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Monoclonal antibodies directed against alpha subunit as probes of the structure and function of *E. coli* RNA polymerase

Riftina, Faina, Ph.D.

City University of New York, 1989

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MONOCLONAL ANTIBODIES DIRECTED AGAINST ALPHA SUBUNIT AS PROBES OF THE STRUCTURE AND FUNCTION OF E. COLI RNA POLYMERASE

by

FAINA RIFTINA

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The City University of New York

1989

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Chair of Examining Committee
(Joseph Krakow)

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Date

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Supervisory Committee

The City University of New York

Abstract

MONOCLONAL ANTIBODIES DIRECTED AGAINST ALPHA SUBUNIT AS PROBES OF THE STRUCTURE AND FUNCTION OF E. COLI RNA POLYMERASE

by

Faina Riftina

Adviser: Dr. Joseph S. Krakow

Anti-alpha monoclonal antibodies (mAbs) have been used to study topological arrangements of the two alpha subunits in E. coli RNA polymerase and to elucidate the role of the alpha subunit in transcription initiation. None of the studied anti-alpha mAbs inhibit the $d(A\text{-}T)_n$-directed synthesis of $r(A\text{-}U)_n$. mAb 126C6 strongly inhibits cAMP-CRP-dependent abortive initiation with lac P+ and partially inhibits abortive initiation with lac L8UV5 promoter; mAb 129C4 and mAb 124D1 are without effect. Kinetic analysis of open complex formation of mAb 126C6-polymerase with lac L8UV5 showed that changes in binding and isomerization steps account for the observed inhibition, with the isomerization step affected to a greater extent. After extended lag the rate of nucleotide incorporation by mAb 126C6-polymerase approached the rate observed with polymerase. DNase I footprinting and protection methylation studies indicate

that non-optimal contacts are established between mAb 126C6-polymerase and lac UV5 promoter. The data suggest that for mAb 126C6-polymerase the rate-limiting step in the open promoter complex formation is the proper alignment of the DNA with respect to the catalytic site of the enzyme. Analysis of the DNase I footprints of the mAb 126C6-polymerase complexed with cAMP-CRP-lac P+ suggests that interactions between CRP and polymerase are affected by binding of the mAb to polymerase.

Effects of the mAbs on reassembly of core enzyme from the subunit mixture suggest that at least one of the alpha subunits undergoes conformational changes during core enzyme assembly. The increase in the affinity of mAb 126C6 for assembled alpha compared to free alpha supports this conclusion.

Double antibody binding studies show that the epitopes for the non-inhibitory mAbs are available on only one of the alpha subunits in RNA polymerase and the epitope on each alpha subunit is available for binding by the inhibitory mAb 126C6. The studies also suggest that in the holoenzyme the sigma subunit is positioned close to one of the alpha subunits. Double antibody binding studies indicate that the alpha-beta, but not $alpha_2beta$, complex is the minimal stable subassembly, suggesting that the in vitro assembly of RNA polymerase core enzyme may proceed via the formation of a more stable alpha-beta complex followed by addition of the second alpha subunit.

Dedicated to the memory of my father,

David Vladimirovich Riftin

ACKNOWLEDGEMENTS

For the knowledge I acquired in the course of this work, I would like to thank my adviser Dr. Joseph S. Krakow. His expert guidance spiced with a steady good humor was invaluable in the success of my efforts. I would like to express my gratitude to my committee members Dr. Wlodeck Szer, Dr. Rivka Rudner, Dr. Thomas Haines, and Dr. Peter Lipke for their support and useful suggestions.

Special thanks are given to Mrs. Valia Krakow for her friendly disposition and expert graphical artwork. Thanks are also due Gopal Nair, Ellen Beasley, and Christopher Roman for their excellent technical help. I wish to acknowledge the importance of Elizabeth DeFalco's contribution in the preparation of anti-alpha monoclonal antibodies, without which this work would not be possible. Thanks also to Jesus Angulo, Pat Rockwell, Xiao-miao Li, Yun-ling Ren, Zhao-hui Yang, Jian-ying Luo, and Nicole Dalla Venezia who contributed to a pleasant and stimulating work environment.

Special thanks are due Sophie Weintraub of the Biochemistry Department for her sincere friendship.

Most of all I am grateful to my mother and to my American family, whose belief in me and love were decisive. Special love and gratitude go to my husband, Paul Ross, for his computer expertise and noncomputerized emotional support, and for being with me lovingly every step of the way.

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ABBREVIATIONS

BSA: bovine serum albumin;

cAMP: 3′, 5′ cyclic adenosine monophosphate;

CRP: cyclic AMP receptor protein;

DTT: dithiothreitol;

EDTA: ethylenediamine tetraacetic acid

ELISA: enzyme-linked immunosorbent assay;

lac P+: *lac* wild type promoter;

lac: the lactose operon;

mAb: monoclonal antibody;

PBS: phosphate buffered saline.

SDS: sodium dodecyl sulphate

TCA: trichloroacetic acid;

Tris: Tris-(hydroxymethyl)aminomethane

Introduction.

The DNA-dependent E. coli RNA polymerase (EC 2.7.7.6) is a complex multisubunit enzyme. Two alpha subunits (36.5 kDa each), one beta (150.6 kDa), and one beta' subunit (155 kDa) comprise a transcriptionally competent core enzyme. The core enzyme contains 2 g-atoms of tightly bound Zn ions per mol (Wu et al., 1977). Interaction of the core enzyme with the sigma subunit (70 kDa) results in the formation of a promoter-selective holoenzyme. Transcription is initiated de novo at specific promoter sites and starts almost exclusively with either ATP or GTP. Specificity of nucleotide incorporation is determined by Watson-Crick base pairing to the template. The phosphodiester bonds are formed by the processive addition of the 5'-alpha-phosphate of the incoming nucleoside triphosphate to the 3' hydroxyl end of the growing nascent RNA chain. The intrinsic maximum rate of polymerization has been estimated as 30-60 nucleotides/sec (Chamberlin, 1976). The detailed mechanism of the catalysis and the structure of the active site is not known.

Based on reconstitution of the core enzyme from the individual purified subunits and from subunit mixtures (Ishihama & Ito, 1972; Ito & Ishihama, 1973; Fukuda & Ishihama, 1974; Yarbrough & Hurwitz, 1974; Palm et al., 1975; Harding & Beychock, 1976; Saitoh & Ishihama, 1976;

Saitoh & Ishihama, 1979; Ishihama, 1981) the following stepwise sequence of subunit assembly has been proposed (Ishihama, 1981):

$$a + a \rightarrow a_2 + B \rightarrow a_2B + B' \rightarrow \underset{\text{inactive}}{a_2BB'} \rightarrow \underset{\text{active}}{a_2BB'}$$

Although the formation of the alpha-dimer as the first step of the core enzyme assembly is generally accepted there is no convincing experimental proof for a stable $alpha_2$ complex. Cross-linking studies with bifunctional reagents showed very little (Hillel & Wu, 1977; Ishihama et al., 1979) or no alpha dimers (Coggins et al., 1977). The presence of a highly structured but inactive (premature) core enzyme has been confirmed. In the premature core the subunits are loosely associated. The temperature-dependent maturation is required for the formation of a structurally stable and functionally active RNA polymerase. Activation of the premature core enzyme may also be enhanced by the presence of either DNA or the sigma subunit (Ishihama, 1981).

The functional role of each subunit is only partially understood. The sigma subunit is involved in initiation specificity and at least part of the active site of the enzyme is present in the beta subinit (Zillig et al., 1976; Grachev et al., 1987). The beta subunit also binds

rifampicin, the specific inhibitor of transcription initiation (Zillig et al., 1976). Binding of rifampicin was shown to inhibit the translocation step after the formation of the first phosphodiester bond (McClure & Cech, 1978). The beta' subunit participates in DNA binding and also binds the polyanionic inhibitor, heparin (Zillig et al., 1976). Cross-linking of polymerase lysine residues with DNA phosphates (Chenchick et al., 1981) showed that the sigma, beta and beta' subunits make contacts with the DNA (lac UV5) template spanning the promoter region from +30 to -47 bp. The core and holoenzyme showed the same pattern of contacts only within the region between +30 and +3 bp, but differed in the region between +1 and -37 bp. The -35 region was crosslinked to the beta subunit in the core and to the sigma subunit in the holoenzyme. Simpson (1979) identified thymines at -3 bp and +3 bp on the non-template strand to which the sigma subunit and the beta subunit, respectively, were crosslinked. No crosslinking between alpha subunit and the DNA template was observed. The role of the alpha subunit remains to be defined (von Hippel et al., 1984; Burgess et al., 1987).

The structural organization of the E. coli polymerase has been studied by electron microscopy (Lubin, 1969), small angle neutron scattering (Stockel et al. 1980a, 1980b), and small angle X-ray scattering (Meisenberger et al., 1980a, 1980b, 1980c, 1981), as well as by cross-linking with

bifunctional reagents (Hillel & Wu, 1977; Coggins et al., 1977, Ishihama et al., 1979), and by partial proteolysis (Lill & Hartman, 1975; Lowe & Malcolm, 1976; Fisher & Blumenthal, 1980). Subunit-specific polyclonal antibodies have been used to investigate the accessibility and function of the subunits in RNA polymerase (Stender, 1980, 1981) and to study the mode of subunit interaction (Tichelaar et al., 1983). In the electron microscope the molecules of RNA polymerase appear porous and of irregular shape, some molecules resemble a wedge, others an oblong (Lubin, 1969). The core and holoenzyme were found to have approximately the same dimensions, 9 nm x 16 nm (Tichelaar et al., 1983).

Limited proteolysis of the individual subunits of RNA polymerase, subunit subassemblies, polymerase alone or complexed with DNA (Lowe & Malcolm, 1976) indicated the presence of exposed polypeptide loop on the surface of the RNA-polymerase. The data suggested that the exposed loop is a part of the beta' subunit. The limited proteolysis of the beta subunit, but not the beta' subunit, was inhibited by the presence of the alpha subunit in the mixture and confirmed the alpha-beta association reported previously (Ito & Ishihama, 1973; Fukuda & Ishihama, 1974; Palm et al., 1975). Analysis of RNA polymerase by trypsin cleavage pointed to a specific association between the beta and sigma subunit during the closed to open complex transition (Fisher & Blumenthal, 1980). Cross-linking with bifunctional

reagents yielded beta-beta' as a major product. The other products were: alpha-beta, alpha-beta', sigma-beta, sigma-beta', alpha-sigma, alpha-sigma-beta, alpha-sigma-beta', sigma-beta-beta', and very little (Hillel & Wu, 1977; Ishihama et al., 1979) or no alpha dimers (Coggins et al., 1977). Based on their studies Hillel and Wu (1977) presented a tetrahedron-like model of the core enzyme in which the alpha-dimer interacts with both beta and beta' subunits; in the holoenzyme the sigma subunit interacts with beta and beta', and with one alpha subunit. A similar but more detailed model was proposed on the basis of small-angle neutron scattering data (Stockel et al., 1980a, 1980b). In this model the elongated and slightly curved beta and beta' subunits interact to form the two sides of nearly equilateral triangle, the third side is formed by the putative alpha-dimer in which one of the alpha subunits projects beyond the beta subunit with which it interacts, hence, it is more exposed. The asymmetric position of the two alpha subunits within RNA polymerase is also suggested by the selective ADP-ribosylation of one of the alpha subunits in the enzyme (Rohrer et al., 1975). Another evidence is dissociation of only one alpha subunit from the polymerase in the presence of 0.1 mM para-chloro-mercuribenzoate (Ishihama, 1972). The Y-shape of the sigma subunit proposed by Meisenberger et al. (1980a) fits well with the above model. In the holoenzyme the sigma subunit

interacts with beta and beta' and with one of the alpha subunits. However, the precise orientation of the sigma subunit with respect to core enzyme is not yet known. Using small angle X-ray scattering Meisenberger et al. (1980a, 1980b, 1980c, 1981) proposed a different arrangement of the polymerase subunits in the core enzyme. In their model beta and beta' subunits have a conical shape and are lying side by side with the thick ends in the same direction. The alpha dimer, a curved elongated disc with a deep crevice in the middle, is attached to the thick ends of the beta-beta' complex. This model is also in conflict with the quaternary structure of the E. coli RNA polymerase derived from the immuno electron microscopy studies of Tichelaar et al., (1983). Based on the shape and size of the five characteristic profiles Tichelaar et al. (1983) constructed a model for the core enzyme in which curved elongated beta and beta' subunits are crossing each other at about one-fourth of their lengths with the alpha dimer located at the short ends. The beta and beta' are curved in the same direction forming a convex and concave surface. The sigma subunit contacts the alpha dimer at the concave side of the core enzyme. This model is in better agreement with the model of Stockel et al. (1980a, 1980b) than with that proposed by Meisenberger et al. (1981).

