after treatment with pAb2 [A]. Three α derivatives, αΔN60 (deletion of the N-terminal 60 amino acid residues), αΔN100 and αΔN120, did not crossreact with this antibody. The immunoreactivity also decreased significantly for αΔN10, αΔN20, αΔN30 and αΔN40. All other α derivatives crossreacted with pAb2 as strongly as the wild-type. Thus, pAb2 was inferred to recognize a major region of epitope(s) located in the exteme N-terminus between residues 1 to 10 and a minor region between residues 41 to 60. Regions outside of these two domains showed no detectable immunogenicity.

Three C-terminally truncated mutant α subunits, α-296, α-256, and α-235, lacking the C-terminal 33, 74 and 95 residues, respectively, as well as one internally deleted mutant αΔ221-240 were examined for their immunoreactivity against pAb5. The results shown in Figure 17A clearly indicate that that only α-235 cannot crossreact with this antibody. Thus we concluded that the major region of epitope(s)

recognized by pAb5 is located within a sequence of 16 amino acids in length between residues 240 and 256.

Three N-terminally truncated mutant α proteins, αΔN100, αΔN120 and αΔN160 and three internally truncated α proteins, αΔ121-140, αΔ141-160, and αΔ161-180 were used to determine the immunoreactivity of these proteins with pAb6. Two N-terminally truncated (αΔN120 and αΔN160) as well as one internally deleted (αΔ121-140) α protein did not crossreact with this antibody (Figure 18A). All other α derivatives gave a signal equivalent to wild-type α. We concluded that this antibody recognized epitopes located between residues 112-140.

Five internally deleted α mutant proteins, αΔ141-160, αΔ161-180, αΔ181-200, αΔ201-220, αΔ221-240, were used to assess the binding ability of pAb7. Figure 19A shows the immunoblot pattern against these internal deletion mutants. One mutant, αΔ181-200, bound very weakly, two mutants, αΔ161-180 and αΔ201-220 somewhat strongly, while others bound as strongly as wild-type α with pAb7. Thus it was concluded that the major region of epitope(s) recognized by this antibody is located between residues 181-200, and minor epitopes are also present around residues 161-180 and 201-220. The results suggest that a region constituting residues 161-220 might be exposed at the surface of the α subunit.

Direct Assembly of β'σ Complexes from free Subunits:

Prior results in our laboratory on the assembly of the β' subunit into core and holoenzyme indicated the presence of a β' domain that specifically interacted with

the σ subunit (Luo *et al.,* 1996) suggesting that the β' and σ subunits may directly interact with each other even in the absence of the other RNA polymerase subunits. In order to test this possibility, free β' subunit was incubated with free σ^{70}, using the conditions presented in Materials and Methods; the assembled β'σ complex or σ alone was subjected to gel-filtration HPLC. The results indicated that free σ eluted in fractions 16 to 20 (Figure 20B). However, in the presence of the β' subunit, σ appeared as a faster eluting β'σ complex seen in fractions 1 to 13, in addition to the excess σ that eluted as a free subunit in fractions 16 to 20 (Figure 20A). The results indicated that β' could directly interact with free σ to form a stable β'σ complex. The results were also confirmed by immunoprecipitation of the β'σ complex with the anti-β' monoclonal antibodies even in the presence of excess amounts of tRNA, non-specific DNA or heparin (Figure 21), which are non-specific competitors of β' binding (β', being a basic protein, binds non-spepecifically to polyanions like DNA). In order to assess the ability of other subunits and subassemblies of RNA polymerase to interact with the σ subunit, immunoprecipitation with different subunits was carried out using subunit specific monoclonal antibodies in the presence of σ. The results indicated that $\alpha_2\beta$ failed to coimmunoprecipitate σ, likewise, β'σ failed to interact with the α subunit (Figures 22 A and C) supporting the notion that β' can only be assembled once the obligatory $\alpha_2\beta$ comlex has formed. Free α or β subunits remained unable to coimmunoprecipitate σ (data not shown).

Identification of the β' Domain Interacting with the σ Subunit:

Once it was established that free β' and σ subunits were able to form a stable complex, β' deletion mutant proteins were assayed for assembly with free σ subunit in order to define the region on β' interacting with σ. A schematic representation of these deletion mutant proteins is shown in Figure 23. The ability of a mutant β' to form a complex with σ was assessed by immunoprecipitating the complex with a suitable anti-β' antibody and subsequent detection of the coimmunoprecipitable σ subunit by Western blotting using anti-σ mAb 2D1 as a probe. The results indicated that both wild type β' and JLP6 formed a stable complex with σ while JLP7 as well as JLP7B interacted very weakly with the σ subunit (Figure. 24). The results suggested that σ subunit might be interacting with a region of the β' subunit spanning amino acids 201- 477.

DISCUSSION

The involvement of the α subunit in transcription regulation is a topic of considerable interest. C-terminal truncations which still allow for assembly of the altered α into RNA polymerase are no longer able to support CRP-dependent *lac* transcription (Igarashi and Ishihama, 1991). Likewise, most of the transcription factors that bind upstream of the promoter are unable to activate mutant RNA polymerases containing a C-terminal truncated α (Igarashi *et al.*, 1991). Amino acid substitution mutations within this contact site I region of the C-terminal domain of α render mutant RNA polymerases insensitive to transcription activation by class-I activators (Lombardo *et al.*, 1991; Slauch *et al.*, 1991; Thomas and Glass, 1991; Zou *et al.*, 1992). However, some mutations have also been identified within the N-terminal region. Results obtained using a library of C-terminal and N-terminal random mutations in α showed that most of the Lac^- mutants carried a mutation within a narrow region of α located between amino acids 265-270 (Zou *et al.*, 1992), but about 10% of the Lac^- mutants were found to carry mutations in the N-terminal region (Zou *et al.*, unpublished results). Another study with PCR directed random mutagenesis of *rpoA* gene involved the isolation and sequencing of randomly picked mutants defective in CRP-dependent but not CRP-independent transcription. Remarkably, all the 21 randomly picked mutants showed mutations within a narrow region on α between amino acids 258 and 265. Alanine scanning, using site directed mutagenesis replacing one amino acid at a time, from residue 255 to 270

revealed that Glu-261 was the most important one and Asp-258 or Asp-259 were also defective in CRP-dependent transcription (Tang *et al.*, 1994). Most of the α mutations which made cells defective in *ompR* regulation are located in the C-terminal domain, however, two of the mutations are near the N-terminus (Slauch *et al.*, 1991). One of the mutants, *rpoA*150, is altered at Pro-240 and Leu-28 (it is thought that the significant mutation is Pro-240 and not Leu-28), while the other mutant, *rpoA*53, is mutated in the extreme N-terminus at Gly-3. The apparent *ompR* defect was considered to be a consequence of the overexpression of the mutant α subunit. However, our epitope mapping studies suggest that both the C-terminal and the N-terminal regions may be involved in molecular communication with regulatory proteins.

The epitopes for two Group 3 monoclonal antibodies (mAb 125C6 and mAb 130B1) which inhibit CRP-dependent *lac* P1 transcription are located near the C-terminus of α between amino acids 310-320 (Figure. 12). The epitope for another inhibitory monoclonal antibody (Group 1 mAb 130C2) maps near both the N-terminal region between amino acids 1-23 and the C-terminal region between amino acids 320-329. The ability to inhibit CRP-dependent *lac* transcription is also shown by the Group 2 mAb 124D1 (data not shown) and mAb 121C5 whose epitopes are mapped in the central region of α between amino acids 190-210. This is a region of α for which mutants defective in CRP-mediated regulation have not as yet been demonstrated. Anti-α antibodies which do not inhibit transcription with *lac* P^+ have epitopes which map close to the regions where the epitopes for inhibitory antibodies

reside. Located in close proximity are the epitopes for the inhibitory mAb 124D1 and mAb 121C5 as well as mAb 129C4 which was shown to block assembly of RNA polymerase (Riftina *et al.*, 1989). This suggests that the inhibitory effect of the monoclonal antibodies may be specific rather than merely a steric effect of the monoclonal antibodies binding to α in RNA polymerase. mAb 130C2, mAb 121C5 and mAb 125C6 strongly inhibit initiation when incubated with free RNA polymerase or RNA polymerase in the closed complex. However, they fail to inhibit initiation once the open complex is formed. The resistance of the RP_o complex could be a consequence of the masking of these epitopes by CRP. It is also possible that the antibody can no longer block an essential conformational change in RNA polymerase once the RP_o complex has formed. Recent studies on the three dimensional structure of the core RNA polymerase revealed that it bound tightly to the DNA template closing the channel that has been implicated to be the catalytic center accomodating the DNA bubble. Partial transition of this conformation takes place upon the RP_o formation (Polyakov *et al.*, 1995). Similarly, in a head-on collision between transcribing RNA polymerase and replicating DNA polymerase, RNA polymerase survives without aborting its growing RNA chain. It resumes its activity after a pause indicating a very strong interaction of RNA polymerase-DNA complex (Liu and Alberts, 1994; Liu *et al.*, 1995). Our results clearly demonstrate that the α subunit plays an important role in the interaction of CRP with the holoenzyme for promoters having a CRP binding site upstream of the -35 region. Several other factors have also been shown to interact with α for the transcriptional

activation while several others involve only σ (reviewed in Ishihama, 1993)

Mutations affecting the response to CRP activation of *lac* transcription *in vivo* have been primarily mapped within a narrow region of α located between amino acids 258-270 with Glu-261 being critical in contact with CRP (Zou *et al.*, 1992; Tang *et al.*, 1994). Reconstituted holoenzymes with mutant α subunits, carrying amino acid substitution mutations between position 265 and 270, activated *gal* but not *lac* transcription (Zou *et al.*, 1992). Crosslinking between CRP and the C-terminal region of α has been demonstrated with *lac* promoter (Chen *et al.*, 1994). Proteolytic studies identified two structural domains in α linked to each other with an unstructured flexible linker. The C-terminal domain has the dimerization and DNA binding abilities (Blatter *et al.*, 1994; Negishi *et al.*, 1995). NMR studies of a C-terminal fragment of α suggested that helix 1 and proximal regions are involved in *cis*-acting UP element and *trans*-acting factor binding (Jeon *et al.*, 1995).The inhibitory antibodies bind to epitopes present in regions of α distal to the proposed CRP contact region. Antibody binding may indirectly result in inhibition by blocking an essential conformational change in α consequent to its interaction with CRP. It is also possible that antibody binding could alter the conformation of α such that the proposed contact site (Zou *et al.*, 1992; Tang *et al.*, 1994) is inaccessible to CRP. Benjamin *et al.* (1992) have proposed that an antigen may undergo long range conformational changes distant to the epitopic site upon binding to the antibody. In that case the different monoclonal antibodies upon binding to different α domains may still be inhibitory and hence the actual site on the α subunit that interacts with

CRP on the *lac* P1 type promoter cannot be defined by the epitopes. None of the anti-α monoclonal antibodies inhibit CRP-dependent *gal* P^+ transcription; similarly no α mutants have been presently shown to affect this reaction supporting the notion that the target site of CRP for *gal* activation is located on the σ^{70} subunit (Kumar *et al.*, 1994; Jin *et al.*, 1995).

Five epitopes (for proteins other than α) have been characterized by determining the crystal structure of an antigen:monoclonal antibody Fab complex. All of these epitopes occupy large areas comprised of 15 to 22 amino acid residues and composed of several surface loops, indicating that the antigenicity of the native protein is strictly dependent on its conformation (reviewed in Laver *et al.*, 1990). Epitopes of 4 to 7 amino acids in length mapped by using synthetic peptides may represent only parts of the epitopes exposed on denatured forms. In the present study all of the truncated α proteins have been immunoprecipitated at least by some monoclonal antibodies, indicating that some epitopes can assume the native conformation on these truncated derivatives. Where a particular antibody failed to precipitate a truncated fragment due to the presumed loss of the epitope sequence, a PCR-amplified DNA fragment bearing the target sequence coded for a peptide fragment which was bound by the monoclonal antibody. However, the possibility that the regions mapped represent only a major portion but not all of the epitope sequence cannot be ruled out. One of the monoclonal antibodies, mAb 130C2, binds to epitopes which are located in the N-terminus and C-terminus of the α subunit. Since there is no apparent sequence homology for these two regions of α

it is possible that protein folding may form a functional domain. This would bring some of the 23 N-terminal residues and 9 extreme C-terminal residues in close proximity. Proteolytic and peptide specific polyclonal antibbody studies reveal that at least N-terminal 10 amino acid form a distinct domain (Blatter *et al.*, 1994 ; Sharif *et al.*, 1996 in preparation) while NMR studies reveal that C-terminal region from Trp^{321} to Ile^{326} shows a sharp turn in which Pro^{322} takes on the *cis* configuration at the peptide bond (Jeon *et al.*, 1995).

Polyclonal antibodies are very useful in determining the topological arrangements of their antigens. It is important for a region to be surface exposed on an antigen in order for it to be immunogenically active. Therefore, mapping the regions bearing epitopes of a polyclonal antibody could reveal important information pertaining to the domains that are exposed at the surface and are involved in protein-protein or protein-DNA interactions. In this study four sets of polyclonal antibodies were used to determine the regions that they recognize on the α subunit of *E. coli* RNA polymerase. These polyclonal antibodies were raised by immunizing individual rabbits with smaller α fragments of varying sizes covering the entire region of the α subunit. The α peptide-specific polyclonal antibody (pAb2) was raised against a N-terminal peptide spanning amino acids 1 to 113. This region contains a major region of epitopes in the exteme N-terminus between residues 1-10 and a minor one between residues 41-60. The extreme N-terminal region of α, residues 1-10, might be exposed at the surface, and the region spanning residues 10-40 might be embedded inside, while the portions of the region near residues 41-

60 are exposed at surface, probably in the form of loops and folds which may explain a weaker immunoreactivity. No other continous sequences, long enough to be immunogenic, seem to be exposed to the surface since regions outside of these two domains showed no detectable signal on Western blotting.