The various approaches have provided valuable information regarding the dimensions of RNA polymerase, the gross shape

of the individual subunits and the enzyme and also suggested possible modes of subunit interaction. However, due to the inherent limitations of the methods employed, the regions involved in subunit:subunit interactions and possible changes in subunit conformation attendant to enzyme assembly remain undefined. The development of hybridoma technology (Kohler & Milstein, 1975) has allowed the preparation of site-specific monoclonal antibodies. Being directed against a unique antigenic determinant on the surface of a protein monoclonal antibodies can provide a useful probe in unraveling of the topological arrangement of subunits in a multi-subunit protein. In a protein with a complex quaternary structure the availibility of a particular epitope depends on the mode of subunit interactions as well as on the conformational changes induced by these interactions in the course of the subunit assembly. Direct blockage of an epitope by a neighbouring subunit as well as conformational changes that develop in the protein during the process of a subunit assembly may render an epitope inaccessible. The first part of this thesis describes the use of murine monoclonal antibodies directed against the individual purified subunits of the E. coli polymerase to study the topological arrangements of the two alpha subunits in the enzyme, the conformational changes that occur in the alpha subunit during core enzyme assembly and a pathway for core enzyme assembly alternative to that proposed by

Ishihama (1981). Double antibody binding studies (sandwich assay devised by Pestka et al. 1983) and reconstitution of core enzyme from the subunit mixture in the presence of anti-alpha monoclonal antibodies suggested that the first step in assembly of core enzyme in vitro may proceed via the formation of more stable alpha-beta intermediate, which is then followed by addition of the second alpha and beta' subunits. The second part of the thesis describes the effects of anti-alpha monoclonal antibodies on initiation of transcription from linear lac P+ and lac L8UV5 promoters.

Synthesis of RNA by the E. coli DNA-dependent RNA polymerase is a complex multistep process. The process includes the sequential formation of a promoter-specific initiation complex between RNA polymerase and DNA, transition to a stable elongation complex and termination of transcription with the release of the enzyme and RNA from the DNA template. The functional initiation complex is called the "open" promoter complex since it was shown that the region from -9 to +3 bp becomes single-stranded (Siebenlist et al., 1980). The formation of the open complex is preceeded by the formation of the "closed" promoter complex in which the above region remains double-stranded. Conversion of the initiation complex to a stable elongation mode is associated with the release of the sigma subunit after an 8 to 9 nucleotide long RNA chain is synthesized.

Each step of the pathway is a complex multistep event which has been only partially elucidated. For recent reviews on mechanism and control of transcription initiation in prokaryotes see von Hippel et al. (1984) and McClure (1985).

Transcription in E. coli is initiated at specific sites on the DNA termed promoters. Analysis of the DNA sequences of more than 100 promoters from E. coli has revealed the regions of homology from which two hexameric consensus sequences were derived:

```
5'--TTGaca---------------TAtaaT------- start site--3'OH
    -35    ~17 bp spacer   -10   5-7 bp
```

The -35 and -10 regions are numbered with respect to the transcription start site. The capital letters indicate highly conserved nucleotides. Polymerase-promoter interactions are characterized by the promoter strength which is determined by the intrinsic DNA sequence of the promoter and by activator or repressor proteins that modulate RNA polymerase-promoter interactions. The consensus sequence is correlated with maximal promoter strength. The length of the spacer is also critical for only the proper orientation of the -35 and -10 bp regions can be recognized by polymerase. The strength of a certain promoter is determined by the deviations from the consensus sequence. Mutations that decrease the homology to the consensus

regions weaken polymerase-promoter contacts and are termed down mutations. The up mutations favor polymerase-promoter contacts. The weakest promoters with poor homology to the consensus sequence are positively modulated by ancillary proteins. Lac P+ is an example of a weak promoter which requires cAMP-CRP binding for functional interaction with RNA polymerase. The up mutation (UV5) in the -9 and -8 bp positions results in the cAMP-CRP-independent, moderately strong promoter.

Formation of the promoter-specific preinitiation complex between RNA polymerase and DNA was originally proposed by Walter and Zillig (Walter et al. 1967) and extended by Chamberlin (1974). The first step in transcription initiation is the formation of the "closed" promoter complex that reflects the initial specific binding of RNA polymerase to the double-stranded DNA sequence. Recognition of a specific base-paired DNA by the enzyme is based on the complementarity of the hydrogen bond donor-acceptor matrix in the major and minor grooves of the DNA to the matrix of hydrogen bond acceptors and donors comprising the polymerase binding domain (von Hippel et al., 1984). The 5-methyl groups of two adjacent thymines in the -35 region also play a crucial role. Subsitution of uracil for either thymine resulted in a substantial reduction in the rate of formation of a transcriptionally competent complex whereas substitution at both sites generated an inactive promoter

(Dubendorff et al., 1987). Similar substitution in the -10 region had a negligible effect on transcription. However, substitution of a C*G (or C*I) base pair for the T*A base pair at the -7 bp position resulted in an inactive promoter. It was suggested that functional groups in the major groove of the strongly conserved T*A base pair at the -7 bp position could be the sites of direct interaction with RNA polymerase (Dubendorff et al., 1987). The following experimental data support the existence of the closed promoter complex: kinetic analysis of the RNA synthesis shows a lag phase with double-stranded but not single-stranded DNA (McClure, 1980); binding of E. coli RNA polymerase to the early promoters of phage T7 at 0°C was observed in electron microscope (Williams et al., 1977); DNase I footprinting of polymerase-T7-A3 promoter complexes at 0°C (Kovacic, 1987) revealed some significant differences from the footprints obtained with open complexes. Substitutions in the spacer region suggested that the -10 and -35 regoins contact polymerase simulteneously during closed promoter formation (Auble & de Haseth, 1988). Buc and McClure (1985) postulated the existence of the transcriptionally inactive intermediate complex (RP_i) during the closed to open promoter complex transition. Spassky et al. (1985) correlated a conversion of a closed promoter complex, RP_c, to an intermediate, RP_i, with the strict positioning on the polymerase surface of the -10 and -35

regions of the promoter with respect to each other. Conversion of the RP_i to an open promoter complex occurs rapidly at 37°C and is associated with the cooperative polymerase-DNA interactions and the unstacking of DNA bases at and near the transcription start site. The rate-limiting step in the formation of the open promoter complex in this sequence is the formation of RP_i. An intermediate open promoter complex that favors abortive initiation cycling, proposed by Carpousis and Gralla (1980), and having stronger contacts downstream from -24 bp has been demonstrated by Straney and Crothers (1987). This complex undergoes a temperature-dependent transition to a the next state which is characterized by stronger polymerase-promoter interactions in the upstream from the -24 region and is associated with the stable elongation mode.

In the present study an inhibitory anti-alpha monoclonal antibody has been used to investigate the possible role of the alpha subunit in transcription initiation. The antibody proved to be useful in elucidating intermediate pathways of the open promoter complex formation and in probing RNA polymerase interactions with the cyclic AMP receptor protein during and after the open complex formation.

Materials and methods.

Materials. Reagents were obtained as follows: radioimmunoassay grade bovine serum albumin (BSA), 4-methylumbelliferyl-beta-D-galactopyranoside, papain, cAMP, ApA, Sigma Chemical Co.; goat anti-mouse IgG-beta-galactosidase, Hyclone; Immunoprecipitin, Bethesda Research Laboratories; $d(A\text{-}T)_n$, ribonucleoside triphosphates, Protein A-Sepharose, Sephacryl SF-400, Pharmacia; Bio-Rex 70, Affi-Gel Blue, bisacrylamide, TEMED, Bio-Rad; [^{3}H]UTP, [^{3}H]ATP, [gamma-^{32}P]ATP, and [alpha-^{32}P]ATP, ICN; [^{125}I]NaI, [^{3}H]N-ethylmaleimide, New England Nuclear; T4 polynucleotide kinase, alkaline phosphatase, DNA polymerase I Klenow fragment, restriction endonucleases EcoRI and PvuII, Boeringer Mannheim; DNase I, Cooper Biochemical; urea, Schwarz-Mann; dimethyl sulfate, piperidine, and hydrazine, Aldrich; formamide, Amresco; acrylamide, Serva; Scintisol, Isolab.

Methods. Preparation of RNA polymerase. RNA polymerase was purified from E. coli K12 cells by a modification of the procedure of Burgess and Jendrisak (1975). Holoenzyme and core enzyme were resolved by chromatography on denatured calf thymus DNA covalently linked to sepharose (Lowe et al., 1979). Holoenzyme appeared pure, and core enzyme was more

than 98% pure as judged by 10% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Protein concentration was determined using the following extinction coefficients: core polymerase, $E^{1\%}_{280nm} = 5.8$; holoenzyme, $E^{1\%}_{280nm} = 6.7$.

Preparation of monoclonal antibodies. Subunit-specific monoclonal antibodies (De Falco et al., 1983; Rockwell et al., 1985) were prepared as indicated in Rockwell et al. (1985). Monoclonal antibodies were raised against purified subunits of E. coli RNA polymerase using SJL/J female mice. Spleen cells of the immunized mice were fused with P3x63-Ag 8.653 myeloma cells (Kearney et al., 1979; Oi and Herzenberg, 1980). Antibodies were prepared from spent media of expanded cultures grown to stationary phase and purified by chromatography on protein A-Sepharose (Ey et al.,1978) or DEAE-cellulose (Parham et al., 1982). Immunoglobulin concentration was determined using the extinction coefficient $E^{1\%}_{280nm} = 14.6$ (Ey et al., 1978). The immunoglobulin isotype was determined by using the mouse immunoglobuline subtype identification kit purchased from Boehringer-Mannheim. Each monoclonal antibody could bind only to its respective subunit and to core or holo RNA polymerase as determined by ELISA and Western blotting (De Falco et al., 1983).

Separation of RNA polymerase subunits. Alpha subunit was

separated from beta and beta' subunits by gel-filtration on Sephacryl SF-400 in 10 mM potassium phosphate buffer (pH 7.2) containing, 6 M guanidine hydrochloride, 1 mM dithiothreitol, 0.1 mM EDTA and 5% glycerol. The separated subunits were stored in this buffer at -20°C. For the binding studies alpha subinit was prepared by chromatography of urea-denatured core enzyme on Bio-Rex 70 (Lowe & Malcolm, 1976). The purified alpha subunit was dialyzed against PBS buffer containing 0.1mM DTT and stored at -20°C.