Two of the monoclonal antibodies raised against native α, mAb 123C2 (null antibody) and mAb 130C2 (inhibitory to *lac* transcription) map their epitopes to a comparable region within residues 1-23 (Sharif *et al.*, 1994). Further supporting the notion that the extreme N-terminal region might be exposed at the surface on α, even on the RNA polymerase holoenzyme. The assembly of RNA polymerase proceeds sequentially: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (Ishihama, 1981), in which the α subunit plays a major role in subsequent assembly of the two large subunits. The subunit-subunit assembly sites in α are all located within the N-terminal two-thirds upstream from residue 235 [intact α is composed of 329 amino acid residues] (Igarashi and Ishihama, 1991; Igarashi *et al.*, 1991; Hayward *et al.*, 1991). The major contact site on α for the β subunit is located in the N-terminal proximal region near residue 45 (Kimura and Ishihama, 1995a; 1995b). Mutations in Arg45 make α inactive in binding β even though the mutant α forms dimers (Ishihama *et al.*, 1980; Kawakami and Ishihama, 1980; Igarashi *et al.*, 1990; Kimura and Ishihama, 1995b). This region, normally involved in β binding, must be exposed on isolated α, and thus should be recognized as an antigenic determinant by the immune system.

pAb5 was raised against the C-terminal peptide downstream from residue 230. This region carries the contact sites for both *trans*-acting class I protein factors

(Ishihama, 1992; 1993) and *cis*-acting DNA UP elements (Ross *et al.*, 1994). A region consisting of at least 16 amino acid residues appears to be exposed at the surface of α in the region between residues 240 and 256. We cannot exclude, however, that additional linear epitopes, in general, and conformational epitopes, in particular, may be located downstream from residues 257.

The antigenic region thus identified forms a long linker domain that connects the N-terminal core enzyme assembly domain and the C-terminal activation domain including the contact sites for protein factors and DNA signals (Negishi *et al.*, 1995; Jeon *et al.*, 1995). This region is surface exposed and is very sensitive to proteolytic cleavage (Negishi *et al.*, 1995). This linker seems flexible (≥ 44 Å if fully extended; Blatter *et al.*, 1994) because the actual distance between the N-terminal assembly domain and C-terminal activation domains is subject to variation depending on the distance of the interaction site on a factor or the UP element relative to a promoter (Zhou *et al.*, 1994). Therefore, this region is exposed to solvent even when α is assembled in RNA polymerase (Figure 1).

The region between residues 112-140 shows another immunogenic region recognized by pAb6 which was raised against a fragment corresponding to residues 112 to174 of the α subunit. This region, in intact α, is relatively buried in the folded state, and is not involved in contact with other proteins [however, it may be involved in α dimerization]. Therefore, the antigenic site recognized by pAb6 may only be exposed in the fragment (residue 112-174) used for immunization.

Finally, a region corresponding to residues 161-220 shows another domain

recognized by pAb7, which is raised against an α fragment consisting of residues 159 to 230. Therefore, this domain may be exposed to the solvent and thus recognized by the immune system. The major contact site on α with the β' subunit is located between residues 180 to 200 (Kimura and Ishihama, 1995b), in good agreement with the major antigenic sites in this region. β' is the last subunit assembled into core enzyme in the sequential assembly mechanism. Thus, β' is easily dissociated from the core enzyme during denaturation under mild conditions (Ishihama, 1981). Three anti-α monoclonal antibodies raised against native α (Group 2 antibodies, mAb 129C4, mAb 124D1 and mAb 121C5) have been shown to recognize their epitopes on the α subunit within a comparable region from residues 190 to 210 (Sharif *et al.*, 1994). Among these monoclonal antibodies, mAb 129C4 interferes with the reconstitution of the core RNA polymerase (Riftina *et al.*, 1989). The mAb 129C4 bound to native α subunit (Sharif *et al.*, 1994) but failed to immunoprecipitate RNA polymerase core or holoenzyme (Luo and Krakow, unpublished data) further suggesting that this region is exposed on the surface of the α subunit and is involved in subunit-subunit interaction during core polymerase assembly.

The immunogenic regions recognized by these peptide specific anti-α polyclonal antibodies reveal useful information on the topology of the α subunit. Most regions recognized by these antibodies, and hence exposed at the surface of α, are in good agreement with the structural studies of this subunit using biochemical and molecular biological techniques. For example, the location of β

binding domain in the α N-terminus and that of β' binding domain in the α middle region are in good agreement with the immunogenic regions (Kimura and Ishihama, 1995; Kimura and Ishihama, 1995b; Sharif *et al.*, 1994). However, there are regions shown to be involved in protein-protein or protein-DNA communication and hence presumed to be exposed, yet the polyclonal antibodies failed to recognize such regions. For example, a number of transcription factors have been proposed to interact with α at contact site I between amino acid residues 250 to 329 (Ishihama, 1993), helix1 comprising residues 264 to 273 seems to contact with UP element (Jeon *et al.*, 1995), two monoclonal antibodies (Group 3 mAbs, 130B1 and 125C6), raised against native α, map their epitopes to a region between residues 310 and 320, and another mAb 130C2, shows part of its epitope in the exteme C-terminus between amino acids 320 and 329 (Sharif *et al.*, 1994). The inability of the polyclonal antibodies to recognize these regions might be a result of the regions being exposed yet not recognized by the immune system of the given animal used and hence, anti-sera from several immunized animals would be necessary to identify these regions. It is also possible that part of the exposed regions may not be highly immunogenic or that the exposed regions are composed of α-helices with loops and folds that would result in a pool of antibodies recognizing conformational epitopes. Since the regions recognized by these polyclonal antibodies were identified only by Western blotting, such conformational epitopes will be disrupted once the proteins are SDS denatured and linearized. NMR studies on a C-terminal fragment of α from residues 233 to 329, which also contains *cis*-acting DNA binding

and *trans*-acting factor binding domains, indicated that it is composed of four helices with sharp turns and folds (Jeon *et al.*, 1995).

The assembly mechanism of the RNA polymerase from its individual subunits has been a subject of extensive investigation. Analysis of modified or mutant subunits in the reconstitution process has generated useful information not only on the sequence of events that take place during reconstitution but also on the understanding of the functional role of the subunits and their topological arrangement (reviewed in Kumar, 1981; Zillig *et al.*, 1976). Cloninig of each of the subunits of RNA polymerase into expression vectors has made it possible to investigate this complex enzyme using various mutant subunits assembled and analyzed in cell free systems (Zalenskaya *et al.*, 1990; Igarashi *et al.*, 1990; Hayward *et al.*, 1991; Luo, 1992; Severinov *et al.*, 1995; Kimura and Ishihama, 1995a; 1995b). Prior work done in our laboratory on the immunoprecipitation of various reconstituted RNA polymerase forms containing internally deleted β' subunits indicated that mutant ß' proteins JLE3, JLK3, JLP6 could assemble efficiently into both core and holoenzyme forms. Three other mutant ß' proteins, JLE2, JLP7 and JLP7B which could assemble into core enzyme failed to coprecipitate the σ subunit. This suggested that among the core subunits, β' interacted with σ to form holoenzyme. This interaction seemed to be located on or near the region of β' between residues 201 and 477. The results presented in this study confirmed the interaction between β' and σ as individual subunits mixed under reconstituting conditions made a β'σ complex strong enough to withstand conditions

of HPLC or immunoprecipitation. β', being a basic protein, binds non-spepecifically to polyanions like DNA. Therefore, polyanions are non-specific competitors of β' binding. The β'σ complex was retained even in the presence of increasing amounts of non-specific DNA, tRNA or heparin. (Fig. 21) and also 0.005 % SDS during the incubation and washing steps of immunoprecipitation indicating a specific interaction between the two subunits. Other subunits and $\alpha_2\beta$ subassembly failed to interact with sigma (Figure 22A) further supporting that this σ interaction was specific for the β' subunit. Likewise ß'σ complex did not bind α (Figure 22C). Earlier work had suggested interaction between β' and the σ subunit. For example free β' bound DNA or heparin but addition of free σ resulted in a reduced affinity of β' for nonspecific DNA (Zillig *et al.*, 1970, 1971; Fukuda and Ishihama, 1974). β' dependence for σ binding to core polymerase has been demonstrated (Palm *et al.*, 1975). A stable β'σ complex has been recovered along with $\alpha_2\beta$ from the disassembled *L. curvatus* RNA polymerase after phosphocellulose chromatography (Stetter and Zillig, 1974). Although, crosslinking between σ and all core polymerase subunits has been documented, this approach merely indicate a close proximity and not necessarily protein-protein interaction (McMahan and Burgess, 1994; Hillel and Wu, 1977). Some evidence for a βσ complex had also been documented (Sethi and Zillig, 1970) though this has not been supported by later work (Fukuda and Ishihama, 1974; Palm *et al.*, 1975).

The results presented also support and define the region on β' involved in σ binding. Where deletion of the middle region (aa 449-929) did not affect σ binding

as compared to the wild-type β', deletion in the region between residues 201 and 477 resulted in significant loss of signal for the coimmunoprecipitation of σ (Figure 24). The deletion of the C-terminal β' region (aa 1074-1407) in JLP7B in addition to the N-terminal region (aa 201-477) (Figure 23) did not affect σ binding indicating that the C-terminal region may not be involved. Kashlev et al. (1993) have shown that a β' subunit with six histidine residues fused to its C-terminal end can assemble into a RNA polymerase which performs all vital *in vivo* functions and behaves qualitatively normal *in vitro*. This data is consistent with our suggestion that the N-terminal region of the β' subunit is important for the assembly of the β' subunit into RNA polymerase. The β' domain that interacts with σ appears to be constituted of a portion of the N-terminal region between residues 1-175 in addition to residues 201-477 since both JLP7 and JLP7B proteins retain this region (aa 1-175) (Figure 23) and are still able to bind weakly to σ (Figure 24). The ability of various mutants to assemble to form core polymerase (Luo, 1992; Luo *et al.*, 1996 in preparation) and JLP6 to bind σ infers that the truncated ß' proteins can assume a physiologically relevant conformation. Similar reconstitution assays were also successful for assembly of a functional *E. coli* RNA polymerase containing β subunit fragments (Severinov et al., 1995). The weaker σ binding of the ß' proteins, JLP7 or JLP7B, seems to be directly a result of the deletion of the contact site. However, the improper folding of the truncated β' in the vicinity of the σ intearction domain may still not be ruled out.

Reconstitution of core enzyme from purified subunits takes place in the

following sequence: $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2 \beta\beta'$ (reviewed in Ishihama *et al.*, 1987). An $\alpha_2\beta'$ complex has not been detected in reconstitution assays and assembly of β' subunit into core enzyme is β-dependent. Figure 22C shows that the assembled β'σ comlex was unable to bind α in the absence of the β subunit.

Binding of the free β' subunit to DNA is well established (Sethi and Zillig, 1970; Fukuda and Ishihama, 1974). The σ subunit is involved in the specific binding of the holoenzyme to promoters while the core enzyme binds DNA templates nonspecifically. Interaction of the σ subunit with β' may play a role in DNA binding by RNA polymerase. In contrast, β and $\alpha_2\beta$ show little affinity for the σ subunit in reconstitution experiments (Fukuda and Ishihama, 1974; Palm et al., 1975). The interaction between the β' and σ subunits is important for both assembly of holoenzyme and specific binding of holoenzyme to DNA. The σ subunit is released from the elongation complex when the nascent transcript reaches a critical length (Krakow and von der Helm, 1971; Gerard *et al.*, 1972). It is also possible that the β' subunit may interact with other regulatory proteins subsequent to σ release; A mutation at residue Glu^{402} has been reported to restore rho-dependent termination and suppresses the temperature-sensitive phenotype of NusA11. It also inhibited lambda Q-mediated antitermination without affecting N antitermination (Ito and Nakamura, 1993). Our assigned β'-σ interaction region 201-477 on the β' includes this mutation site as well as a conserved region 348-380 which was found to be homologous to the DNA-binding domain of two prokaryotic DNA polymerases (Allison et al., 1985).

Further Studies:

1. Epitope mapping of the rest of the anti-α monoclonal antibodies in this laboratory as well as those prepared in other laboratories (McMahan and Burgess, 1994) may give new information on the structure as it relates to the function to the α subunit.

2. These monoclonal antibodies can be further characterized for their effects on initiation with a variety of *trans*-acting factor-dependent promoters as well as *cis*-acting UP element-dependent promoters. This will further define the role of α in the initiation process and could identify domains responsible for the specific interactions.

3. Polyclonal antibodies raised against the native α subunit can be used to identify the immunogenic regions using the various α deletion mutants (Igarashi and Ishihama, 1991; Kimura *et al.*, 1994; Kimura and Ishihama, 1995a, 1995b). This may reveal distinct domains exposed on the surface of α. These polyclonal antibodies can also be characterized for their effects on the function of α with the various *trans*-acting factor-dependent promoters as well as *cis*-acting UP element-dependent promoters.

4. In this study we have domonstrated a stable β'σ complex of the *E. coli* RNA polymerase. β' has been shown to bind to nonspecific DNA. This binding is weakened by the addition of σ (Zillig *et al.*, 1970, 1971; Fukuda and Ishihama, 1974). It would be interesting to find if the β'σ complex alone would recognize promoter sequences by gel retardation and DNA footprinting.

5. Our study has demonstrated that the removal of a region constituting residues 201-477 of the β' subunit results in a weak binding with σ. This finding suggests that while this region is involved in σ interaction it is not sufficient to explain highly efficient interaction. Regions further toward the N-terminus seem to be included in the σ binding domian. Deletions in N-terminal region between residues 1 and 201 may better define the location of the domain involved in σ interaction.

Table 1

Primers used for PCR reactions.

Primer name	Sequence*
α-T7RBS 100-107	GAA TTC TAA TAC GAC TCA CTA TAG GGT TAA CTT TAA GAA GGA GAT ATA CAT ATG CTT ACC TTG AAT AAA TCT GGC ATT.
α-185-190R	TTA TTA CGC TGC TTC AAC ATT GTA.
α-205-210R	TTA TTA TGT GCC CGT GGT TTC CAT.
α-T7RBS 210-217	GAA TTC TAA TAC GAC TCA CTA TAG GGT TAA CTT TAA GAA GGA GAT ATA CAT ATG ACA ATC GAT CCT GAA GAG GCG ATT.
α-305-310R	TTA TTA ACG GGA AGC CAG CAC GTC.
α-315-320R	TTA TTA GTT TTC CAG GCG CAT GCC.
α-324-329R	TTA TTA CTC GTC AGC GAT GCT TGC.

* Underlining indicates *rpoA* sequence.

Table 2

Plasmids used in this study

Plasmid	Properties	Source
pTAX185	α-WT gene in pSPT19	Igarashi *et al.* (1991)
pTAD256	α-256 gene in pSPT19	Igarashi *et al.* (1991)
pTAD235	α-235 gene in pSPT19	Igarashi *et al.* (1991)
pTAD150	α-150 gene in pSPT19	Igarashi *et al.* (1991)
pGEMAX185	α-WT gene in pGEMΔXba	Igarashi and Ishihama (1991)
pGEMAD296	α-296 gene in pGEMΔXba	Igarashi and Ishihama (1991)
pGEMAD256	α-256 gene in pGEMΔXba	Igarashi and Ishihama (1991)
pT7α	α-WT gene in pT7-6	Tabor and Richardson (1985)
pHA7	CRP gene in pBR322 derivative	Aiba *et al.* (1982)
pKB252	*lac* UV5 promoter	Kajitani and Ishihama (1983)
pBdC1	*gal* promoter	Taniguchi *et al.* (1989)
pHW104	*lac* promoter	Dr. Crothers, personal communication

Table 3

Antibodies used in this study

Antibodies	Epitope (s)	Inhibition with *lac* promoter*
Anti-α mAb		
123C2	1-23	-
123C2	1-23, 320-329	+
129C4	190-210	ND (**)
124D1	190-210	+
121C5	190-210	+
125C6	310-320	+
130B1	310-320	+/- ?
Anti-α pAb		
pAb2	1-10, 41-60	ND
pAb5	240-256	ND
pAb6	112-140	ND
pAb7	161-220	ND
Anti-β'		
311G2	1047-1072	+ (***)
340F11	817-876	-

* The inhibitory effects are prior to RP_o formation.

** The mAb inhibited reconstitution of the core enzyme.

***The mAb also inhibited transcription with the *lac* UV5 promoter.

Table 4

Properties of *E. coli* RNA polymerase subunits*

Subunits	α	β	β'	σ
Gene	*rpoA*	*rpoB*	*rpoC*	*rpoD*
Size in amino acids	329	1342	1407	613
MW (daltons)	36,511	150,615	155,159	70,262
Map position	73	90	90	67
Charge at pH 7	-14	-34	+6	-40

*(from Burgess *et al.*, 1987)

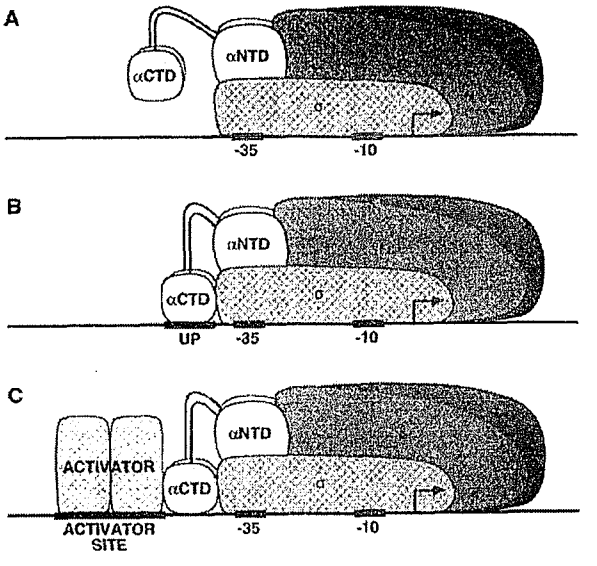

Figure 1. A model showing the possible mechanism of transcription activation by promoter upstream elements and upstream binding activator proteins mediated through the α subunit or RNA polymerase (After Blatter *et al.*, 1994). [A] Promoter lacking an upstream element or upstream activator site. [B] Promoter with an upstream element. [C] Promoter with an upstream-binding activator protein (e.g. *lac* P1 where CRP binds upsteam of the promoter at -61.5).

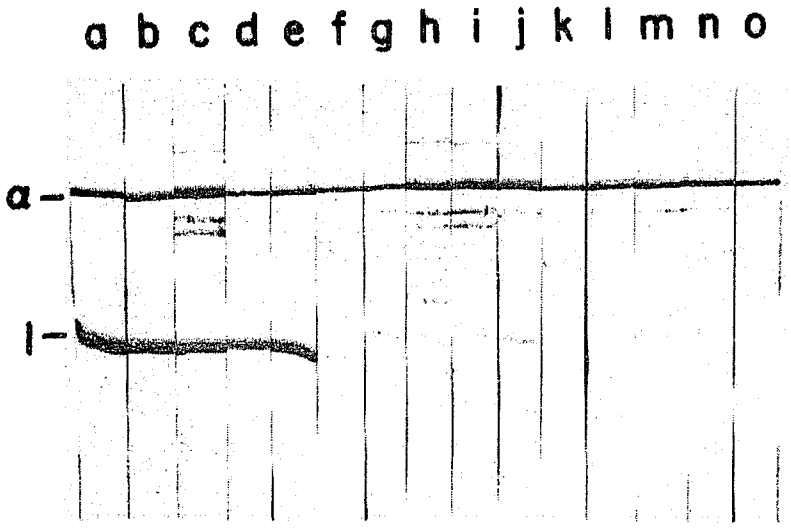

Figure 2A. Western blotting of chymotrypsin-digested α-subunit: Purified α-subunit was partially digested with chymotrypsin and subjected to Western blotting with anti-α monoclonal antibodies as indicated in Materials and Methods. Lane a, mAb 125C6; lane b, mAb 126C6; lane c, mAb 128D5; lane d, mAb 130C2; lane e, mAb 130B1; lane f, mAb 121C2; lane g, mAb 123C7; lane h, mAb 21D1; lane I, mAb 129C4; lane j, mAb 123C2; lane k, mAb 131B1; lane l, mAb 127B3; lane m, mAb 124D1B3E3; lane n, mAb 121C5; lane o, mAb 24D1.

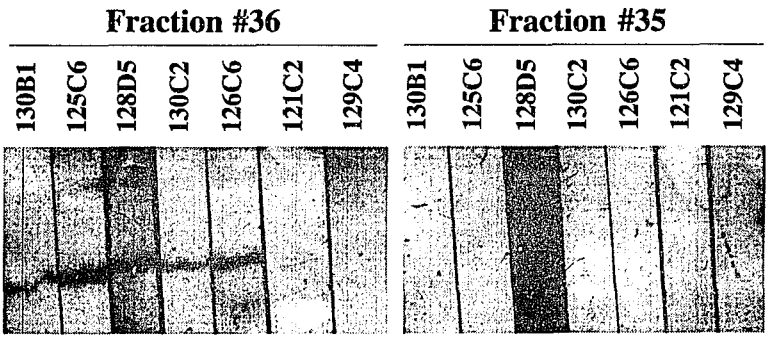

Figure 2B. Western blotting of chymotryptic fragments of the α-subunit resolved by reverse-phase HPLC : Purified α-subunit was partially digested with chymotrypsin and subjected to Western blotting after HPLC with anti-α monoclonal antibodies as in Materials and Methods.

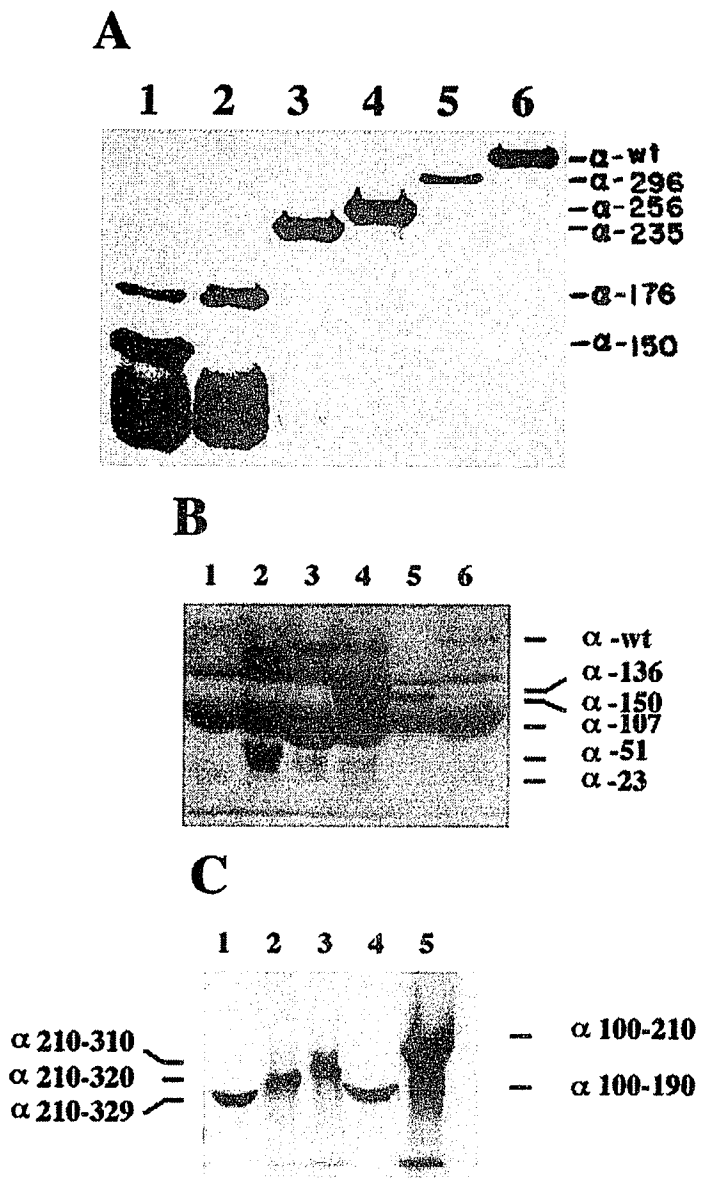

Figure 3. *In vitro* translation of C-terminal or internally truncated fragments of the α subunit. 2 μg of each of the DNA templates generating the indicated truncation were transcribed using T7 RNA polymerase and translated using a rabbit reticulocyte lysate translation system [A and B] or using S-30 coupled transcription-translation [C, lanes 2,3 and 5]. The ^{35}S-methionine or ^{3}H-leucine labeled proteins were resolved by SDS-PAGE and autoradiographed. [A] Lane 1, α-150; lane 2, α-176; lane 3, α-235; lane 4, α-256; lane 5, α-296; lane 6, α-wt. [B] Lane 1, α-23; lane 2, α-51; lane 3, α-107; lane 4, α-136; lane 5, α-150; lane 6, α-wt. [C] Lane 1, α210-329; lane 2, α210-320; lane 3, α210-310; lane 4, α100-190; lane 5, α100-210. Panel C, 1 and 4 are ^{3}H-leucine labeled, all others are labeled with^{35} S-methionine.

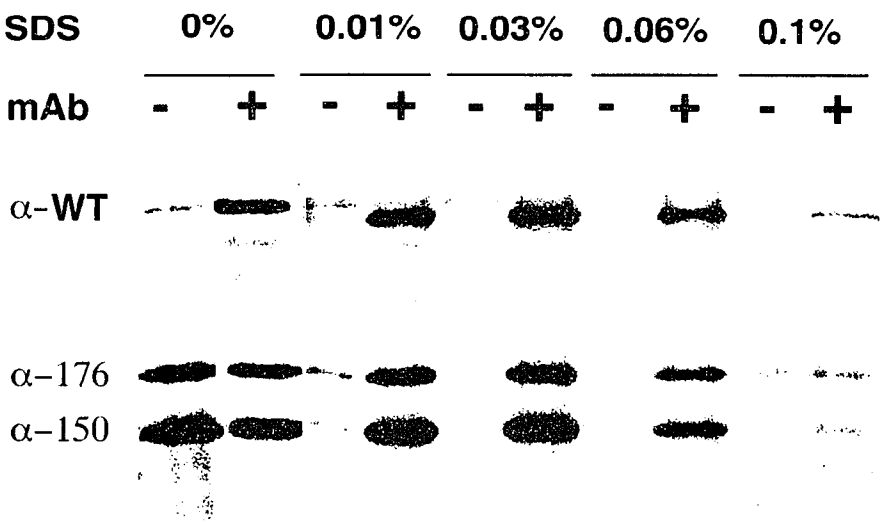

Figure 4. Immunoprecipitation of ^{35}S-labeled α-wt, α-176, and α-150 with the anti-α monoclonal antibody (mAb 130C2) in the presence of varying amounts of SDS as described in Materials and Methods.

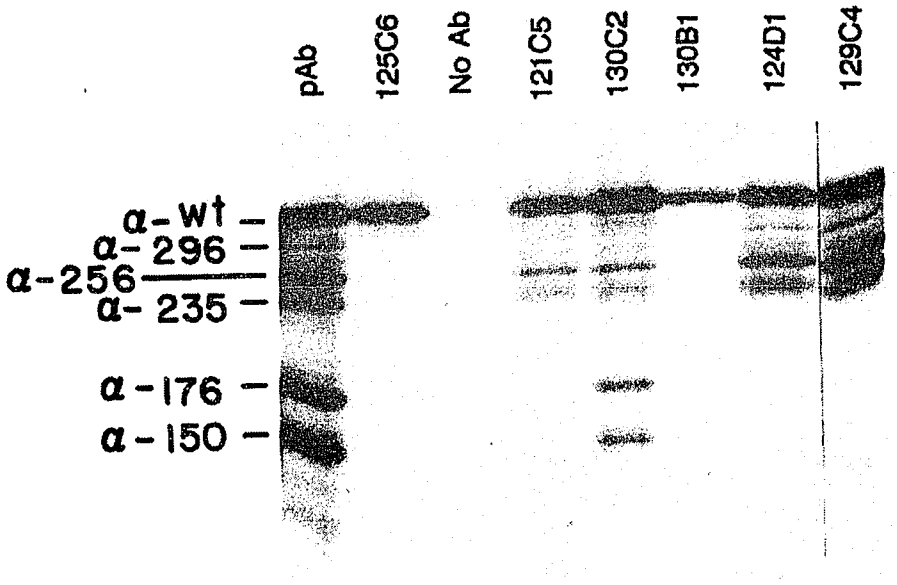

Figure 5. Immunoprecipitation of the C-terminal truncated α fragments with anti-α monoclonal antibodies. 4 µl each of ^{35}S-labeled α-150, α-176, α-235, α-256, 8 µl of α-296, and 6 µl of α-wt were mixed in 0.5 ml of DMEM containing 5% fetal bovine serum and 0.02% SDS, and then immunoprecipitated with the indicated monoclonal antibodies as described in Materials and Methods.