Preparation of $alpha_2beta$ subassembly. $Alpha_2beta$ subassembly was obtained by chromatography of urea-denatured core enzyme on either Affi-Gel Blue (Wu, et. al., 1977), or Bio-Rex 70 (Lowe & Malcolm, 1976). Fractions containing alpha plus beta subunits were pooled and dialyzed against storage buffer containing 20 mM potassium phosphate (pH 7.5), 150 mM NaCl, 60% glycerol, 1 mM dithiothreitol and 0.1 mM EDTA and stored at -20°C.

Preparation of F_{ab} fragments of mAb 129C4 and mAb 126C6. F_{ab} fragments of anti-alpha mAb 129C4 and mAb 126C6 were prepared by proteolytic digestion of the intact mAbs with papain (Mage, 1980). To stop the reaction and to eliminate the possibility of papain reactivation by dithiothreitol routinely present in reconstitution buffer the reaction mixture was treated with 0.1 M glycine-HCl buffer (pH 2.5)

for 10 minutes at room temperature (instead of treatment with iodoacetamide). Papain is rapidly and irreversibly inactivated at acid pH values (Glazer & Smith, 1971). The cleaved F_c region was removed by treatment with protein A-sepharose. The generation of F_{ab} fragments was confirmed by SDS gel electrophoresis.

Gel Electrophoresis. SDS gel electrophoresis was performed by the method of Laemmli (1970). Good separation of beta and beta' subunits was achieved by electrophoresis of core enzyme on sodium tetradecyl sulfate (STS) gel (10% acrylamide, 0.1% bisacrylamide) as described by Fisher and Blumenthal (1980).

Reconstitution of core enzyme. The conditions used for reconstitution were essentially those described by Palm et al. (1975). 100 pmol of alpha and 50 pmol each of beta and beta' subunits (obtained by gel-filtration and stored in the presence of 6 M guanidine-HCl) were combined and the total volume of the mixture was adjusted to 250 ul with reconstitution buffer (50 mM Tris-HCl, pH 8, 0.2 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol) to obtain the optimal core enzyme concentration of 0.2 mg/ml. Alternatively, core enzyme was dissociated by dialysis against 10 mM potassium phosphate (pH 7.2) containing 6 M guanidine-HCl, 0.1 mM EDTA, 1mM

dithiothreitol and 5% glycerol for 2 hours at 4°C after which the protein concentration was adjusted to 0.2 mg/ml. Reconstitution mixtures in the presence or absence of monoclonal antibodies were dialyzed against reconstitution buffer at 4°C overnight and then incubated for 30-40 minutes at 30°C. Protein concentration after reconstitution was determined by the method of Schaffner and Weissmann (1973). To determine the activity of the reconstituted core enzyme, 25 ul aliquots of the reconstituted mixtures were used. The activity of the reconstituted core enzyme was assayed by $d(A-T)_n$ directed synthesis of $r(U-A)_n$. Approximately 400 nmol of [^{3}H]UMP was incorporated per nmol of core enzyme after incubation for 10 minutes at 37°C. Alpha was dialysed against PBS containing 0.1 mM dithiothreitol before running reconstitution assays with mAb 124D1. The activity of reconstituted core was not effected whether alpha had been stored in the presence of 6 M guanidine-HCl or in PBS.

Assay of $d(A-T)_n$-directed $r(A-U)_n$ synthesis. Monoclonal antibody-polymerase complexes were formed by incubation for the time and temperature indicated in the legends. After addition of 3 nmol of $d(A-T)_n$ the mixture was incubated for 10 min at 37°C. Synthesis of $r(A-U)_n$ was carried out in a reaction mixture (90 ul) which contained RNA polymerase holoenzyme (2 pmol) or core enzyme (4 pmol), or a mAb-polymerase complex, 400 nmol ATP, 100 nmol [^{3}H]UTP

(44,000 cpm/nmol), 40 mM Tris-HCL (pH 8), 10 mM $MgCl_2$, 100 mM KCl, 10 mM mercaptoethanolamine, 0.1-0.25 mM dithiothreitol and 5.5% of glycerol. After incubation at 37°C for the time indicated, the $r(A-U)_n$ was precipitated with 5% trichloroacetic acid, collected on glass fiber filters (Whatman GFC) and counted in Scintisol.

Abortive initiation assay. A modification of the abortive initiation assay of Malan et al. (1984) was used to determine the effect of the monoclonal antibodies on transcription from lac L8UV5 and lac P+ promoters. The reaction mixture (final volume 50 ul) contained: 40 mM Tris-HCL (pH 8), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5% glycerol, 3 nM lac P+ (plus 40 nM CRP and 0.1 mM cAMP) or lac L8UV5 DNA fragment, and the amount of RNA polymerase or polymerase-mAb complex indicated in the legends. Monoclonal antibody-polymerase complexes were formed by incubation of a mAb with polymerase for 30 min at 37°C or for 1 hour on ice at the molar ratio indicated in the legends. After additional preincubation with the template for 10 min at 37°C 1 mM ApA, and 50 uM [^{3}H]UTP (190 cpm/pmol) were added. The reaction was allowed to proceed at 37°C for the time indicated and was terminated by addition of 10 ul 0.5 M EDTA. The radioactive products were resolved by ascending paper chromatography in WASP solvent: water, saturated ammonium sulfate, 2-propanol (18:80:2)

adjusted to pH 8 with ammonium hydroxide (Hansen & McClure, 1979). The amount of ApApUpU synthesized was estimated by determining the radioactivity of appropriate 1 cm segments in Scintisol.

Modification of sulfhydryl groups of the alpha subunit with [^{3}H]N-ethylmaleimide. Sulfhydryl groups of the alpha subunit were labeled according to a following procedure: 31 ug of alpha subunit in 500 ul of a buffer containing 10 mM Tris-HCl, pH 7.5, 0.01 mM dithiothreitol, 0.5 M NaCl, and 50% glycerol was mixed with 375 ul of 9.16 mM [^{3}H]N-ethylmaleimide (specific activity 17,969 cpm/nmol) and incubated for 12 minutes at 37^{o}C. The reaction was terminated by addition of 20 ul of 1 M mercaptoethanol. Under these conditions 4.1 nmole of N-ethylmaleimide (73,673 cpm) was incorporated per nmol of alpha subunit as determined after precipitation with trichloroacetic acid.

Preparation of ^{125}I-labeled monoclonal antibodies. 20 ug of a monoclonal antibody was labeled with 1 mCi of sodium ^{125}Iodide (specific activity approximately 17 Ci/mg iodine) by the chloramine T procedure (Hunter, 1967). The specific activities of the [^{125}I]labeled monoclonal antibodies were determined by TCA precipitation and are indicated in the legends.

Double antibody binding assays. A modification of the method of Pestka (Pestka et al. 1983) was used to assay for binding of the mAbs to free and polymerase-associated alpha. Costar 96-well EIA polystyrene plates were coated with antibody by incubation overnight at 4°C. Each well received 500 ng of the indicated monoclonal antibody in 100 ul PBS (10 mM potassium phosphate (pH 7.4) and 150 mM NaCl). Remaining protein binding sites were blocked by incubation with 200 ul per well of PBT: PBS containing 2 mg/ml bovine serum albumin, 0.05% Tween-80, and 0.04% NaN_3. The plates were then washed twice with PBT. Binding of RNA polymerase or the indicated subunit to the immobilized monoclonal antibody was carried out by incubation for one hour at 37°C or for 16 hours at 4°C in a PBS solution (100 ul) containing: 365 ng alpha or 700 ng $alpha_2beta$ or 1 ug core or 1 ug holoenzyme plus 2 mg/ml bovine serum albumin. After washing three times with PBT, 100 ul of a solution containing a saturating amount (determined from Scatchard plots) of the indicated [^{125}I]mAb in PBS + 2mg/ml BSA was added. After incubating for 90 minutes at 37°C or 16 hours at 4°C, the plates were washed with PBT. [^{125}I]-monoclonal antibody bound was determined after incubation for 30 min with 140 ul of 1 N NaOH followed by transfer to 10 x 75 mm tubes. Radioactivity was determined in a LKB Autogamma counter.

When Staph. aureus cells (Immunoprecipitin) with formalin-

fixed Protein A were used (in place of polystyrene plates) the binding assays were performed as follows: 1 mg of S. aureus cell suspension (binding capacity 12 ug of IgG per mg of cell suspension) was incubated with 15 ug of the indicated monoclonal antibody in 100 ul of PBS-BSA for 15 min at room temperature. To saturate unoccupied binding sites on the S. aureus cells bovine IgG (20ug) was then added and the mixture was incubated for an additional 10 min at room temperature. Unbound antibody was removed by centrifugation of the suspension at 15,000 rpm for 2 min and the S. aureus-monoclonal antibody complex was washed two times with 1 ml of PBS-BSA. The [^{125}I]mAb 129C4 (10 pmol, 1.5x10^6 cpm/pmol) was incubated for 30 min at 37^{o}C with 1.1 ug of alpha$_2$beta, or 2.4 ug core enzyme, or 3 ug holoenyzme in PBS-BSA (final volume of 250 ul for alpha$_2$beta, 300 ul for core or holoenzyme). After addition of 50 ul of the indicated [^{125}I]-mAb 129C4-antigen complex to 10 ul of the S. aureus-monoclonal antibody suspension the mixture was incubated for 30 min at 37^{o}C. Unbound [^{125}I]-monoclonal antibody was removed by washing two times with 1 ml of PBT followed by centrifugation at 15,000 rpm for 2 min. After transfer of precipitate to 10 x 75 tubes, radioactivity was determined in a LKB Autogamma counter.

Determination of monoclonal antibody affinity constants. The K_d values for mAb 126C6 and alpha, core and holoenzyme,

and for mAbs 129C4 and 124D1 and alpha were determined by the method of Friguet et al. (1985) with 4-methyl-umbelliferyl-beta-D-galactopyranoside (4-MUG) as the fluorescent substrate for beta-galactosidase conjugated to goat anti-mouse IgG. The plates (MicroFluoro "B" flat bottom plates, Dynatech Corp.) were coated with core enzyme overnight at $4^{o}C$. Monoclonal antibody (at a fixed concentration) was incubated in a test tube with an antigen (at a varied concentration) overnight at $4^{o}C$. The fixed concentrations of the mAs were: mAb 126C6, 2×10^{-9} M; mAb 129C4 and mAb 124D1, 1×10^{-9} M. The antigen concentration was varied from 1×10^{-9} M to 5×10^{-7} M. Aliquots (100 ul) were removed from the test tube mixtures for binding to the plate-immobilized core enzyme; the binding was carried out for 1 hour at $37^{o}C$ followed by incubation with goat anti-mouse IgG-beta-galactosidase and then with 4-MUG. Fluorescence of the product was determined with a Dynatech MicroFluoro Reader.

Dissociation constants (K_d) for mAb 129C4 and mAb 124D1 and alpha, core and holoenzyme were calculated from Scatchard plots obtained with ^{125}I-labeled monoclonal antibodies in a double antibody (sandwich) binding assay (Pestka et al., 1983) on polystyrene plates (Costar) and double antibody binding assay on S. aureus cells. For the plate-binding assay each well received 500 ng of the indicated monoclonal antibody in 100 ul PBS (10 mM potassium

phosphate (pH 7.4) and 150 mM NaCl) and the plate was incubated overnight at 4°C. Remaining protein binding sites were blocked by incubation for 1 hour at 37°C with 200 ul per well of PBT. The plates were then washed twice with PBT. Binding of RNA polymerase or alpha subunit to the immobilized monoclonal antibody was carried out by incubation for one hour at 37°C in a PBS solution (100 ul) containing: 365 ng alpha or 1 ug core or 1 ug holoenzyme plus 2 mg/ml bovine serum albumin. After washing three times with PBT, 100 ul of a solution containing the increasing amount of the indicated [^{125}I]mAb in PBS + 2mg/ml BSA was added. After incubating for 90 minutes at 37°C the plates were washed with PBT. [^{125}I]-monoclonal antibody bound was determined after incubation for 30 min with 140 ul of 1 N NaOH followed by transfer to 10 x 75 mm tubes. Radioactivity was determined in a LKB Autogamma counter. For [^{125}I]mAb 129C4 (1,312 cpm/fmol) the immobilized antibody was mAb 124D1, and for [^{125}I]mAb 124D1 (1,275 cpm/fmol) the immobilized antibody was mAb 129C4. The concentrations of the [^{125}I]mAbs was varied from 2 x 10^{-10} M to 12 x 10^{-9} M.