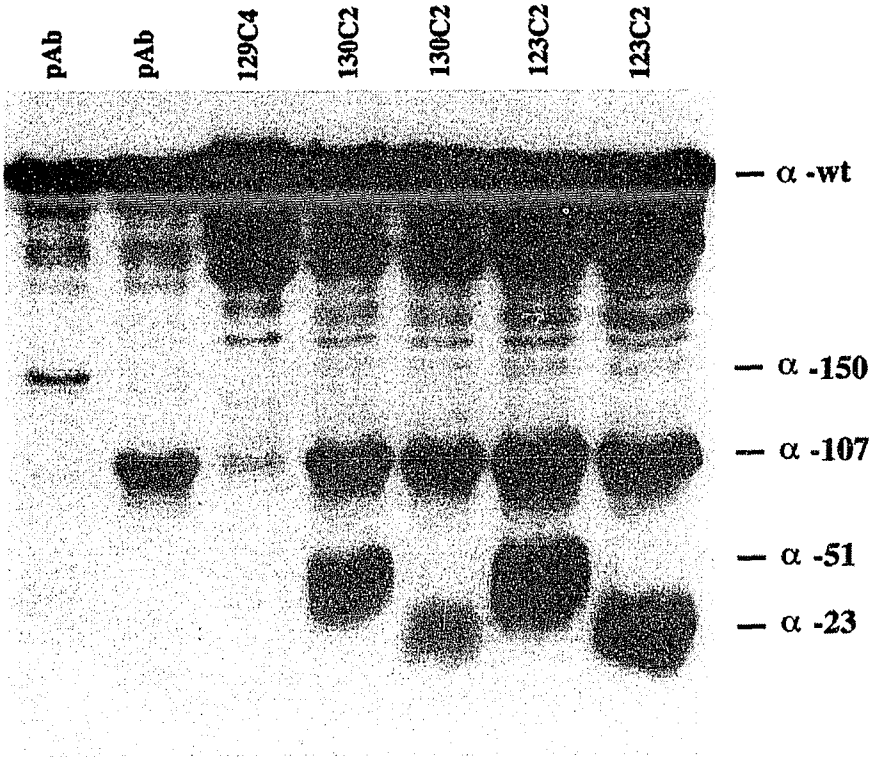

Figure 6. Immunoprecipitation of the C-terminal truncated α fragments with the indicated anti-α monoclonal antibodies or anti-α polyclonal antibodies. Immunoprecipitation was carried out as described in Methods and Materials.

Schematic Representation of PCR Generated Templates Coding for Smaller Alpha Fragments

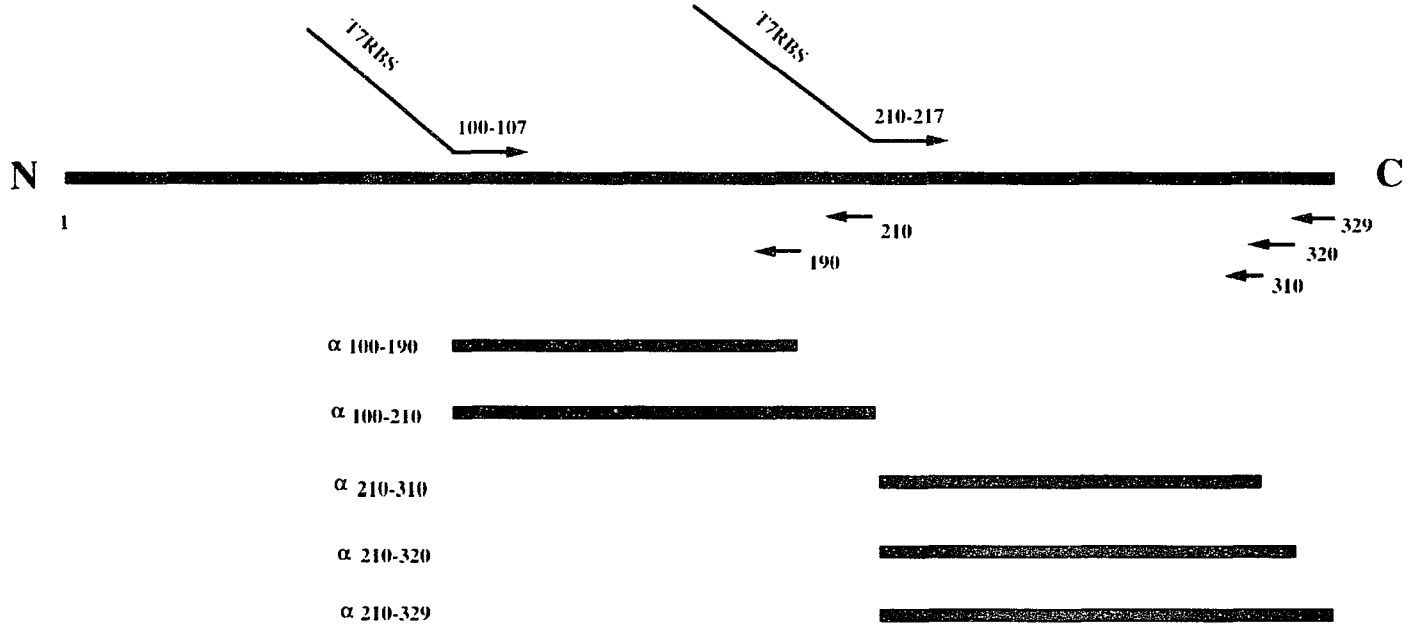

Figure 7. Schematic representation of the PCR amplified templates coding for smaller α fragments. The numbers correspond to the amino acid residues in α. T7RBS represents the T7 promoter and the ribosmal binding sequences which were present in the upstream primers as a 5' flanking region.

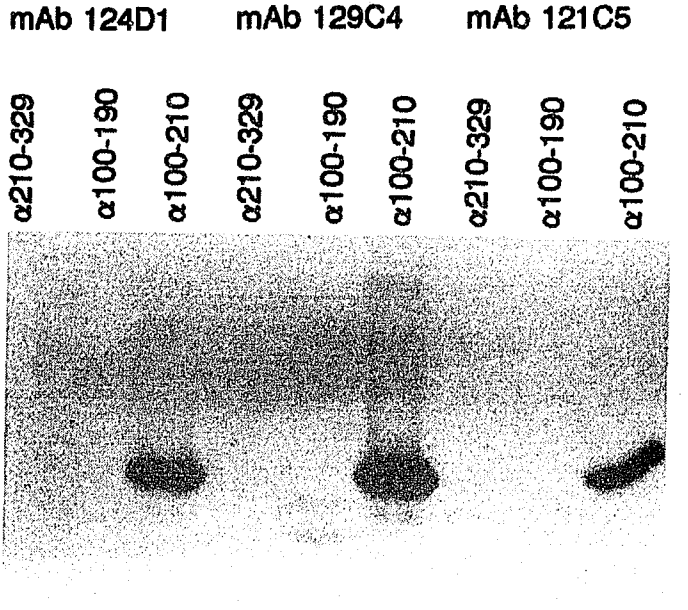

Figure 8. Immunoprecipitation of the internally truncated α fragments with anti-α monoclonal antibodies. The α fragment spanning amino acid 100-329, was produced by coupled transcription-translation of PCR-generated templates. Equal amounts of TCA-precipitable radioactivity for α100-190, α100-210, and α210-329 in 0.3 ml of DMEM containing 5% FBS were treated with the indicated monoclonal antibodies as described in Materials and Methods.

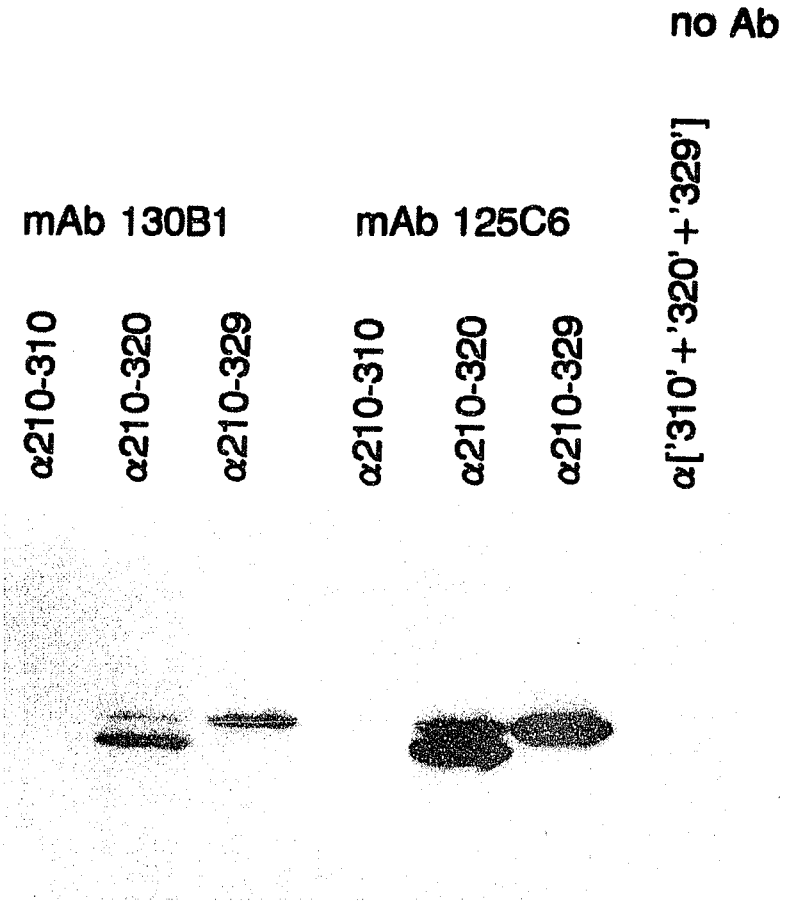

Figure 9. Immunoprecipitation of the internally truncated α fragments with anti-α monoclonal antibodies. The α fragment spanning amino acid 210-329, was produced by coupled transcription-translation of PCR-generated templates. Equal amounts of TCA-precipitable radioactivity for α210-310, α210-320, and α210-329 in 0.3 ml of DMEM containing 5%FBS were treated with the indicated monoclonal antibodies as described in Materials and Methods.

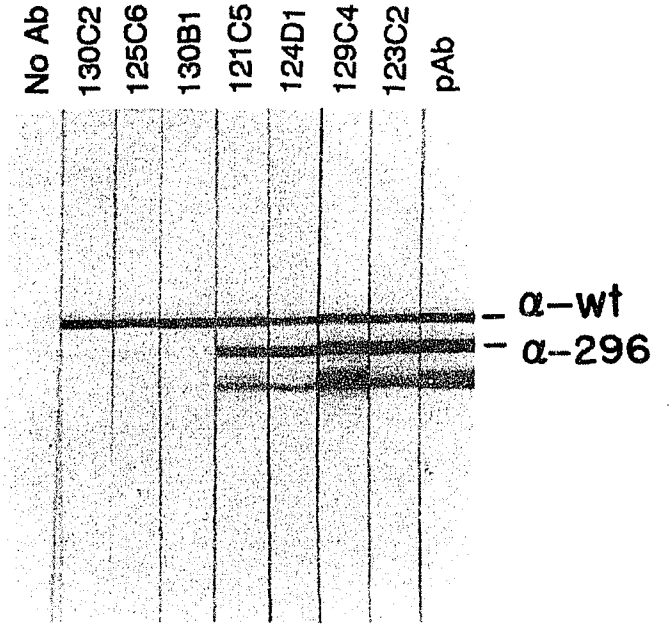

Figure 10. Western blotting analysis of intact α and α-296. Western blotting was done as described in the Materials and Methods. The more rapidly migrating band is probably a degraded form of α-296.

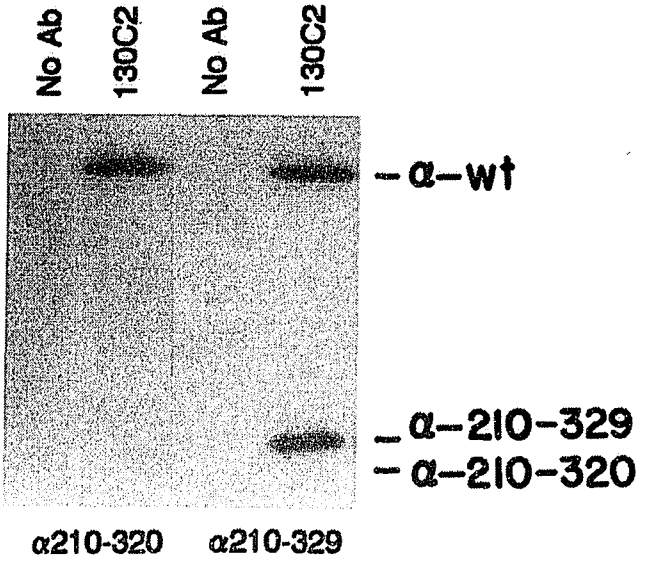

Figure 11. Immunoprecipitation of the internally truncated α fragments with anti-α monoclonal antibody 130C2. The α fragment spanning amino acid 210-329, was produced by coupled transcription-translation of PCR-generated templates. Equal amounts of TCA-precipitable radioactivity for α210-320 and α210-329 in 0.3 ml of DMEM + 5%FBS were treated with the indicated monoclonal antibodies as described in Materials and Methods.

Epitope Map of Anti-α Monoclonal Antibodies

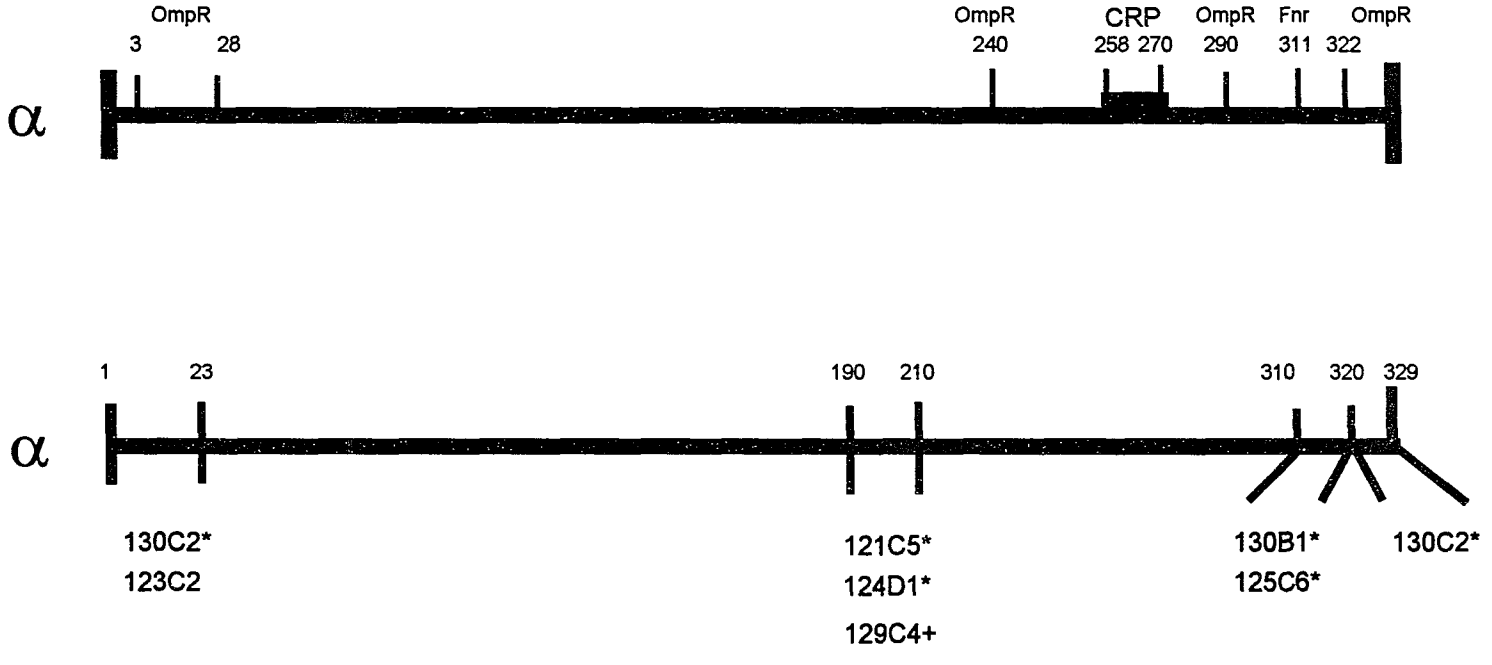

Figure 12. Epitope map for the anti-α monoclonal antibodies. The numbers correspond to the amino acid residues in the α subunit; * indicates inhibitory antibodies; + indicates monovlonal antibody which inhibits reconstitution. The upper bar shows a map of some mutant α proteins defective for different activator proteins.

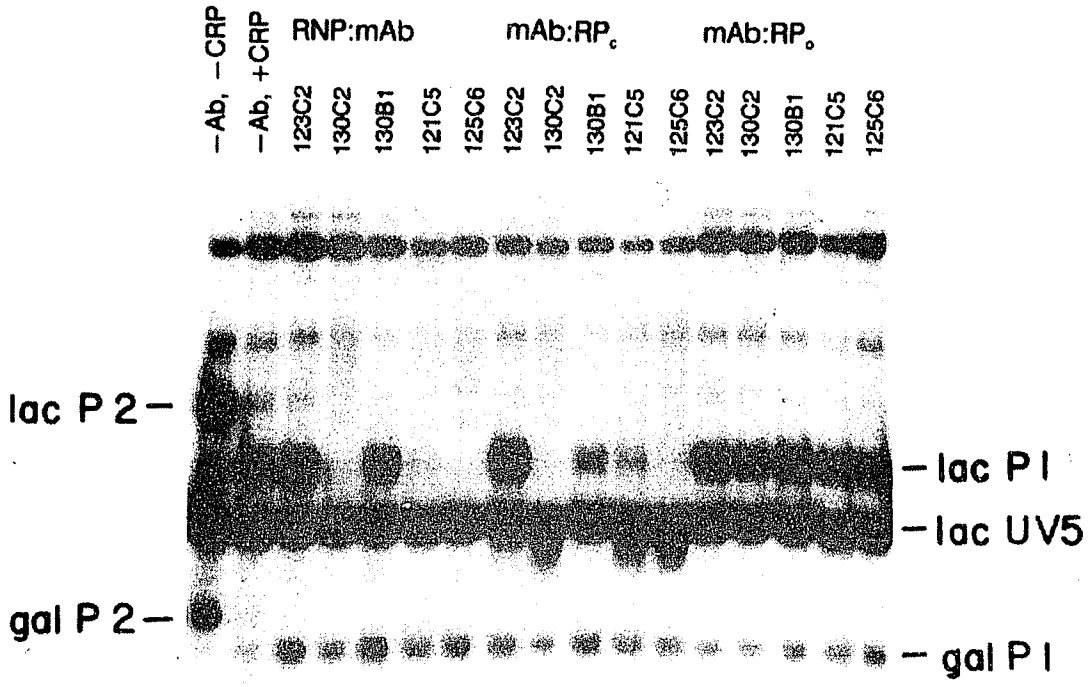

Figure 13. Effects of the anti-α monoclonal antibodies on transcription from the CRP-dependent *lac* and *gal* promoters. The transcription reaction was carried out as indicated in Materials and Methods. Each of the monoclonal antibodies was incubated with RNA polymerase, or RP_c overnight at 5° C, or with RP_o , in the presence of cAMP-CRP, for 30 minutes at 37°C to form antigen-antibody complexes.

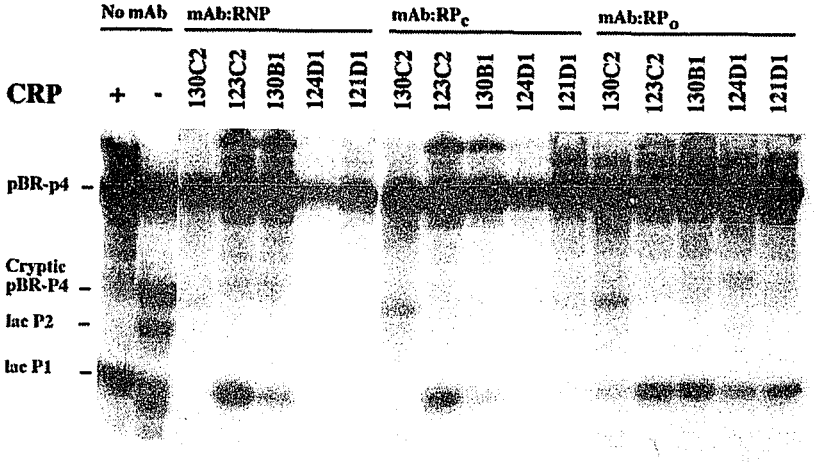

Figure 14. Effects of the anti-α monoclonal antibodies on transcription from the *pBR* P4 promter that is activated by CRP. Transcription reaction was carried out as indicated in Materials and Methods. Each of the monoclonal antibodies was incubated with RNA polymerase, or RP_c overnight at 5° C, or with RP_o , in the presence of cAMP-CRP, for 30 minutes at 37° C to form antigen-antibody complexes.

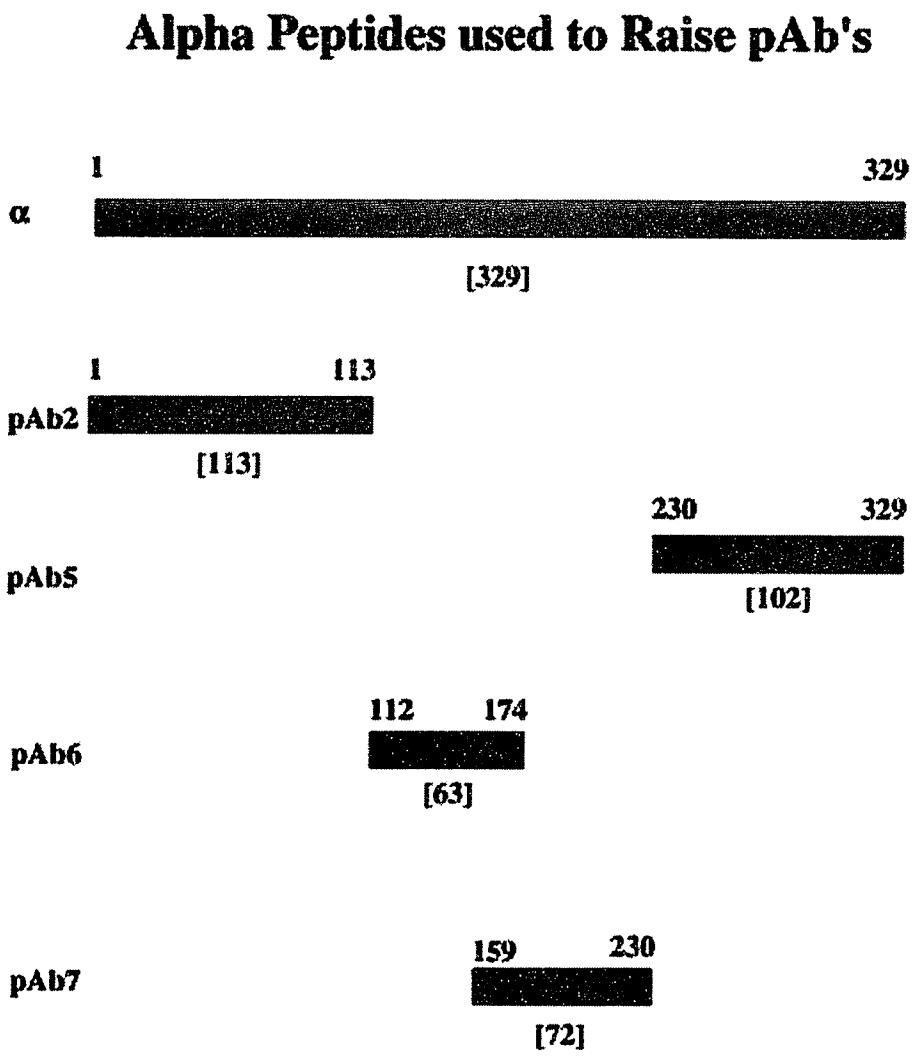

Figure 15. Schematic representation of the α peptides used to raise ploYclonal antibodies. The numbers above the bars correspond to the α subunit amino acid residues present in the indicated protein fragment. The numbers in brackets under the bars represent the length of the peptide.

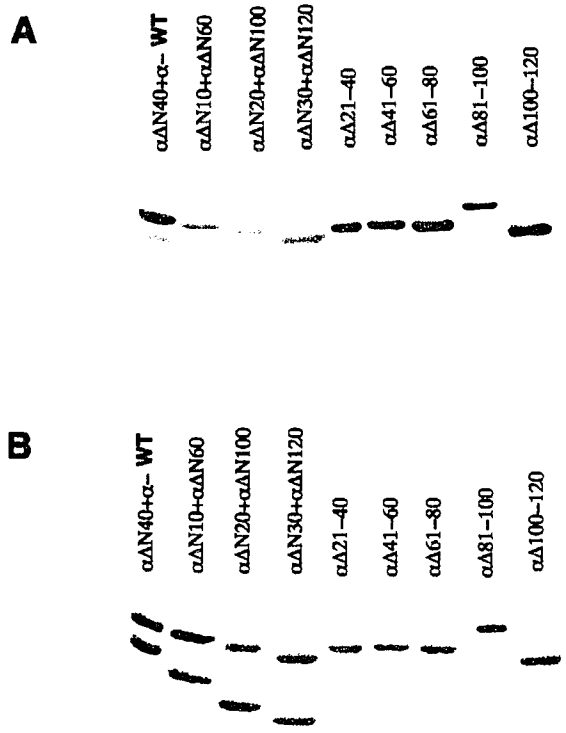

Figure 16. Epitope analysis of anti-α polyclonal antibody 2 (pAb 2) using N-terminal and internally truncated α proteins spanning amino acids 1-120. [A] Western blot analysis [B] Coomassie stained gel of the protein

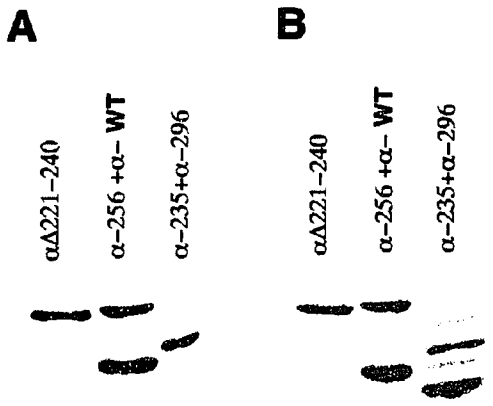

Figure 17. Epitope analysis of anti-αpolyclonal antibody 5 (pAb 5) using both N- and C-terminal as well as internally truncated αproteins spanning amino acids 220-329. [A] Wetern blot analysis [B] Coomassie stained gel of the α proteins.

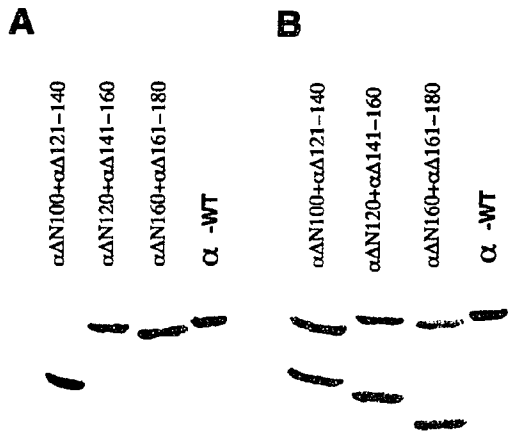

Figure 18. Epitope analysis of anti-α polyclonal antibody 6 (pAb 6) using N-terminal and internally truncated α proteins spanning amino acids 100-180. [A] Western blot analysis [B] Coomassie stained gel of the α protein.

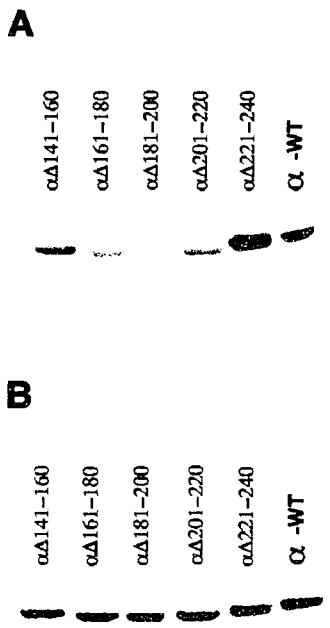

Figure 19. Epitope analysis of anti-α polyclonal antibody 7 (pAb 7) using N-terminal and internally truncated α proteins spanning amino acids 141-240. [A] Western blot analysis [B] Coomassie stained gel of the α proteins.