The determination of the K_d values for mAb 124D1 and alpha subunit or core enzyme in a double antibody assay with S. aureus cell suspension was as follows: The S. aureus cell suspension (1 mg) was incubated with 2 pmol (300 ng) of mAb 129C4 in 40 ul PBS-BSA for 15 min at room

temperature. The unbound mAb was removed by centrifugation (15,000 rpm, 5 min) and washing with PBT buffer (1 ml x 2 times). The remaining antibody-binding sites were saturated with 20 ug of bovine IgG. After addition of 1 pmol of alpha or 0.5 pmol core enzyme in 100 ul PBS-BSA the mixture was incubated for 30 min at 37°C. After unbound antigen was removed by washing with PBT (1 ml x 2 times), 100 ul of [^{125}I]mAb 124D1 (1,275 cpm/fmol) was added and incubated for 30 min at 37°C. The concentration of the [^{125}I]mAb 124D1 varied from 2.5 x 10^{-10} M to 12 x 10^{-9} M. The unbound [^{125}I]mAb was removed by washing with PBT buffer (1 ml x 3 times). The precipitated [^{125}I]-immunocomplex was resuspended in the 0.5 ml PBS-BSA, transferred to 10 x 75 mm tubes and the bound radioactivity was determined in the Autogamma counter (LKB).

Determination of the binding and isomerization constants for mAb 126C6-RNA polymerase and lac L8UV5. The kinetic analysis of abortive initiation devised by McClure (1980) was used for evaluation of the binding (K_B) and isomerization (k_2) constants for mAb 126C6-RNA polymerase and lac L8UV5. The analysis is based on the simplified reaction scheme for the formation of an RNA polymerase open promoter complex:

$$R + P \underset{k_{-1}}{\overset{k_1}{<==>}} RP_c \underset{k_{-2}}{\overset{k_2}{<==-->}} --> ... --> RP_o$$

where R and P represent free polymerase and promoter, respectively; RP_c and RP_o correspond to the closed and open complex, respectively. For the in vitro experiments the pseudo-first-order conditions were applied where the polymerase concentration was in excess to the total promoter concentration. Steady state was assumed for $[RP_c]$. It was also assumed that the rate of the forward reaction (k_2) is much greater than the rate of the reverse reaction (k_{-2}). Under these conditions RNA polymerase and a promoter combine at a rapid equilibrium to form a closed promoter complex. The rate-limiting step is a slow transition of RP_c to RP_o ($k_2 >> k_{-2}$). The solution of the rate equation $d[RP_o]/dt = k_2[RP_c] - k_{-2}[RP_o]$ yields an equation for the average time required for open complex formation, tau_{obs}:

$$tau_{obs} = 1/k_2 + (k_{-1}+k_2)/k_1[R]k_2$$

where the reciprical of $(k_{-1}+k_2)/k_1$ represents the binding constant K_B. Thus the plot of tau_{obs} versus $[R]^{-1}$ will yield $1/k_2$ and $1/K_B$ on the intercepts with ordinate and abscissa, respectively. The lag time (tau_{obs}) for each concentration of the polymerase is estimated from the plot of the product

formation vs time as a distance between the origin and the intercept of the best fit line drawn through the points of the established steady state for a given reaction. This approach was used in the present study to evaluate the K_B and k_2 for mAb 126C6-polymerase complex and lac L8UV5. Two protocols that differed in the initiation step were employed. Steady-state control values were obtained by preincubating polymerase with the promoter fragment for 15 min at 37°C to allow for the formation of the RP_o; the reaction was initiated by addition of a mixture of ApA and [^{3}H]UTP and aliquots (50 ul) were taken for paper chomatography at the times indicated. Lag time measurements in the presence and absence of mAb 126C6 were obtained by preincubating the mixture of the template and the substrates at 37°C and initiating transcription by addition of the preincubated at 37°C polymerase or mAb 126C6-polymerase complex. Aliquots (50 ul) were removed at the times indicated and the reaction products were resolved by ascending paper chromotography as described above. The conditions for the abortive initiation are described in the section on Abortive initiation assay.

Preparation of lac P+ and lac L8UV5 DNA fragments. pMB9 lac P+ and pMB9 lac L8UV5 constructed by Dr. F. Fuller were obtained from Dr. A. Revzin (Michigan State University, East Lansing). The plasmid DNA was purified on

pZ523 column obtained from 5 Prime-3 Prime, Inc. The 203 bp promoter fragments were excised with EcoRI and purified by 7.5% polyacrylamide gel electrophoresis. DNA concentrations were determined with ethidium bromide according to Le Pecq and Paoletti (1966).

Preparation of uniquely labeled lac L8UV5 and lac P+ DNA. DNA polymerase I Klenow fragment and [alpha-^{32}P]dATP were used to label the 3′ end of the non-template strands of the lac promoters. The 5′ end of the template strand was labeled with [gamma-^{32}P]ATP by T4 polynucleotide kinase following treatment with alkaline phosphatase. The reactions were terminated by addition of 0.5 M EDTA. The labeled fragments were precipitated with ethanol - 2 M ammonium acetate (3.75 : 1) and restricted with PvuII which cuts the DNA at -123 yelding uniquely labeled promoter fragments. Routinely 1 ug of the DNA was used for the end labeling. The restricted labeled lac fragments were stored in 40 ul 10 mM Tris-HCl (pH 8) and 1mM EDTA at 4^{o}C.

DNase I footprinting. Footprinting with DNase I was carried out using conditions similar to those used for the abortive transcription assay. The standard binding mixture contained (final volume 50 ul): 40 mM Tris-HCl (pH 8), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5% or 10% glycerol, 3 nM 3′ or 5′ [^{32}P]-lac fragment and where indicated 50 nM RNA

polymerase, 40 nM CRP, 0.1 mM cAMP, and the amount of monoclonal antibody indicated in the legends. The times of incubation are given in the figure legends. Digestion with 1 ul (4 ng) of DNase I was for 45 seconds at 37°C. The reaction was terminated by adddition of a solution (200 ul) containing 3 M ammonium acetate, 25 mM EDTA, and 63 ug/ml tRNA followed by phenol extraction, ethanol precipitation, and reprecipitation. After drying the pellets under vacuum, 10 ul of loading buffer containing 80% deionized formamide, 10 mM NaOH, 1mM EDTA, 0.1% bromphenol blue and 0.1% xylene cyanol was added. The samples were loaded on an 8% denaturing sequencing gel according to Maxam and Gilbert (1980). After electrophoresis the gel was autoradiographed at -70°C using Kodak XAR-5 film and a Cronex H-Plus intensifying screen.

Methylation of guanine and cytosine residues with dimethyl sulfate. Probing the accessibility of N-7 in guanine (Maxam & Gilbert, 1980; Spassky et al., 1985) and N-3 in cytosine (Kirkegaard et al., 1983) residues was carried out using conditions similar to those used for the abortive transcription assay. The standard binding mixture contained (final volume 50 ul): 40 mM Tris-HCl (pH 8), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5% or 10% glycerol, 3 nM 3′ or 5′ [^{32}P]-lac fragment and where indicated 50 nM RNA polymerase, 40 nM CRP, 0.1 mM cAMP, and the amount of

monoclonal antibody indicated in the legends. The times of incubation are given in the figure legends. Methylation of guanine residues ("G" reaction) was carried at 100 mM dimethyl sulfate for 1 min at 37°C. Methylation of cytosines ("C" reaction) with 200 mM dimethyl sulfate was for 2 minutes at 37°C. The reactions were terminated by addition of a solution (200 ul) containing 3 M ammonium acetate, 25 mM EDTA and 63 ug/ml tRNA followed by ethanol precipitation and reprecipitation. The "G" samples were redissolved in 100 ul of 1 M piperidine, incubated for 30 min at 90°C, and lyophilized. To obtain preferential cleavage at N-3 methylated cytosines the "C" samples prior to the piperidine cleavage were treated with 20 ul of cold hydrazine, 20 ul dioxane, and 10 ul of water for 7 minutes on ice. The lyophilized samples were resuspended in an appropriate amount (8-10 ul) of loading buffer containing 80% deionized formamide, 10 mM NaOH, 1mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol. The samples were loaded on an 8% denaturing sequencing gel according to Maxam and Gilbert (1980). After electrophoresis the gel was autoradiographed at -70°C using Kodak XAR-5 film and a Cronex H-Plus intensifying screen.

Results

Effect of anti-alpha mAbs on activity of RNA polymerase. Shown in Table I are properties of the monoclonal antibodies raised against purified alpha subunit used in this study. The monoclonal immunoglobulins contain G2a or G2b heavy chains and kappa light chains. Alpha specificity of the monoclonal antibodies was verified by immunoblotting using RNA polymerase holoenzyme resolved by polyacrylamide gel electrophoresis and by solid phase ELISA using purified alpha, beta, and beta' subunits (data not shown). None of the four monoclonal antibodies inhibit the $d(A\text{-}T)_n$-directed synthesis of $r(A\text{-}U)_n$. mAb 126C6 strongly inhibits cAMP-CRP-dependent initiation with lac P+ and to a lesser extent inhibits initiation with the lac UV5 promoter; mAb 129C4, mAb 124D1, and mAb 121C2 are without effect. The data indicate that the reactions involving a functional promoter are more sensitive to inhibition by mAb 126C6 than are reactions directed by $d(A\text{-}T)_n$.

Effect of anti-alpha mAbs on reconstitution of core enzyme. The lack of effect on the $d(A\text{-}T)_n$-directed synthesis of $r(A\text{-}U)_n$ was used to assay for possible effects of the anti-alpha mAbs 129C4, 124D1, and 126C6 on recovery of active polymerase following reconstitution of core enzyme from

alpha and beta + beta' subunits separated by gel filtration, or from the dissociated subunit mixture. The data presented in Fig. 1 show that mAb 124D1 and bovine IgG have no effect on reconstitution. When reconstitution is carried out in the presence of mAb 129C4 or mAb 126C6 recovery of active polymerase is inhibited. Incubation of an equimolar concentration of mAb 129C4 and alpha subunit results in complete inhibition of reconstitution. At a mAb 126C6 to alpha ratio of four to one approximately 10% of the core polymerase activity is recovered. Since mAb 129C4 is a very potent inhibitor of RNA polymerase reconstitution at a low molar ratio of mAb to alpha it was important to establish whether or not the potential ability of one mAb molecule to bind two alpha subunits was the cause of inhibition of reconstitution. To answer this question monovalent F_{ab} fragments of mAb 129C4 were prepared by proteolytic digestion of the mAb with papain (Mage, 1980) (Fig.2). It was found that when added to the subunit mixture the mAb 129C4-F_{ab} had the same strong inhibitory effect on reconstitution of core enzyme as intact mAb 129C4 (Table II).

Ishihama (1981) proposed that the assembly of the RNA polymerase core enzyme proceeds in a sequential fashion; the last step involves the temperature-dependent conversion of a premature core to the active core enzyme. The data presented in Table II compare the effects of the anti-alpha mAbs on

recovery of core enzyme activity at various stages of reconstitution. The data indicate that incubation of the subunit mixture with mAb 129C4 completely blocks reconstitution of active core enzyme. When intact mAb 129C4, or F_{ab}129C4 is incubated at 0°C with premature core only an incomplete inhibition of the conversion to the mature core occurs. The mAb 124D1 is without apparent effect. Only 22% of the reconstituted core enzyme activity was lost when mAb 124D1 was preincubated with alpha subunit prior to reconstitution. In contrast mAb 126C6 inhibits reconstitution when incubated with either the subunit mixture or the premature core. The data reflect some critical changes that involve the alpha subunits initially during assembly into premature core enzyme and subsequently during the temperature-dependent conversion of the premature core enzyme to the transcriptionally active form.

Dissociation constants of the anti-alpha mAbs for alpha subunit and RNA polymerase core and holoenzyme. The affinity of mAb 129C4, mAb 124D1, and mAb 126C6 for their respective epitopes on the free and RNA polymerase-associated alpha subunit was determined using the indirect ELISA assay (Table III, Fig.3) devised by Friguet et. al. (1985) or by direct binding with ^{125}I-labeled mAbs to an antigen in a double antibody sandwich assay (Table III, Fig. 4, and 5) developed by Pestka et al. (1983). In these assays both an antigen and

a mAb are in their native conformation and the K_d values obtained reflect the true affinity between a mAb and its epitope. The data for mAb 129C4 and mAb 124D1 indicate that the affinity of these antibodies for their respective epitopes in free alpha is similar to or slightly lower than that for polymerase-associated alpha. In contrast the data obtained with mAb 126C6 indicate that this antibody shows a higher affinity for alpha when it is part of polymerase core enzyme and an even greater affinity when alpha is a constituent of polymerase holoenzyme (Fig. 3). Similar results for mAb 126C6 were obtained in a competition assay with ^{125}I-labeled alpha (DeFalco et al. (1983). The results indicate that conformational changes in alpha resulting in an increased affinity for mAb 126C6 occur during the course of RNA polymerase assembly. Binding of mAb 126C6 to a partially oxidized alpha subunit (alpha was dialyzed against PBS buffer and stored without addition of dithiothreitol or mercaptoethanol) is remarkably decreased. The same preparation of the alpha stored under reducing conditions showed about 25 times greater affinity for mAb 126C6 (Table III). The data imply that SH-groups of the alpha subunit are reduced when alpha is a part of RNA polymerase. Whether the alpha subunit was reduced or partially oxidized the affinities of mAbs 129C4 and 124D1 for their epitopes were not affected.

Effect of the modification of SH-groups of alpha subunit on the reconstitution of RNA polymerase core enzyme. To investigate the state of oxidation and the role of sulfhydryl groups of the alpha subunit in maintaining the active conformation of the E. coli RNA polymerase the alpha subunit was modified with [^{3}H]N-ethylmaleimide and used in reconstituting core enzyme. The results of these experiments demonstrate (Table IV) that the transcriptional activity of the reconstituted core in which all four SH-groups of alpha are modified is not significantly affected. The data show that there are no disulfide bridges within the alpha subunits of the E. coli RNA polymerase.

Binding of ^{125}I-anti-alpha mAbs to RNA polymerase core and holoenzyme denatured with urea.

The data presented in Table V indicate that the anti-alpha monoclonal antibodies bind to their respective epitopes in free alpha and RNA polymerase immobilized on the surface of polystyrene microtiter wells. Inherent in such solid phase assays are the difficulties in determining the amount of the antigenic protein adsorbed to the polystyrene surface and knowing to what extent adsorption to the polystyrene affects the native conformation of the proteins (Friguet, Djavadi-Ohaniance and Goldberg, 1984). Both of the mAbs shown to be noninhibitors of RNA polymerase activity show relatively greater binding to the free alpha subunit compared to alpha

assembled in RNA polymerase core or holoenzyme. In contrast the inhibitory mAb 126C6 binds equally well to free alpha and to alpha present in RNA polymerase. Incubation of the immobilized core enzyme with 4 M urea results in an approximately two-fold increase in the amount of [^{125}I]mAb 124D1 and [^{125}I]mAb 129C4 bound. This is not due to a possible effect on the conformation of alpha since the prior incubation of the immobilized alpha with 4 M urea does not increase binding by [^{125}I]mAb 124D1 and [^{125}I]mAb 129C4. mAb 126C6 behaves differently from the other two mAbs in that urea treatment of immobilized alpha increases binding by about 25% while urea treatment of immobilized core (or holoenzyme) decreases binding of [^{125}I]mAb 126C6 by about 50%. The data suggest that particular regions of alpha may be conformationally altered and/or sterically blocked when alpha is assembled in RNA polymerase.

Double antibody binding studies of the topological arrangements of epitopes for mAb 129C4, mAb 124D1, and mAb 126C6 on the alpha subunit, $alpha_2$beta subassembly, and in RNA polymerase core and holoenzyme.

Depending on the topological arrangement of the two alpha subunits within RNA polymerase the accessibility of a particular epitope for binding by a particular monoclonal antibody may be 0, 1, or 2. To determine the accessibility of a pair of the individual epitopes with respect to each

other a solid phase double antibody (sandwich) binding assay developed by Pestka (Pestka et al. 1983) was used. In this assay an unlabeled mAb is adsorbed onto the surface of the wells of microtiter polystyrene plates; subsequently an antigen is incubated with the immobilized mAb and finally the immune complexes which have formed are tested for binding to the identical or heterologous ^{125}Iodine-labeled mAb. In our experiments all ^{125}I-labeled mAbs were used at their saturating concentrations to assure the most complete occupancy of the accessible epitopes. The data presented in Table VI show that when the alpha subunit is used there is an obvious difference between homologous and heterologous [^{125}I]monoclonal antibody binding. The data is consistent with the predominant species being the alpha monomer since the homologous pairs of the immobilized mAb and soluble [^{125}I]mAb show only low alpha-binding activity. The lack of interference for the binding of the labeled antibody to alpha bound to a heterologous antibody indicates that each monoclonal antibody binds to an independent antigenic domain on the alpha subunit.

The results of the binding of [^{125}I]-anti-alpha mAbs to $alpha_2beta$, core and holoenzyme bound to immobilized anti-alpha, and anti-beta mAbs presented in Tables VII and VIIa. When core or holoenzyme were used as soluble antigens in an anti-alpha homologous system a strong interaction with [^{125}I]mAb 126C6 indicated that the mAb specific epitope is

available on each of alpha subunits in RNA polymerase. Conversely, no binding above a threshhold level was observed with ^{125}I-labeled mAb 129C4, mAb 124D1 or mAb 121C2 in the respective homologous systems. The data imply that the epitopes for mAb 129C4, mAb 124D1, and mAb 121C2 are available only on one of the alpha subunits in the enzyme.

When an $alpha_2beta$ preparation (obtained according to Lowe and Malcolm, 1976) was assayed with each of the homologous anti-alpha antibody pairs the binding of all four [^{125}I]mAbs was very low. Only [^{125}I]mAb 126C6 is able to bind to the immune complex formed between anti-beta mab 210E8 (the inhibitory mAb characterized by Rockwell et al. 1985, 1988) and $alpha_2beta$ preparation while no binding is observed in the homologous anti-alpha system. This suggests that a stable alpha-beta subassembly but not $alpha_2beta$ is present. The binding of [^{125}I]mAb 126C6 to core or holoenzyme actually increased about two fold with respect to $alpha_2beta$ preparation when the antigens were complexed with anti-beta mAb 210E8, providing further evidence for an alpha-beta rather than $alpha_2beta$ association. Considering the reports on the existence of an $alpha_2beta$ subassembly (Ishihama & Ito, 1972; Palm et al., 1975; Lowe & Malcolm, 1976) and given that the epitopes for mAb 126C6 are available on each alpha subunit in polymerase attempts were made to stabilize $alpha_2beta$ subassembly by addition of either rifampicin or an excess of alpha, or both. This had no effect on the

binding in homologous anti-alpha double antibody system and resulted only in a slight increase in the binding of [^{125}I]mAb 126C6 to alpha-beta complexed with immobilized anti-beta mAb 210E8. Similar results were obtained with another anti-beta antibody, 221C7 (data not shown). It would appear that in the $alpha_2$beta subassembly one of the alpha subunits is weakly bound and may have dissociated from the subassembly following binding of the monoclonal antibody.

Core or holoenzyme bound to the plate-fixed mAb 126C6 does not permit the binding of either ^{125}I-mAb 129C4 or ^{125}I-mAb 124D1 to its epitope on the available alpha subunit (Table VII). It is possible that polymerase bound to the plate-immobilized mAb 126C6 does not allow access of the soluble mAb 129C4 or mAb 124D1 to its site due to the proximity of the polystyrene surface. Binding of [^{125}I]mAb 126C6 to the plate-fixed polymerase-mAb 129C4 (or mAb 124D1) complex is not inhibited. The data suggest that the two available epitopes for mAb 126C6 have an unequal exposure in RNA polymerase. Such an arrangement of each of the mAb epitopes would account for the fast and preferential binding of the plate-immobilized mAb 126C6 to its more exposed epitope in the enzyme and explain the subsequent interference of the plate surface for the binding of ^{125}I-labeled mAb 129C4 and mAb 124D1 to the core- (or holoenzyme)-mAb 126C6 complex.

To ascertain this possibility S. aureus cells, that allow a greater degree of spatial freedom, were used in place of

polystyrene plates. The results (Table VIII) show that the binding of [^{125}I]mAb 129C4-core (or holoenzyme) complex to the mAb 126C6 adsorbed to S. aureus cells was not inhibited. Binding of [^{125}I]mAb 129C4-core enzyme to the Protein A bound mAb 210E8 although is low but evident.

Binding of [^{125}I]mAb 124D1, and [^{125}I]mAb 129C4 to alpha$_2$beta, core and holoenzyme complexed with the plate-fixed anti-beta mAb 210E8 is either very low or does not occur (Table VII). Binding of anti-beta [^{125}I]mAb 210E8 to alpha$_2$beta, core and holoenzyme complexed with a plate-immobilized mAb (Table IX) indicates that the epitopes for mAb 129C4 and mAb 124D1 are not blocked in the alpha-beta subassembly since binding of mAb 210E8 does occur. This suggests that steric factors may account for the low simultaneous binding of these mAbs to their respective epitopes in the alpha-beta subassembly and polymerase. The binding pattern of the inhibitory anti-beta' [^{125}I]mAb 311G2 to RNA-polymerase complexed with the plate-fixed anti-alpha mAbs is very similar to the binding pattern of anti-beta [^{125}I]mAb 210E8 (Table IX) suggesting that the epitopes for anti-alpha mAb 129C4 and mAb 124D1 may be located in the vicinity of the anti-beta and anti-beta' epitopes. Simultaneous binding of anti-beta mAb 210E8 and anti-beta' mAb 311G2 to polymerase core and holoenzyme is not inhibited (Table IX).

In anti-alpha double antibody assays the binding of

[^{125}I]mAbs to the core enzyme (where it occurred) was 2 to 3 times greater than in the corresponding assays with holoenzyme (Table VII). The data suggest that in the holoenzyme the sigma subunit may be positioned close to one of the two alpha subunits. The data are consistent with the studies of Hillel and Wu (1977) and Tichelaar et al. (1983) who showed that in the RNA polymerase holoenzyme at least one of the alpha subunits is adjacent to sigma.

Effect of anti-alpha mAb 126C6 on interaction of RNA polymerase with lac P+ and lac UV5 promoters.