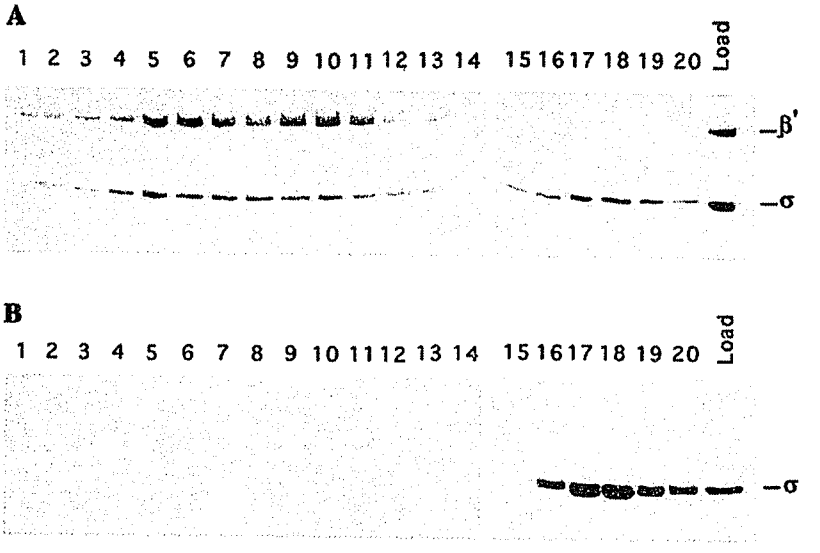

Figure 20. SDS-PAGE analysis of the β'σ complex resolved by size-exclusion HPLC. 100 µl of the reconstituted β'σ mixture [A] or σ alone [B] was loaded onto an HPLC gel-filtration column as described in Materials and Methods. The lane numbers correspond to the collected fractions.

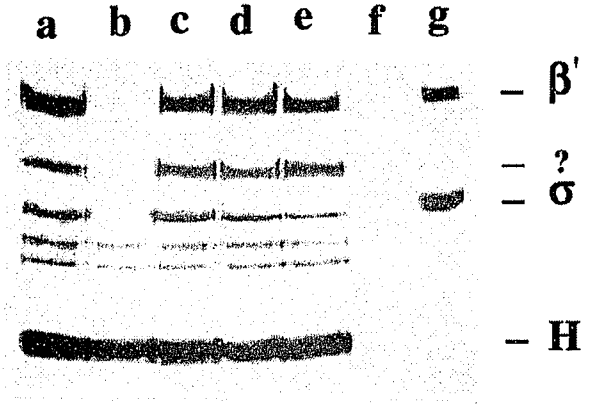

Figure 21. SDS-PAGE analysis of the immunoprecipitation of the β'σ complex using anti-β' monoclonal antibody 311G2. Heavy chains of the mAb are labeled "H". Lane a, β'σ complex + mAb 311G2; lane b, σ alone + mAb 311G2, lane c, β'σ complex + mAb 311G2 + 4 μg tRNA; lane d, β'σ complex + mAb 311G2 + 70 μg plasmid pTAX185; lane e, β'σ complex + mAb 311G2 + 3 μg heparin; lane f, β'σ complex, no antibody control; lane g, reconstitution mixture.

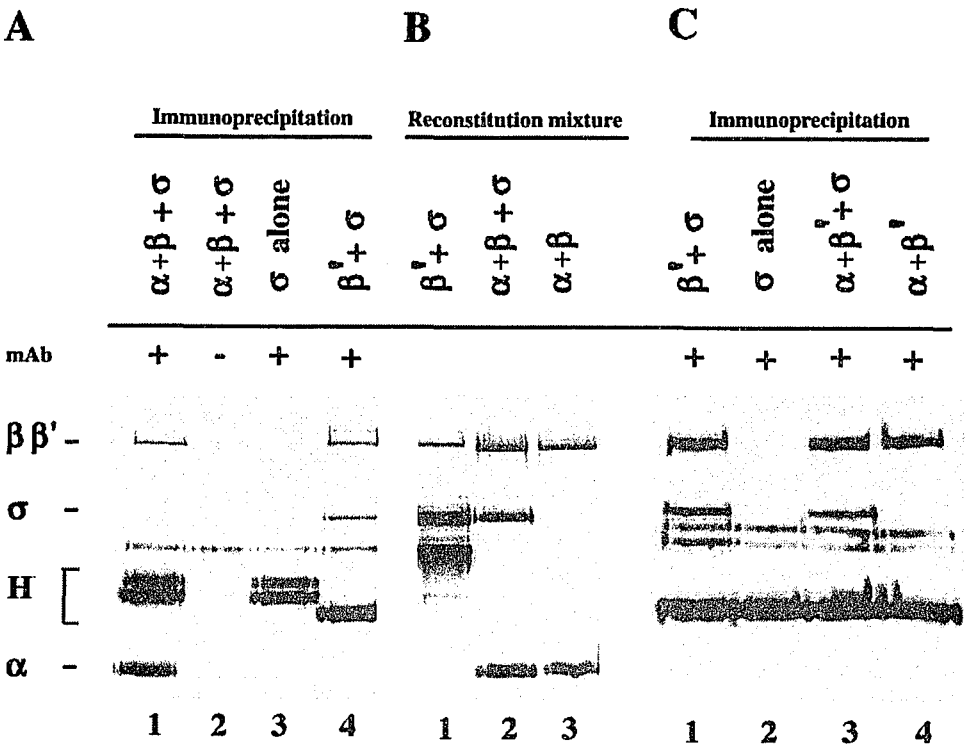

Figure 22. SDS-PAGE analysis of the immunoprecipitation of the subassemblies using subunit specific monoclonal antibodies. Heavey chains of the monoclonal antibodies are labeled "H". Anti-α mAb 125C6 was used to immunoprecipitate the $\alpha_2\beta$ complex (panel A, lanes 1-3), for other complexes anti-β' mAb 311G2 was used. [A and C] immunoprecipitation of reconstitution mixtures; [B] SDS-PAGE analysis of the reconstitution mixtures. [C] immunoprecipitation of α, β' and σ mixtures.

Schematic Representation of β' Deletion Mutants

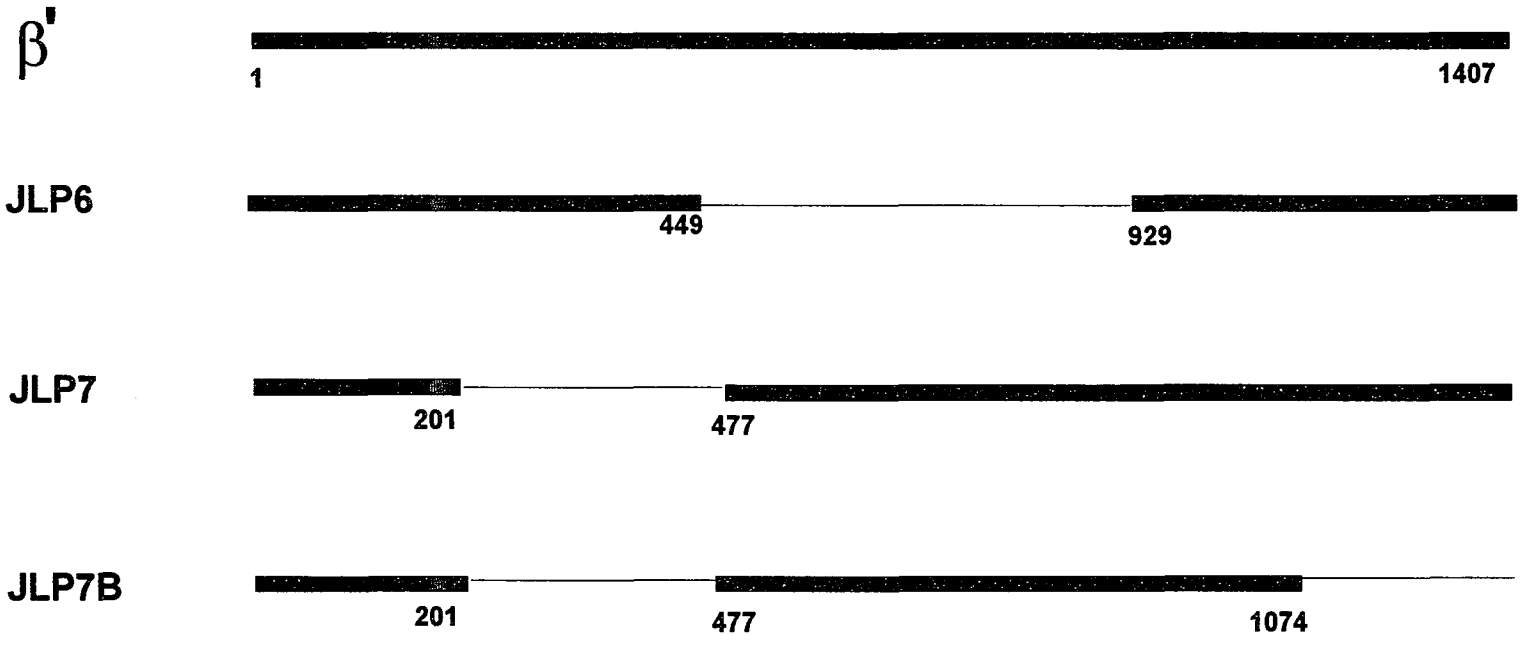

Figure 23. Schematic representation of β' deletion mutants. The numbers under the bars correspond to the amino acid residues in the β' subunit.

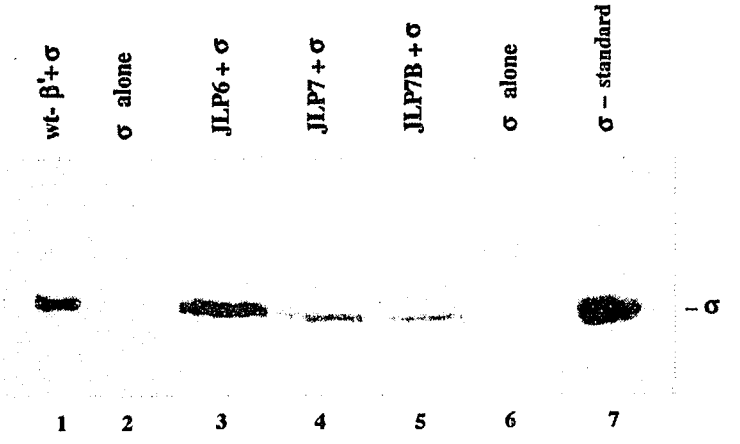

Figure 24. Western blot analysis of the immunoprecipitation of the β'σ complexes containing either wild-type or internally deleted β' mutant proteins and using anti-β' monoclonal antibody 311G2 (lanes 1-5) or 340F11. Identification of σ on Western blot was done by anti-σ monoclonal antibody 2D1.

REFERENCES

Ahearn, J. M., Bartolomei, M. S., Wast, M. L., Cisek, L. J., and Corden, J. L. (1987). Cloning and sequence analysis of the mouse genomic locus encoding the largest subunit of RNA polymerase II. *J. Biol. Chem.* **262**, 10695-10705.

Aiba, H. Fujimoto, S. And Ozaki, N. (1982) Molecular cloning and nucleotide sequencing of the gene for the *E. coli* cAMP receptor protein. *Nucl. Acids Res.* **10,** 1345-1361.

Allison, L. A., Moyle, M., Shales, M., and Ingles C. J. (1985). Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. *Cell* **42**, 599-610.

Benjamin, D. C., Williams Jr., D. C., Smith-Gill, S. J., and Rule, G. S. (1992) Long-range changes in a protein antigen due to antigen-antibody interaction. *Biochemistry* **31**, 9539-9545.

Biggs, J., Searles, L. L., and Greenleaf, A. L. (1985). Structure of the eukaryotic transcription apparatus: features of the gene for the largest subunit of Drosophila RNA polymerase II. *Cell* **42**, 611-621.

Binnie, C., Lampe, M., and Losick, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5943-5947.

Blatter, E., Ross, W., Tang, H., Gourse, R. and Ebright, R. (1994) Domain Organization of RNA polymerase α subunit: C-terminal 85 amino acids constitute a domain capable of dimeriztion and DNA binding. *Cell*, **78**, 889-896.

Boylan, S. A., Suh, J.-W., Thomas, S. M., and Price, C. W. (1989) *J. Bacteriol.* **171**, 2553-2562.

Burgess, R. R., Erickson, B., Gentry, D., Gribskov, M., Hager, D., Lesley, S., Strickland, M. and Thompson, N. (1987) Bacterial RNA polymerase subunits and genes. In RNA polymerase and the regulation of transcription (Reznikoff, W. S., Burgess, R. R., Dahlberg, J. E., Gross, C. A., Record, Jr., M. T., Wickens, M. P. Eds.)Elsvier Science Publishing Co. Inc. NY., pp3-16.

Busby, S. and Ebright, R. H. (1994) Promoter structure, promoter recognition, and transcription activation in prokaryotes. *Cell*, **79** 743-746.

Chamberlin, M. J. (1974) The selectivity of transcription. *Ann. Rev. Biochem.* **43**, 721-775.

Chen, Y., Ebright, Y. and Ebright, R. (1994) Identification of the target of a transcription activator protein by protein-protein photocrosslinking. *Science*, **265**, 90-92.

Clerget, M., Jin, D. J. and Weisberg, R. A. (1995) A zink-binding rregion in the beta' subunit of RNA polymerase is involved in antitermination of early transcription of phage HK022. *J. Mol. Biol.* **248**, 768-780.

Coggins, J. R., Lumsden, J., and Malcolm, A. D. B. (1977). A study of the quaternary structure of *E. coli* RNA polymerase using bis (imido esters). *Biochemistry* **16**, 1111-1116.

Coppo, A., Manzi. A., Pulitzer, J. F., and Takahashi, H. (1975) Host mutant (tabD)-induced inhibition of bacteriophage T4 late transcription. I. Isolation and phenotypic characterization of the mutants. *J. Mol. Biol.* **96**, 579-600.

Dale, E. C., Christie, G. E., and Calendar, R. (1986) Organization and expression of the satellite bacteriophage P4 late gene cluster. *J. Mol. Biol.* **192**, 793-803.

Dalla Venezia, N., and Krakow, J. S. (1990). Effects of anti-α monoclonal antibodies on initiation and elongation by the *Escherichia coli* RNA polymerase. *J. Biol. Chem.* **265**, 8122-8126.

Darst, S. A., Kubalek, E. W., and Kornberg, R. D. (1989). Three-dimensional structure of *Escherichia coli* RNA polymerase holoenzyme determined by electron crystallography. *Nature* (London), **340**, 730-732.