Kinetic analysis of the steady-state synthesis of ApApUpU (with ApA as the varied substrate) by mAb 126C6-polymerase from lac UV5 promoter indicates that the K_m and V_{max} are not significantly affected by the binding of mAb 126C6 to RNA polymerase (Fig.6). The slight inhibition observed is non-competitive (Fig.6). Formation of the open promoter complex (RP_o) is the rate-determining step for transcription initiation. Kinetic analysis of abortive initiation as devised by McClure (1980) allows for an evaluation of the binding and isomerization constants during the formation of the open promoter complex. This procedure was used to study the effect of mAb 126C6 on the formation of the RP_o with the linear lac L8UV5 fragment. The results presented in Fig.7 indicate that an extended lag time was required before a steady-state rate of UMP incorporation was achieved by the

mAb 126C6-RNA polymerase-lac L8UV5 complex. A plot of tau_{obs} (the time required for RP_o formation) versus the reciprocal of the RNA polymerase concentration yields the binding constant (K_B) and isomerization constant (k_2). A tau plot showing the effects of mAb 126C6 on the rate of RP_o formation is presented in Fig.8. Both binding and isomerization are affected. The data indicate that the rate of isomerization (k_2) is more sensitive to mAb 126C6 than the initial rate of binding (K_B) of RNA polymerase to the promoter.

To determine more specifically how anti-alpha mAb 126C6 effects polymerase interaction with lac fragments the binary complexes (mAb-polymerase-lac P+ or lac UV5) were probed for susceptibility of the sugar-phosphate backbone to the pancreatic DNase I attack and the reactivity of N-7 groups of guanines and N-3 groups of cytosines to the alkylating reagent dimethyl sulfate.

Specific interaction of RNA polymerase with the lac P+ promoter is cAMP-CRP-dependent. DNase I footprinting shows that the productive binding of RNA polymerase to lac P+ is strongly affected by mAb 126C6 (Fig.9, lane h; Fig.10, lane h). This observation explains the inhibitory effect of mAb 126C6 on abortive initiation from lac P+ promoter. When lac P+ is incubated with the preformed polymerase-mAb 126C6 complex (Fig.9, lane h; Fig.10, lane h) the protection of sugar-phosphate backbone by the enzyme on both template and

non-template strands of the promoter is not seen. Also, there is no enhancement at positions -23 bp and -24 bp (non-template strand) and -25 bp and -38 bp (template strand). In addition, in the CRP-binding domain of the lac P+ promoter protection at -57 and -58 bp is somewhat lower. The data suggest that the preformed mAb 126C6-polymerase complex does not bind to the lac P+ fragment presumably due to an inhibitory effect of the mAb on the interaction of RNA polymerase with cAMP-CRP-lac P+. Preincubation of the open promoter complex (cAMP-CRP-lac P+-polymerase) with mAb 126C6 resulted in an almost complete inhibition of abortive initiation (Table I). The time course of binding of mAb 126C6 to the lac P+ open promoter complex (Fig.9 and Fig.10, lanes e,f,g) shows an increase in susceptibility to DNase I attack after 10 minutes of incubation of RP_o with the mAb. However, the enhancement at -23 bp, and -24 bp on the non-template strand persists even after 40 minutes of incubation of the RP_o with mAb 126C6 implying that the enzyme had not dissociated from the DNA. In contrast, on the template strand the enhancement at -38 bp is not seen after 10 minutes of incubation and the intensity at -25 bp gradually declines till its complete loss. The data suggest that the interaction of RNA polymerase with lac P+ is asymetric, the enzyme appears to associate more strongly with the non-template strand. This is supported by the greater protection of the non-template strand by polymerase against DNase I

attack in the absence of mAb 126C6. Effects of the anti-alpha mAb 126C6 are also seen in the CRP-binding domain of the lac promoter. The enhancement at -52 and -54 bp is reduced to that seen with cAMP-CRP alone (Fig.10, lanes e,f,g) and protection at -57 and -58 bp is decreased. Since both polymerase and cAMP-CRP remain bound to the promoter the data imply that the interactions between the polymerase and CRP are affected by the bound mAb 126C6.

RNA polymerase interaction with lac L8UV5 is cAMP-CRP independent. Because of the L8 mutation in the CRP binding site, interaction of cAMP-CRP with lac L8UV5 is RNA polymerase-dependent. Fig.11 and 12 illustrate the effect of mAb 126C6 on protection of lac L8UV5 by polymerase from DNase I attack. As with the lac P+ promoter, protection of the non-template strand of lac L8UV5 (Fig. 11) appears to be more effective. The footprints show that the enhancement at -45 bp and -47 bp (non-template strand) (Fig. 11) and at -38 bp and -49 bp (template strand) (Fig. 12, and 13a) is lost upon interaction of the lac fragment either with the preformed mAb 126C6-polymerase complex or following incubation of the open promoter complex with mAb 126C6. The data show that mAb 126C6 lowers the overall interaction of polymerase with the lac L8UV5 fragment and has a somewhat stronger effect on the upstream region of the promoter. These distortions apparently do not significantly interfere with the rate of transcription after the RP_o is formed

(compare K_m and V_{max} values for polymerase and mAb 126C6-polymerase Fig. 6). When the open promoter complex is formed with cAMP-CRP the pattern of cAMP-CRP binding to its site on the non-template strand is comparable to that on the lac P+ fragment as judged by the protection against DNase I attack. In the polymerase-binding domain the phosphodiester bonds at -45 and -47 bp on the non-template strand are also protected (Fig.13, lane b). The polymerase-dependent cAMP-CRP protection of the template strand of lac L8UV5 is less evident (Fig. 13a, lane f). Nevertheless the effect of cAMP-CRP on polymerase interaction with the template strand of lac L8UV5 is seen as a partial suppression of the enhancement at -38 and -49 bp (Fig. 13a, lanes f, h, i). Interaction of the open promoter complex (cAMP-CRP-lac L8UV5-polymerase) with mAb 126C6 results in dissociation of cAMP-CRP from its site with a concomitant loss of both the protection and the enhancement in those regions of the promoter that are effected by the binding of the mAb 126C6 in the absence of cAMP-CRP (Fig. 13 and 13a). The enhancements at -23 bp, and -24 bp on the non-template strand and at -25 bp on the template strand are not significantly affected by mAb 126C6. Similar results are obtained with F_{ab} fragments of mAb 126C6 (Fig.13, lanes f, g; Fig. 13a, lane j). The data indicate that mAb 126C6 lowers RNA polymerase protection of the lac promoter upstream from -41 bp on the non-template strand and

abolishes the enhancement at -38 and -49 bp on the template strand. At the same time it affects cAMP-CRP-DNA contact by presumably interfering with CRP-polymerase interaction. Alternatively, dissociation of the cAMP-CRP may be a consequence of the distortion by the mAb 126C6 of interaction of polymerase with the upstream promoter region adjacent to the CRP binding site.

The time course of incubation of the mAb 126C6-polymerase complex with lac L8UV5 followed by DNase I treatment showed that the enhancement at -25 bp (template strand) develops gradually in contrast with the fully expressed enhancement of this band after a short (10 seconds or 1 minute) incubation of polymerase or the polymerase-mAb 129C4 complex with lac L8UV5 (Fig.12, lanes b-e). The enhancement at -38 bp and -49 bp was not seen even after 60 minutes of incubation of mAb 126C6-polymerase complex with the promoter (Fig. 12, lane e). The data are consonant with the extended lag time shown for formation of the open promoter complex with polymerase-mAb 126C6 and with the strong effect of the mAb on the isomerization constant (k_2).

mAb 129C4 and mAb 124D1 can bind simultaneously to RNA polymerase (Tables VII and VIII). The binding is efficient with K_d values of 2.77 nM and 3.85 nM for mAb 129C4 and mAb 124D1, respectively (Table III). The DNase I footprint shows (Fig. 13, lane c) that cAMP-CRP remains bound to its site on the lac L8UV5 promoter in the presence of the mAb 129C4-RNP-

mAb 124D1 complex.

mAb 126C6-RNA polymerase protection of the N-7 position of guanine of lac L8UV5 promoter was probed with dimethyl sulfate as described by Spassky et al. (1985). The guanine protection pattern by polymerase correlated well with the pattern previously described (Johnsrad, 1978; Siebenlist et al., 1980; Spassky et al., 1985). Fig. 14 A, lane c shows that, like polymerase alone, mAb 126C6-polymerase protects guanine -24 on the non-template strand and facilitates reactivity of the -14 guanine on the template strand (Fig. 14 B, lane c). However, in contrast to polymerase alone the mAb 126C6-polymerase complex does not protect guanines in position -6, and -13 on the non-template strand and -32 on the template strand (Fig.14). Guanine in the -17 position (non-template strand) is not enhanced by mAb 126C6-RNP. Incubation of mAb 126C6 with the RP_o followed by treatment with dimethyl sulfate had a greater effect on guanine -32 contact with polymerase than on contacts in the downstream region. The overall protection pattern of guanines of the non-template strand by mAb 129C4-polymerase is similar to the protection displayed by polymerase (Fig.14 A, lanes b, and e). However, subtle differences can be seen: the relative intensity of the guanines at +9 and +11 bp (non-template strand) is higher and closer to the intensity displayed with mAb 126C6-polymerase than with polymerase alone.

Methylation of N-3 of cytosine and N-1 of adenine by dimethyl sulfate can occur if these groups are not hydrogen bonded in a Watson-Crick double helix. The observation that N-3 methylcytosine is more reactive to hydrazine than cytosine (Peattie and Gilbert, 1980) was employed by Kirkegaard et al.(1983) to map cytosines in the single-stranded regions of RNA polymerase-promoter complexes. Cytosine residues of the template strand at positions -1, -2, -4, and -6 were found to be in an unpaired region. The method of Kirkegaard et al. (1983) was applied to determine the effect of mAb 126C6 on the rate of accessibility of the N-3 position of cytosines during incubation of the mAb 126C6-polymerase complex with lac L8UV5. The data show that methylation of N-3 in cytosines near the transcripton start site occurs after 1 minute of incubation the of mAb 126C6-polymerase with lac L8UV5 (Fig. 15).

The data presented in Fig.16 compare the accessibility of the cytosines in cAMP-CRP-lac P+-RNP preincubated with mAb 126C6 and in cAMP-CRP-lac P+ preincubated with the mAb 126C6-polymerase complex. The data show that even after 40 minutes of incubation of the RP_o with mAb 126C6 the cytosines near the start site of transcription remain accessible to dimethyl sulfate (Fig. 16, lanes b, c, d). mAb 126C6-polymerase inhibits the formation of the open complex (Fig.16, lane e). The latter finding correlates with the lack of initiation from lac P+ by mAb 126C6-polymerase. The

former results indicate that although the strands of the DNA near the transcription start site remain separated, this is not sufficient for initiation of transcription (Table I).

The effect of mAb 126C6 on protection of lac L8UV5 against DNase I attack during abortive transcription is shown in Fig. 17. The footprints demonstrate the shift in the enhancement at -23 and -24 bp that occurs during transcription and also indicate that mAb 126C6 remains bound to polymerase after 30 minutes of abortive transcription.

Discussion

Reconstitution of the E. coli RNA polymerase core enzyme from dissociated subunit mixtures as well as from the isolated individual subunits has been extensively studied (Ishihama & Ito, 1972; Ito & Ishihama, 1973; Fukuda & Ishihama, 1974; Yarbrough & Hurwitz, 1974; Palm et al., 1975; Harding & Beychock, 1976; Saitoh & Ishihama, 1976; Ishihama, 1981). The sequence proposed for assembly of RNA polymerase core enzyme (Ishihama, 1981) is:

$$a + a \rightarrow a_2 + B \rightarrow a_2B + B' \rightarrow a_2BB'$$

Reassembly at 4°C results in an inactive premature core enzyme; conversion of the premature core to the catalytically active core requires incubation at 30-37°C. The physical and enzymatic properties of the active form of the reconstituted enzyme closely resemble those of the native RNA polymerase (Harding & Beychock, 1976; Ishihama et al., 1979). Analysis of the absorbance spectra, far- and near-UV CD spectra and tritium exchange rate of the premature and reconstituted active polymerase indicates that although most of the secondary structure of the premature core is similar to that of the active enzyme there are minor but significant differences. Studies using sensitivity to

trypsin digestion, crosslinking with bifunctional reagents, sedimentation velocity and elution profiles from phosphocellulose and DEAE-Sephadex indicate that the subunits in the premature core are weakly associated (Ishihama et al., 1979). The results of glycerol gradient centrifugation suggested that the premature core is in rapid equilibrium with the $alpha_2$beta subassembly and beta' subunit.