Davis, L. G., Dibner, M. D., and Battey, J. F. (1986) Basic Methods in Molecular Biology, Elsevier, New York.

Din, S., Brill, S. J., Fairman, M. P., and Stillman, B. (1990) Cell-cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. *Genes Dev.* **4**, 968-977.

Dombroski, A., Walter, W., Record, M., Siegele, D., and Gross, C. (1992) Polypeptides containing highly conserved regions of transcription initiation factor σ^{70} exhibit specificity of binding to promoter DNA. *Cell*, **70**, 501-512.

Eilen, E. And Krakow, J. S. (1977) Cyclic AMP-mediated intersubunit disulfide crosslinking of the cyclic AMP receptor protein of *E. coli. J. Mol. Biol.* **114,** 47-60.

Fujita, N., and Ishihama, A. (1987) Mol. *Gen. Genet.* **210**, 10-15.

Fukuda, R., Iwakura, Y., and Ishihama, A. (1974). Hetrogeneityof RNA polymerase in *Escherichia coli,* I. A new holoenzyme containing a new sigma factor. *J. Mol. Biol.* **83**, 353-367.

Gardella, T., Moyle, T., and Suskind, M. M. (1989) A mutant *Escherichia coli* σ^{70} subunit of RNA polymerase with altered promoter specificity. *J. Mol. Biol.* **206**, 579-590.

Garrett, S., and Silhavy, T. M. (1987) Isolation of mutations in the α operon on *Escherichia coli* that suppress the transcriptional defect conferred by a mutation in the porin regulatory gene *envZ*. *J. Bacteriol.* **169**, 1379-1385.

Gerard, G. F., Johnson, J. C., and Boezi, J. A., (1972) Release of the σ subunit of Pseudomonas putida deoxyribonucleic acid dependent ribonucleic acid polymerase. *Biochemistry* **11**, 989.

Giffard, P. M., and Booth, I. R. (1988) The rpoA341 allele of *Escherichia coli* specifically impairs the transcription of a group of positively-regulated operons. Mol. *Gen. Genet.* **214**, 148-152.

Giladi, H., Igarashi, K., Ishihama, A., and Oppenheim, A. B. (1992) Stimulation of the phage λ pL promoter by integration host factor requires the carboxy terminus of the α-subunit of RNA polymerase. *J. Mol. Biol.* **227**, 985-990.

Glass, R. E., Jones, S. T., Nene, V., Nomura, T., Fujita, N., and Ishihama, A. (1986a). Genetic studies on the β subunit of *Escherichia coli* RNA polymerase. VIII. Localization of a region involved in promoter selectivity. *Mol. Gen. Genet.* **203**, 487-491.

Glass, R. E., Honda, A., and Ishihama, A. (1986b). Genetic studies on the β subunit of *Escherichia coli* RNA polymerase. IX. The role of the carboxy-terminus in the enzyme assembly. *Mol. Gen. Genet.* **203**, 492-495.

Glass, R. E., Ralphs, N. T., Fujita, N., and Ishihama, A. (1988). Assembly of amber fragments of the ß subunit of *Escherichia coli* RNA polymerase. *Eur. J. Biochem.* **176**, 403-407.

Gribskov, M., and Burgess, R. R. (1983). Overexpression and purification of the sigma subunit of *Escherichia coli* RNA polymerase. *Gene (Amst.)* **26**, 109-118.

Gross, G., Fields, D. A., and Bautz, E. K. F. (1976). Characterization of a ts ß' mutant RNA polymerase of *Escherichia coli*. *Mol. Gen. Genet.* **147**, 337-341.

Gussin, G. N., Olson, C., Igarashi, K., and Ishihama, A. (1992) Activation defects caused by mutations in *Escherichia coli rpoA* are promoter specific. *J. Bacteriol.* **174**, 5156-5160.

Hayward, R. S., Igarashi, K., and Ishihama, A. (1991) functional specialization within the α subunit *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **221**, 23-29.

Heil, A., and Zillig, W. (1970) Reconstituion of bacterial DNA-dependent RNA polymerase from isolated subunits as a tool for the elucidation of the role of the subunits in transcription. *FEBS Letters* **11**:165.

Helmann, J. D., and Chamberlin, M. J. (1988) Structure and function of bacterial sigma factors. *Annu. Rev. Biochem.* **57**, 839-872.

Heyduk, T., Lee, J. C., Ebright, Y. W., Blatter, E. E., Zhou, Y., and Ebright, R. H. (1993) CAP interacts with RNA polymerase in solution in the absence of promoter DNA. *Nature* **364**, 548-549

Hillel, Z., and Wu, C-W. (1977). Subunit topography of RNA polymerase from *Escherichia coli.* A crosslinking study with bifunctional reagents. *Biochemistry* **16**, 3334-3342.

Hinkle, D., and Chamberlin, M. (1971) The role of sigma subunits in template site selection by *E. coli* RNA polymerase. *Cold Spring Harbor Symp. Quant. Biol.***35**, 65.

Hinkle, D., and Chamberlin, M. (1972) Studies of the binding of *E. coli* RNA polymerase to DNA. II. The kinetics of the binding reaction. *J. Mol. Biol.* **70**, 187.

Igarashi, K., Fujita, N., and Ishihama, A. (1990) Sequence analysis of two temperature-sensitivemutations in the alpha subunit gene (*rpoA*) of *Escherichia coli* RNA polymerase. *Nucl. Acids Res.* **18**, 5945-5948.

Igarashi, K., Fujita, N., and Ishihama, A. (1991a). Identification of a subunit assembly domain in the alpha subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **218**, 1-6.

Igarashi, K., Hanamura, A., Makino, K., Aiba, H., Aiba, H., Mizuno, T., Nakata, A., and Ishihama, A. (1991b) Functional map of the α subunit of *Escherichia coli* RNA polymerase: two modes of transcription activation by positive factors. *Proc. Natl. Acad. Sci. USA* **88**, 8958-8962.

Igarashi, K., and Ishihama, A. (1991) Bipartite functional map of the *E. coli* RNA

polymerase α subunit: involvment of the c-terminal region in transcription activation by cAMP-CRP. *Cell* **65**, 1015-1022.

Ishihama, A. (1981). Subunit assembly of *Escherichia coli* RNA polymerase. *Adv. Biophys.* **14**, 1-35.

Ishihama, A. (1988). Promoter selectivity of prokaryotic RNA polymerases. *Trends in Genetics.* **4**, 282-286.

Ishihama, A. (1991) Protein-protein communications within the transcription apparatus. *Mol. Cells*, **1**, 381-384.

Ishihama, A. (1992) Role of the RNA polymerase α subunit in transcription activation. *Mol. Microbiol.* **6**, 3283-3288.

Ishihama, A. (1993) Protein-protein communications within the transcription apparatus. *J. Bacteriol.* **175**, 2483-2489.

Ishihama, A., and Ito, K. (1972) Subunits of RNA polymerase in function and structure. II. Reconstitution of *Escherichia coli* RNA polymerase from isolated subunits. *J. Mol. Biol.* **59**:111.

Ishihama, A., Shimamoto, N., Aiba, H., Kawakami, K., Nashimoto, H., Tsugawa, A., and Uchida, H. (1980) Temperature-sensitive mutations in the α-subunit gene of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **242**, 107-115.

Ishihama, A., Fujita, N., and Glass, R. E. (1987) Subunit assembly and metabolic stability of *E. coli* RNA polymerase. *PROTEINS: Structure, Function, and Genetics* **2**, 42-53.

Ito, K., Egawa, K., and Nakamura, Y. (1991). Genetic interaction between the ß' subunit of RNA polymerase and the arginine-rich domain of *Escherichia coli nus*A protein. *J. Bacteriol.* **173**, 1492-1501.

Ito, K., and Nakamura, Y. (1993) Pleiotropic effects of the rpoC10 mutation affecting the RNA polymerase beta' subunit of *Escherichia coli* on factor-dependent transcription termination and antitermination. *Mol. Microbiol.* **9**, 285-93.

Iwakura, Y., Ishihama, A. and Yura, T. (1973) RNA polymerase mutants of *Escherichia coli.* II. Streptoldigin resistance and its relation to rifampicin resistance. *Mol. Gen. Genet.* **133**, 1-23.

Iwakura, Y., Ito, K., and Ishihama, A. (1974) Biosynthesis of RNA polymerase in

Escherichia coli. I. Control of RNA polymerase content at various growth rates. *Mol. Gen. Genet.* **133**, 1-23.

Jeon, Y. H., Negishi, T., Shirkawa, M., Yamazaki, M., Fujita., N., Ishihama, A., and Kyogoku, Y. (1995) Solution structure of the activator contact domain of the RNA polymerase α subunit. *Science*, **270**, 1495-1497.

Jin, D. J., and Gross, C. A. (1988). Mapping and sequencing of mutations in the *Escherichia coli rpo*B gene that lead to rifampicin resistance. *J. Mol. Biol.* **202**, 45-58.

Jin D. J., and Gross, C. A. (1989). Three *rpo*BC mutations that suppress the termination defects of rho mutants also affect the functions of nusA mutants. *Mol. Gen. Genet.* **216**, 269-275.

Jin, R., Sharif, K. A., and Krakow, J. S. (1995) Evidence for Contact between the Cyclic AMP Receptor Protein and the σ^{70} Subunit of *Escherichia coli* RNA Polymerase. *J. Biol. Chem.* **270**, 19213-19216.

Kabata, H., Kurosawa, O., Arai, I., Washizu, M., Margarson, S. A., Glass, R. E. and Shimamoto, N., (1993) Visualization of single molecules of RNA polymerase sliding along DNA. *Science*, **262**, 1561-1663.

Kajitani, M., and Ishihama, A. (1983) Determination of promoter strength in the mixed transcription system: promoter of lactose, tryptophan, and ribosomal protein L10 operons from *Escherichia coli. Nucl. Acids Res.* **11**, 671-686.

Kashlev, M. A., Lee, J., Zelenskaya, K., Nikiforov, V., and Goldfarb, A. (1990) Blocking of the initiation-to-elongation transition by a transdominant RNA polymerase mutation. *Science* **248**, 1006-1009.

Kawakami, K., and Ishihama, A. (1980) Defective assembly of RNA polymerase subunits in a temperature-sensitive α-subunit mutant of *Escherichia coli. Biochemistry,* **19**, 3491-3495.

Kimura, M. and Ishihama, A. (1995a) Functional map of the alpha subunit of *Escherichia coli* RNA polymerase: Insertion analysis of the amino-terminal assembly domain. *J. Mol. Biol.* **248**, 756-767.

Kimura, M. and Ishihama, A. (1995b) Functional map of the alpha subunit of *Escherichia coli* RNA polymerase: Amino acid substitution within the amino-terminal assembly domain. *J. Mol. Biol.* **254**, 342-349.

Kimura, M. Fujita, N., and Ishihama, A. (1994) Functional map of the alpha subunit of *Escherichia coli* RNA polymerase: Deletion analysis of the amino-terminal assembly domain. *J. Mol. Biol.* **242**, 107-115.

Krakow, J. S. and von der Helm, K. (1971) Azotobacter RNA polymerase transitions and release of sigma. *Cold Spring Harbor Symp. Quant. Biol.* **35**, 73-83.

Kumar, S. A. (1981). The structure and mechanism of action of bacterial DNA-dependent RNA polymerase. *Prog. Biophys. Molec. Biol.* **38**, 165-210.

Kumar, A., Grimes, B., Fujita, N., Makino, K., Malloch, R. A., Hayward, R. S., and Ishihama, A. (1994) Role of the sigma^{70} subunit region 4 of the *Escherichia coli* RNA polymerase in transcription initiation and activation. *J. Mol. Biol.* **235**, 405-413.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**, 680-685.

Landick, R., Colwell, A., and Stewart, J. (1990). Insertional mutagenesis of a plasmid-borne *Escherichia coli rpo*B gene reveals alterations that inhibit ß-subunit assembly into RNA polymerase. *J. Bacteriol.* **172**, 2844-2854.

Laver, W. G., Air, G. M., Webster, R. G., and Smith-Gill, S. J. (1990). Epitopes on protein antigens: misconceptions and realities. *Cell* **61**, 553-556.

Lazcano, A., Fastag, J., Gariglio, P., Ramirez, C., and Oro, J. (1988). Synthesis on the early evolution of RNA polymerase. *J. Mol. Evol.* **27**, 365-376.

Lederer, H., Mortensen, K., May, R. P., Baer, G., Crespi, H. L., Dersch, D., and Heumann, H. (1991). Spatial arrangement of σ-factor and core enzyme of *Escherichia coli* RNA polymerase. A neutron solution scattering study. *J. Mol. Biol.* **219**, 747-755.

Lesley, S. A., and Burgess, R. R. (1989). Characterization of the *Escherichia coli* transcription factor σ^{70}: Localization of a region involved in the interaction with core RNA polymerase. *Biochemistry* **28**, 7728-7734.

Lesley, S. A., Brow, M. D., and Burgess, R. R. (1991) Use of *in vitro* protein synthesis from polymerase chain reaction-generated templates to study interaction of *Escherichia coli* transcription factors with core RNA polymerase and for epitope mapping of monoclonal antibodies. *J. Biol. Chem.* **266**, 2632-2638.

Lesley, S. A., Thompson, N. E., and Burgess, R. R. (1987) *J. Biol. Chem.* **262**, 5404-5407.

Levine, B. J., Orphanos, P. D., Fischmann, B. S., and Beychok, S. (1980). Physiochemical properties and interactions of *Escherichia coli* ribonucleic acid polymerase holoenzyme, core enzyme, subunits, and subassembly α_2ß. *Biochemistry* **19**, 4808-4814

Lill, U. I., and Hartman, G. R. (1970) Reactivation of denatured RNA polymerase from *E. coli. Biochem. Biophys. Res. Comm.* **38**:930.

Lisitsyn, N. A., Monastyrskaya, G. A., and Sverdlov, E. D. (1988) *Eur. J. Biochem.* **177**, 363-369.

Lisitsyn, N. A.,Sverdlov, E. D., Moiseyeva, E. P., Danilevsaya, O N., and Nikiforov, V. (1984) *Mol. Gen. Genet.* **196**, 173-174.

Lisitsyn, N. A.,Sverdlov, E. D., Moiseyeva, E. P., and Nikiforov, V. G. (1985) *Bioorg. Khim.* **11**, 132-133.

Liu, B., and Alberts, B. (1995) Head-on collision between a DNA replication apparatus and RNA polymerase transcription complex. *Science*, **267**, 1131-1137.