Only one of the alpha subunits of RNA polymerase is bound by mAb 124D1. The affinity of mAb 124D1 for its epitope on free alpha and polymerase-associated alpha is similar. While these properties are also seen for mAb 129C4, incubation of polymerase subunits with mAb 124D1 does not inhibit reconstitution. This lack of inhibition may result from a conformational change in one of the alpha subunits during the reassembly process. The proposed conformational change in the affected alpha would take place during assembly of the premature core enzyme since incubation of the alpha subunits with mAb 124D1 prior to addition of beta and beta' results in 78% recovery of polymerase activity (Table II). The antibody binding data indicate that the minimal stable complex is alpha-beta and that reassembly of core enzyme in the presence of mAb 124D1 may proceed as follows:

2[mAb 124D1-a] + B -> mAb 124D1-aB + mAb 124D1-a;

mAb 124D1-aB + mAb 124D1-a + B'-> mAb 124D1-aaBB'+ mAb 124D1.

Concerted interactions between mAb 124D1-alpha, mAb 124D1-alpha-beta and beta' may cause the dissociation of mAb 124D1 from one of the alpha subunits. Subsequently the alpha domain in which the epitope for mAb 124D1 resides may be blocked by interaction with the other polymerase subunits. The inferred conformational change must take place in the affected domain of only one of the polymerase alpha subunits since the affinity of mAb 124D1 for free alpha and the available alpha subunit in the core enzyme is similar. The data also indicate that the in vitro assembly of functional RNA polymerase core enzyme can take place without the involvement of the alpha dimer intermediate postulated by Saitoh and Ishihama (1976) and may proceed as follows:

$$a + B \rightarrow aB + a \rightarrow aaB + B' \rightarrow aaBB'.$$

The postulated by Saitoh and Ishihama (1976) pathway of core enzyme subunit assembly was questioned by Coggins et al. (1977) who studied RNA polymerase subunit proximity by chemical cross-linking; the major products were beta-beta', alpha-beta, and alpha-beta', no alpha-alpha species were found. Hillel and Wu (1977) in addition to the crosslinked subunits shown by Coggins et al. (1977) found a small amount of crosslinked alpha dimer. Incubation of core enzyme with 2 M urea resulted in the release of alpha-beta and alpha-

beta' complexes (Ishihama, 1972). It is possible that one of the alpha subunits interacts more strongly with the beta' subunit than with the alpha-beta subassembly within the polymerase. Nevertheless, the alpha-beta' subassembly appears to be quite unstable because no functional alpha-beta' intermediate was observed upon sequential subunit addition during reconstitution (Palm et al., 1975), and the subunits did not co-sediment during the glycerol gradient centrifugation (Ito & Ishihama, 1973).

Coggins et al. (1977) proposed a model of the core enzyme in which alpha subunits do not form a dimer, but rather are symmetrically positioned on the outer sides of the beta and beta' subunits. Hillel and Wu (1977) suggested that the alpha subunits may lie in an adjacent position in polymerase. Using the monoclonal antibodies we were unable to demonstrate the presence of a stable $alpha_2$beta; the antibody binding data clearly indicate the existence of an alpha-beta complex. However, a destabilizing effect of the monoclonal antibodies on subunit interaction cannot be ruled out. It is possible that in the $alpha_2$beta subassembly one of the alpha subunits is weakly associated with the more stable alpha-beta complex and is released on binding of the monoclonal antibody. Efficient binding of the anti-alpha mAb 129C4 (or mAb 124D1) to $alpha_2$beta complexed with anti-beta mAb 210E8 could not be demonstrated. It does not appear that in $alpha_2$beta complexed with the immobilized mAb 210E8 the

epitope for mAb 129C4 (or mAb 124D1) on one of the alpha subunits is already blocked and the binding of the mAb to its epitope on the second alpha subunit is sterically hindered. That this is not the case becomes evident when the binding of [^{125}I]mAb 126C6 to alpha-beta complexed with the anti-beta mAb 210E8 is compared with the binding of [^{125}I]mAb 129C4 or [^{125}I]mAb 124D1 to alpha$_2$beta in the heterologous anti-alpha assays. If a stable alpha$_2$beta was present in which one epitope for mAb 129C4 (or mAb 124D1) is blocked then the magnitude of [^{125}I]mAb 129C4 (or mAb 124D1) binding to the subassembly in the heterologous anti-alpha assays should be similar to the binding of the mAb 126C6 to alpha-beta complexed with the anti-beta mAb 210E8. The binding of mAb 129C4 (or mAb 124D1) to alpha$_2$beta in the heterologous anti-alpha assays is about 2 times greater than the binding of mAb 126C6 to the alpha-beta-mAb 210E8 complex. The data indicate that alpha is released from the alpha$_2$beta subassembly upon binding of the complex to the plate-fixed anti-alpha mAb. That the epitopes for mAbs 129C4 and 124D1 are not blocked in alpha-beta is also demonstrated by the binding of the anti-beta [^{125}I]mAb 210E8 to the alpha-beta complexed with plate-immobilized mAb 129C4 or mAb 124D1.

Simultaneous binding of anti-beta′ mAb 311G2 and anti-alpha mAb 129C4 (or mAb 124D1) to polymerase does not occur. The binding of anti-beta mAb 210E8 to polymerase complexed

with the anti-alpha mAbs is also inhibited. However, simultaneous binding of mAb 311G2 and mAb 210E8 to polymerase core or holoenzyme is not inhibited. The data suggest that on the surface of the E. coli RNA polymerase the available epitopes for anti-alpha mAb 129C4 and mAb 124D1 may be located in the vicinity of epitopes for anti-beta mAb 210E8 and anti-beta' mAb 311G2. Since relative orientation of the bound mAbs with respect to each other is not known it is impossible to say whether the epitopes are located close to each other or apart. A steric hindrance originating from the adjacent or even overlapping location of the epitopes is possible considering that actual antigen-antibody combining region may represent only 30% of the total surface area of the complimentarity determining region(s) in antibody (Smith-Gill et al., 1987; Davis et al., 1988). The maximum possible distance between anti-alpha and anti-beta (or anti-beta') epitopes could be within the range of the length of F_{ab} or F_c arms of a mAb.

Incubation of mAb 129C4 with the subunit mixture results in an almost complete inhibition of reconstitution. Incubation of the premature core enzyme with mAb 129C4 results in only a partial (20 to 40%) inhibition of the temperature-dependent conversion to the active enzyme core enzyme. A model in which $alpha_2$beta and beta' are in rapid equilibrium with the premature core (Ishihama et al., 1979) is not consonant with the data obtained using mAb 129C4. If

$alpha_2$beta was formed it would be bound by mAb 129C4 with its consequent dissociation into mAb 129C4-alpha and mAb 129C4-alpha-beta resulting in an inhibition of polymerase assembly. It is possible that premature core is composed of a mixture of intermediates of varying stability to dissociation into subassemblies. Alternatively during reconstitution one of the alpha subunits may undergo conformational changes that result in only partial accessibility of the mAb 129C4 epitope. This is suggested by the increased concentration of mAb 129C4 and the prolonged time of incubation with mAb 129C4 required to show the partial inhibition of conversion of the premature core enzyme to the active core enzyme. During the course of the transition to the active enzyme the epitope for mAb 129C4 present on one of the alpha subunits becomes totally inaccessible. This may be due to conformational changes induced in the alpha subunit and/or steric effects resulting from interactions with the other subunits in the core enzyme. The affinity of mAb 129C4 for free alpha and the available alpha present in RNA polymerase is similar suggesting that the available mAb 129C4 epitope in polymerase has the same conformation as that present in the free alpha subunit.

Inhibition of $d(A-T)_n$-directed activity is not observed when mAb 126C6 is incubated with the reconstituted mature core enzyme. However, about 90% of the recoverable enzyme

activity is lost when mAb 126C6 is incubated with either the subunit mixture or premature core enzyme. In native RNA polymerase the mAb 126C6 epitope is available on each of the alpha subunits. Inhibition of the maturation of the premature core enzyme by mAb 126C6 may be due to antibody-induced conformational changes in alpha. If mAb 126C6 induces strong conformational changes in the native enzyme it may have a stronger affect on the overall structure of the premature core enzyme and may possibly cause subunit dissociation. It appears that in the premature core enzyme the alpha subunit(s) is less conformationally stabilized by interactions with neighboring subunits than in mature or native core enzyme. If this is the case then the distortions induced by binding of mAb 126C6 to the alpha subunit(s) in the premature core enzyme can not be counteracted by the stabilization resulting from the temperature-dependent transition to the mature form. The observed increase in the affinity of mAb 126C6 for alpha present in RNA polymerase also implies that the alpha domain in which the epitope for mAb 126C6 resides undergoes conformational changes during enzyme assembly.

It would appear that there are at least local differences in the conformation of each of the polymerase-associated alpha subunits. This is clearly seen in the increased affinity of mAb 126C6 for alpha present in polymerase. The relative affinity of mAb 126C6 for alpha is: $a_2BB's > a_2BB'$

> a and its epitope is exposed on both of the polymerase alpha subunits. In contrast the domains in which the epitopes for mAb 129C4 and mAb 124D1 reside are exposed on only one of polymerase alpha subunits. A small-angle X-ray study suggested that the a_2 complex may have an approximately symmetrical shape (Meisenberger et al., 1980a). The authors also concluded that there are only slight structural differences between the alpha dimer in the isolated state or when incorporated into polymerase. However there is evidence indicating that the alpha subunits in RNA polymerase are not equivalent. Following infection of E. coli with phage T4, initially only one of the alpha subunits is ADP-ribosylated (Rohrer et al., 1975) indicating that the alpha subunits are differentially arranged in RNA polymerase. Treatment of RNA polymerase with 0.1 mM para-chloromercuribenzoate results in the release of one of the alpha subunits (Ishihama, 1972; Ishihama, 1981).

The unequal exposure of the two alpha subunits in RNA polymerase is also indicated by the data obtained with the monoclonal antibodies. It would appear that one of the alpha subunits is relatively exposed while the other alpha is positioned in such a way that much of its surface is shielded from the environment by the much larger beta and beta' subunits. This is consistent with the observations of Stender (1980) regarding the low accessibility of core enzyme to polyclonal anti-alpha antibodies and Tichelaar et

al. (1983) who used immunoelectron microscopy to show that almost half of the alpha surface is covered by beta and beta'. The proposal that the alpha subunits differ in their exposure may also explain the low extent of binding of mAb 129C4 and mAb 124D1 to core or holoenzyme bound to mAb 126C6 adsorbed to the polystyrene surface (Table VII). The alpha subunit which is more shielded by the beta and beta' subunits may bind poorly or not at all to the plate-fixed mAb 126C6. Binding of polymerase to the plate-fixed mAb 126C6 would presumably occur through its epitope on the more exposed alpha subunit. It is assumed that the available epitopes for mAb 129C4 and mAb 124D1 are also located on the alpha subunit bound by immobilized mAb 126C6. The impaired binding of mAb 129C4 and mAb 124D1 to the mAb 126C6-polymerase complex may be a consequence of a steric hindrance resulting from the particular orientation of the enzyme with respect to the plate surface. When Protein-A containing S. aureus cells are used in place of polystyrene plates the formation of a mAb 126C6-polymerase-mAb 129C4 complex is seen.