Liu, B., Wong, M. L., and Alberts, B. (1994) A transcribing RNA polymerase molecule survives DNA replication without aborting its growing RNA chain. *Proc. Natl. Acad. Sci. USA.* **91**, 10660-10664.

Lombardo, M.J., Bagga, D., and Miller, C. G. (1991). Mutations in *rpo*A affect expression of anaerobically regulated genes in *Salmonella typhimurium. J. Bacteriol.* **173**, 7511-7518.

Lonetto, M., Gribskov, M., and Gross, C. A. (1992) The σ^{70} family: sequence conservation and evolutionary relationship. *J. Bacteriol.* **174**, 3843-3849.

Luo, J., and Krakow, J. S. (1992). Characterization and epitope mapping of monoclonal antibodies directed against the ß' subunit of the *Escherichia coli* RNA polymerase. *J. Biol. Chem.* **267**, 18175-18181.

Luo, J., Sharif, K. A., Fujita, N., Ishihama, A., and Krakow, J. S. (1996) *J. Biol. Chem.* (In preparation).

Matsuyama, S., and Mizushima, S. (1987) Novel *rpoA* mutation that interferes with the function of OmpR and EnvZ, positive regulators of the *ompF* and *ompC* genes that code for outer-membrane proteins in *Escherichia coli* K12. *J. Mol. Biol.* **195**, 847-853.

McClure, W. R. (1985) Mechanism and control of transcription initiation in prokaryotes. *Annu. Rev. Biochem.* **54**, 171-204.

McMahan, S. A., and Burgess, R. R. (1994) Use of aryl azide cross-linkers to investigate protein-protein interactions: an optimization of important conditions as applied to *Escherichia coli* RNA polymerase and localization of a σ^{70}-α cross-link to the C-terminal region of α. *Biochemistry*, **33**, 12092-12099.

Merrick, M. J. (1993) In a class of its own-the RNA polymerase sigma factor σ^{54} (σ^{N}). *Mol. Microbiol.* **10**, 903-909.

Meisenberger, O., Piltz, I., and Heumann, H. (1980a). Small-angle X-ray study of DNA-dependent RNA polymerase subunit σ from *Escherichia coli. FEBS Lett.* **112**, 39-41.

Meisenberger, O., Piltz, I., and Heumann, H. (1980b). Small-angle X-ray study of DNA-dependent RNA polymerase subunit α_2 from *Escherichia coli. FEBS Lett.* **120**, 57-60.

Meisenberger, O., Heumann, H., and Piltz, I. (1980c). Small-angle X-ray study of DNA-dependent RNA polymerase core enzyme and partial complex $\beta\alpha_2$ from *Escherichia coli. FEBS Lett.* **122**, 117-120.

Nawrath, C., Schell, J., and Koncz, C. (1990). Homologous domains of the largest subunit of eucaryotic RNA polymerase II are conserved in plants. *Mol. Gen. Genet.* **223**, 65-75.

Negishi, T., Fujita, N., and Ishihama, A. (1995) Structural map of the alpha subunit of *Escherichia coli* RNA polymerase: Structural domains identified by proteolytic cleavage. *J. Mol. Biol.* **248**, 723-728.

Nielsen, D.A., and Shapiro, D. J. (1986) Preparation of capped RNA transcripts using T7 RNA polymerase. *Nucl. Acids Res.* **14**, 5936.

Nikiforov, V. G., Yakubov, L. Z., Bogachova, G. T., Lebedev, A. N., and Rokhlin, O. V. (1983). Monoclonal antibodies inhibiting RNA polymerase from *Escherichia coli. FEBS Lett.* **158**, 113-115.

Palm, P., Heil, A., Boyd, D., Grampp, B., and Zillig, W. (1975). The reconstitution of *E. Coli* DNA-dependent RNA polymerase from its isolated subunits. *Eur. J. Biochem.* **53**, 283-291.

Panny, S. R., Heil, A. Mazus, B., Palm, P., Zillig, W., Mindlin, S. Z., Ilyina, T. S., and

Khesin, R. B. (1974) A temperature sensitive mutation of the β'-subunit of DNA-dependent RNA polymerase from *E. coli* T16. *FEBS Letters* **48**:241.

Pilz, I., Kratky, O. and Rabussay, D. (1972) Studies on the conformation of DNA-dependent RNA polymerase in solution by small-angle X-ray measurements. *Eur. J. Biochem.* **28**, 205.

Platt, T. (1986) Transcription termination and the regulation of gene expression. *Annu. Rev. Biochem.* **55,** 339-372.

Polyakov, A., Severinova, E., and Darst, S. A. (1995) Three-dimensional structure of *E. coli* RNA polymerase: Promoter binding and elongation conformations of the enzyme. *Cell*, **83**, 365-373.

Puhler, G., Leffers, H., Gropp, F., Palm, P., Klenk, H-P., Lottspeich, F., Garrett, R. A., and Zillig, W. (1989). Archaebacterial DNA-dependent RNA polymerases testify to the evolution of the eukaryotic nuclear genome. *Proc. Natl. Acad. Sci. USA* **86**, 4569-4573.

Rabussay, D., and Zillig, W. (1969) A rifampicin resistant RNA polymerase from *E. coli* altered in the β-subunit. *FEBS Letters*, **5**:104.

Reznikoff, W. S., Siegele, D. A., Cowing, D. W., and Gross, C. A. (1985) The regulation of transcription initiation in bacteria. *Annu. Rev. Genet.* **19**, 355-387.

Riftina, F., DeFalco, E., and Krakow, J. S. (1989). Monoclonal antibodies as probes of the topological arrangement of the α subunits of *Escherichia coli* RNA polymerase. *Biochemistry* **28**, 3299-3305.

Riftina, F., DeFalco, E., and Krakow, J. S. (1990). Effects of an anti-α monoclonal antibody on interaction of *Escherichia coli* RNA polymerase with *lac* promoters. *Biochemistry* **29**, 4440-4446.

Rockwell, P. (1986) Characterization of effects of anti-beta and anti-beta' monoclonal antibodies on the activity of the RNA polymerase from *Escherichia coli.* Ph.D. thesis, City University of New York, New York.

Rockwell, P., and Krakow, J. S. (1988) Effects of anti-beta monoclonal antibody on the interaction of the *Escherichia coli* RNA polymerase with *lac* and TAC promoters. *Biochemistry* **27**, 3512-3520.

Rockwell, P., and Krakow, J. S. (1985). Characterization of effects of anti-ß and anti-ß' monoclonal antibodies on the activity of the RNA polymerase from

Escherichia coli. Biochemistry, **24**, 3240-3245.

Ross, W., Gosink, K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K. and Gourse, R. (1993) A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science*, **262**, 1407-1413.

Russo, F. D., and Silhavy, T. J. (1992). Alpha: the cinderella subunit of RNA polymerase. *J. Biol. Chem.* **267**, 14515-14518.

Sethi, V. S., and Zillig, W. (1970). Dissociation of DNA-dependent RNA polymerase from *E. Coli* in lithium chloride. *FEBS Lett.* **6**, 339-342.

Severinov, K., Fenyö, D., Severinova, E., Mustaev, A., Chait, B. T., Goldfarb, A., and Darst, S. A. (1994) The σ subunit conserved region 3 is part of "5'-face" of active center of *Escherichia coli* RNA polymerase. *J. Biol. Chem.* **269**, 20826-20828.

Severinov, K., Kashlev, M., Severinova, E., Bass, I., McWilliams, K., Kutter, E., Nikiforov, V., Snyder, L., and Goldfarb, A. (1994) A non-essential Domain of *Escherichia coli* RNA polymerase required for the action of the termination factor Alc. *J. Biol. Chem.* **269**, 14254-14259.

Severinov, K., Mustaev, A., Severinova, E., Bass, I., Kashlev, M., Landick, R., Nikiforov, V., Goldfarb, A., and Darst, S. A. (1995) Assembly of functional *Escherichia coli* RNA polymerase containing β-subunit fragments. *Proc. Natl. Acad. Sci. USA.* **92**, 4591-4595.

Severinov, K., Soushko, M., Goldfarb, A., and Nikiforov, V. (1993) Rifampicin region revisited. New rifampcin-resistant and streptolygidin-resistant mutants in the beta subunit of *Escherichia coli* RNA polymerase. *J. Biol. Chem.* **268**, 14820-14825.

Sharif, K. A., Fujita, N., Jin, R., Igarashi, K., Ishihama, A., and Krakow, J. S. (1994) Epitope mapping and functional characterization of monoclonal antibodies specific for the α subunit of *Escherichia coli* RNA polymerase. *J. Biol. Chem.* **269**, 23655-23660.

Sharif, K. A., Kimura, M., Glass, R. E., Krakow, J. S., and Ishihama, A. (1996) Epitope mapping of the polyclonal antibodies raised against smaller fragments of the alpha subunit of the *Escherichia coli* RNA polymerase. Manuscript in preparation.

Sharif, T. R., and Igo, M. M. (1993) Mutations in the alpha subunit of RNA polymerase that affect the regulation of porin gene transcription in *Escherichia coli* K-12. *J. Bacteriol.* **175**, 5460-5468.

Siegele, D. A., Hu, J. C., Walter, W. A., and Gross, C. A., (1989) Altered promoter recognition by mutant forms of the σ^{70} subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **206**, 591-603.

Slauch, J. M., Russo, F. D., and Silhavy, T. J. (1991). Suppressor mutations in *rpo*A suggest that *Omp*R controls transcription by direct interaction with the α subunit of RNA polymerase. *J. Bacteriol.* **173**, 7501-7510.

Stender, W. (1980). Antibodies against the subunits of *E. coli* RNA polymerase as probes for subunit-specific binding of DNA and other ligands. *Nucleic Acids Res.* **8**, 1405-1420.

Stender, W. (1981) Inhibition of *E. coli* RNA polymerase by F_{ab} fragments from subunit specific antibodies. *Biochem. Biophys. Res. Commun.* **100**, 198-204.

Stetter, K. O., and Zillig, W. (1974). Transcription in lactobacillaceae. DNA-dependent RNA polymerase from *Lactobacillus curvatus. Eur. J. Biochem.* **48**, 527-540.

Stockel, P., May, R., Strell, A., Cejka, Z., Hoppe, W., Heumann, H., Zillig, W., and Crespi, H. L. (1980a). The core subunit structure in RNA polymerase holoenzyme determined by neutron small-angle scattering. *Eur. J. Biochem.* **112**, 411-417.

Stockel, P., May, R., Strell, A., Cejka, Z., Hoppe, W., Heumann, H., Zillig, W., and Crespi, H. L. (1980b). The subunit position within RNA polymerase holoenzyme determined by triangulation of center-to-center distances. *Eur. J. Biochem.* **112**, 419-423.

Studier, F. W. and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189,** 113-130.

Sugiura, M., Segawa, K., Yoshinaga, K., Yu, F., Ito, N., Yashuda, S., and Hirota, Y. (1977) *Biochem. Biophys. Res. Comm.* **76**:739.

Sunshine, M. G., and Sauer, B. (1975) A bacterial mutation blocking P2 phage late gene expression. *Proc. Natl. Acad. Sci. USA* **72**, 2770-2774.

Tang, H., Severinov, K., Goldfarb, A., Fenyo, D., Chait, B., and Ebright, R. H. (1994) Location, Structure, and function of the target of a transcriptional activator protein. *Genes Dev.* **243**, 603-610.

Taniguchi, T., O'Neill, M., and de Crombrugghe, B. (1989) *Proc. Natl. Acad. Sci. USA* **76,** 5090-5094.

Tao, K., Fujita, N., and Ishihama, A. (1993) Involvement of the RNA polymerase α-subunit C-terminal region in cooperative interaction and transcription activation with OxyR. *Mol. Microbiol.* **7**,859-864.

Thomas, M. S., and Glass, R. E. (1991). *Escherichia coli rpo*A mutation which impairs transcription of positively regulated systems. *Mol. Microbiol.* **5**, 2719-2726.

von Hippel, P. H., Bear, D. G., Morgan, W. D., and McSwiggen, J. A. (1984) Protein-nucleic acid interactions in transcription: a molecular analysis. *Ann. Rev. Biochem.* **53**, 389-446.

Waldburger, C., Gardella, T., Wong, R., and Susskind, M. M. (1990) Changes in conserved region 2 of *Escherichia coli* σ^{70} affecting promoter recognition. *J. Mol. Biol.* **215**, 267-276.

Walter, G., Zillig, W., Palm, P., and Fuchs, E. (1967) Initiation of DNA-dependent RNA synthesis and the effect of heparin on RNA polymerase. *Eur. J. Biochem.* **3**:194.

Woychik, N. A., and Young, R. A. (1990). RNA polymerase II: subunit structure and function. *Trends Biochem. Sci (TIBS)* 15, 347-351.

Wu, F. Y.-H., Yarbrough, L. R., and Wu, C. W. (1976) Conformational transition of *Escherichia coli* RNA polymerase induced by interaction of sigma subunit with core enzyme. *Biochmistry* **15**, 3254.

Yager, T. D., and von Hippel, P. H. (1987) in *Escherichia coli* and *Salmonella typhimurium* (Neidhardt, F. C., ed.) *Am. Soc. Microbiol.*, Washington, DC, p. 1241.

Zalenskaya, K., Lee, J., Gujuluva, C. N., Shin, Y. K., Slutsky, M., and Goldfarb, A. (1990). Recombinant RNA polymerase: inducible overexpression, purification, and assembly of *Escherichia coli rpo* gene products. *Gene (Amst.)* **89**, 7-12.

Zillig, W., Fuchs, E., Palm, P., Rabussay, D., and Zechel, K. (1970) *Lepetit Colloquim on RNA Polymerase and Transcription* (ed. L. Silvestri), **1**, 151-157. North-Holland, Amsterdam.

Zillig, W., Palm, P., and Heil, A. (1976). Function and reassembly of subunits of DNA-dependent RNA polymerase. In *RNA polymerase*, (R. Losick and M. J. Chamberlin, eds.) Cold Spring Harbor Laboratory, pp. 101-125.

Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W. (1971) On the role of different subunits of DNA-dependent RNA

polymerase from *E. coli* in the transcription process. *Cold Spring Harbor Symp. Quant. Biol.* **35**: 47.

Zhou, Y., Pendergrast, P. S., Bell, A., Williums, R., Busby, S., and Ebright, R. H. (1994) The functional subunit of a dimeric transcription activator protein depends on promoter architecture. *EMBO J.* **13**, 4549-4557.