The model of RNA polymerase in which one of the alpha subunits is shielded from the environment by beta and beta' subunits can also explain the two-fold decrease in the binding of mAb 126C6 to the urea-treated plate-immobilized core or holoenzyme. If the proposed subunit arrangement is correct then upon random binding of polymerase to the plate

during the coating process only one of the two alpha subunits will bind to the plate surface. The unadsorbed alpha subunit is therefore sandwiched between beta and beta'; while most of its surface is sterically blocked it still has a favorable conformation for the binding of mAb 126C6. Treatment of the immobilized enzyme with urea weakens the interactions between the subunits of the RNA polymerase. The subsequent binding of mAb 126C6 to its epitope on the shielded alpha appears to further distort the weakened interactions between beta-alpha-beta'. The shielded alpha apparently dissociates and is removed during the washing procedure. The dissociating effect of mAb 129C4 and mAb 124D1 is observed only after treatment of polymerase with 6 M urea.

Results obtained using small angle neutron scattering (Stockel et al., 1980a,b) or circular dichroism (Levine et al., 1980) did not reveal any major differences between the core enzyme and the core component of the holoenzyme. Chemical cross-linking of the holoenzyme indicated that sigma interacts with beta and beta' and at least one of the alpha subunits (Hillel and Wu, 1977). Only minimal differences in the profile of core and holoenzyme were discerned by electron microscopy (Tichelaar et al., 1983). Analysis of the tryptic cleavage of RNA polymerase provided evidence for association between beta and sigma subunits (Fisher and Blumenthal, 1980). Based on studies with

subunit-specific polyclonal antibodies Tichelaar et al. (1983) proposed that the sigma subunit resides at the concave side of the core where it also interacts with the alpha dimer. The steric effect of the sigma subunit was observed in the double antibody assays when the binding of identical anti-alpha antibodies to core and holoenzyme was compared. The reduced binding of the anti-alpha monoclonal antibodies to the holoenzyme may be attributable to a combination of a steric effect of the sigma subunit and a particular orientation of the antibody-bound polymerase with respect to the polystyrene plate surface. This is consistent with the data indicating that there is no significant difference in the affinity of mAb 129C4 and mAb 124D1 for core and holoenzyme and that the affinity of mAb 126C6 for holoenzyme is greater than for core enzyme. The steric effect of the sigma subunit is only seen in the double anti-alpha antibody assays. This indicates that the sigma subunit does not impede binding of mAb 126C6 to its epitope on either of the alpha subunits when there is no steric hindrance imposed by the plate surface. The data are consistent with the studies of Hillel and Wu (1977) and Tichelaar et al. (1983) which indicated that at least one of the alpha subunits in the holoenzyme is located close to sigma.

An indication of a role of alpha subunit in specific

promoter recognition was derived from the studies of T4-modified E. coli RNA polymerase in which the alpha subunit is ADP-ribosylated. In the cell-free system the T4-modified enzyme does not support expression from a number of E. coli genes. Expression of T4 genes is not affected (Mailhammer et al. 1975) implicating alpha in some step in promoter interaction. There is also evidence showing a reduced affinity of ADP-ribosylated core enzyme for the sigma subunit (Walter, Seifert & Zillig, 1968; Seifert et al., 1969) suggesting that altered interactions of core with sigma could also contribute to the effect on promoter selectivity.

In the present study the effects of the anti-alpha monoclonal antibody, mAb 126C6, on formation of the open promoter complex have been characterized. This mAb completely inhibits abortive initiation from CRP-dependent lac P+, partially inhibits abortive initiation from lac UV5 and is without apparent affect on the $d(A-T)_n$-directed synthesis of $r(U-A)_n$.

Kinetic analysis of open complex formation of mAb 126C6-polymerase with lac L8UV5 showed that the observed inhibition by the mAb is a function of effects on the binding and isomerization steps with the isomerization step affected to a greater extent.

A current multistep model of RNA polymerase transcription initiation (Buc & McClure, 1985; Spassky et al.,1985;

Straney & Crothers, 1987)

$$R + P \Leftrightarrow RP_c \rightarrow RP_i \Leftrightarrow RP_o + NTP's \rightarrow \rightarrow ... \rightarrow RNA$$

binding isomerization

has evolved from the two-step model for open promoter complex formation originally proposed by Walter and Zillig (Walter et al., 1967) and extended by Chamberlin (1974):

$$R + P \overset{K_B}{\Longleftrightarrow} RP_c \overset{k_2}{\dashrightarrow} RP_o + NTP's \rightarrow \rightarrow ... \rightarrow RNA$$

Spassky et al. (1985) correlated the conversion of a closed promoter complex (RP_c) to an intermediate (RP_i) with the strict positioning on the polymerase surface of the -10 and -35 regions of the promoter with respect to each other. The postulated intermediate, RP_i, detected at low temperature, was characterized in temperature shift experiments (Buc & McClure, 1985; Spassky et al.,1985) and separated from RP_o by gel electrophoresis (Straney & Crothers, 1987). The complex is resistant to $d(A\text{-}T)_n$ challenge, is transcriptionally inactive and the cytosines near the start site remain hydrogen-bonded (Buc & McClure, 1985; Spassky et al.,1985). The rate-limiting step in the formation of the RP_o in this sequence is the formation of the RP_i. The conversion of the RP_i to the RP_o occurs

rapidly at 37°C and is associated with the unstacking of DNA bases and cooperative polymerase-DNA interactions. Straney and Crothers (1987) reported the separation by gel electrophoresis of two open promoter complexes, O_l and O_u, using the lac UV5 promoter. Formation of the O_l complex precedes the O_u. The unwinding of DNA within O_l is relatively temperature-independent, whereas unwinding in O_u is temperature-dependent. Temperature-independent unwinding in O_l represents a forced unwinding by polymerase which is followed by temperature-dependent unwinding within the O_u complex. The functional difference between the two complexes resides in the strength of the polymerase-lac promoter interaction in the downstream and upstream promoter regions: O_l complex shows strong polymerase-promoter contacts in the -24 bp and downstream region. As a result transcription from the O_l does not efficiently escape abortive cycling (Carpousis & Gralla, 1980). In contrast, the O_u complex has stronger contacts upstream from the -24 bp region that apparently allow the complex to escape abortive transcription cycling (Carpousis & Gralla, 1980) and shift into productive transcription. The escape from abortive cycling to productive binding is correlated with a loss in both the -24 domain binding and methylation protection characteristic of O_l complex.

An alternate mode of polymerase-lac operon contacts was demonstrated in studies of the effects of the anti-beta mAb

210E8 on the formation of RP_o with linear and supercoiled lac UV5 promoters (Rockwell & Krakow, 1988). mAb 210E8 inhibited abortive initiation from linear lac UV5. Abortive transcription by mAb 210E8-polymerase from supercoiled lac UV5 following a lag approached the steady-state rate of nucleotide incorporation comparable to that of the control reaction. Binding of mAb 210E8 to polymerase did not significantly alter the positioning of the polymerase with respect to the backbone of the linear lac UV5 promoter but prevented methylation of the cytosines at positions -6, -4, -2, and -1 suggesting that the open promoter complex was not formed. In addition the essential guanine contacts with the promoter were affected. It was proposed that non-optimal contacts were established with the linear lac UV5 promoter and that mAb 210E8 trapped polymerase in a conformation that hindered the final conversion to a stable RP_o. Supercoiling has been shown to favor the unwinding and the melting of DNA (Davidson, 1972) and this apparently alleviated a constraint imposed by the binding of the anti-beta mAb 210E8 to polymerase.

The lac UV5 complex preceding formation of the stable mAb 126C6-RP_o differs from the postulated RP_i and from the intermediate described by Rockwell and Krakow (1988). Time course data (Fig. 15) show that the mAb 126C6 bound to polymerase did not ultimately hinder linear lac UV5 melting and the consequent strand separation near the transcription

start site. The proper alignment of the DNA with respect to the catalytic site appears to be the rate-limiting step. The extended lag (Fig. 7) indicates that polymerase and DNA in the mAb 126C6-RNP-lac UV5 complex undergo some cooperative conformational changes in an approach to a stable RP_o. Even after the steady state is achieved the optimal contacts between the mAb 126C6-RNA polymerase and lac UV5 promoter are not established. This is demonstrated by the DNase I footprints during abortive transcription from lac UV5 (Fig. 17) and by the methylation of guanine residues in the mAb 126C6-RP_o complex (Fig. 14, panel A). This effect of the mAb 126C6 on polymerase interaction with the promoter apparently does not dramatically affect the transcriptional activity of the enzyme after the open promoter complex has formed but may contribute to the observed decreased rate of RP_o formation. It is possible that the mAb 126C6-polymerase-RP_o complex is related to the O_1 intermediate described by Straney and Crothers (1987) since the mAb-polymerase complex interacts less efficiently with the upstream promoter region but initiates abortive transcription with ApA and UTP. In addition, it appears that the mAb-polymerase complex cannot efficiently support the synthesis of a 63 nucletiode long RNA from lac UV5 (Dalla Venezia & Krakow, in preparation). The results suggest that only a small fraction of the mAb-polymerase open complex can escape abortive cycling and enter the elongation mode when all four nucleotides are

present.

mAb 126C6 does not inhibit transcription from $d(A-T)_n$. Lack of the inhibition of transcription from $d(A-T)_n$ by mAb 126C6 apparently reflects an increased frequency of the productive binding of polymerase to the $d(A-T)_n$ and a lowered duplex stability of the template as compared with lac UV5. These factors, presumably, contribute to the rapid formation of an open complex between mAb 126C6-polymerase and $d(A-T)_n$.

The precise mechanism of transcription stimulation by cAMP-CRP remains to be clearly defined. A direct CRP-polymerase interaction was initially proposed by Majors (1975) and there are experimental data in support of this proposal (Li & Krakow, 1987, 1988; Pinkney & Hogget, 1988). It has been speculated that the bending of the DNA by the CRP bound to its site facilitates polymerase-CRP interactions (Wu & Crothers, 1984; Liu-Johnson et al., 1986). Evidence for direct protein-protein interaction is provided by fluorescence polarization studies (Pinkney & Hogget, 1988). The data showed that at physiological ionic strength in the absence of DNA the cyclic AMP receptor protein binds to RNA polymerase holoenzyme in a cAMP-dependent manner. Studies of the effects of monoclonal antibodies directed against CRP suggested that CRP-polymerase contact occurs when both proteins are bound to the lac P+ promoter (Li & Krakow, 1987). The results of

DNase I footprinting indicate that when the mAb 126C6-polymerase complex is preincubated with cAMP-CRP-lac P+ the interactions between the promoter and polymerase are not established. This would explain the inhibition of transcription from the lac P+ promoter. Since the primary binding site for cAMP-CRP on lac P+ (-50 to -70 bp) is adjacent to the polymerase binding site on the promoter a steric interference between mAb 126C6 bound to polymerase and cAMP-CRP appeared possible. This was tested by addition of the mAb to the preformed open promoter complex. The footprints show that binding of anti-alpha mAb 126C6 occurs and that as a result of this binding interactions between the DNA and polymerase are severely affected. Nevertheless, the mAb-polymerase complex remains bound to the promoter as indicated by the enhancement at -23 bp and -24 bp on the non-template strand. These footprints resemble the footprints of lac P+-polymerase formed in the absence of cAMP-CRP (Fig.9 and 10, lane b). cAMP-CRP also remains bound to the promoter. However, the mutual effect of polymerase and CRP on the DNA conformation in the CRP binding site of the promoter as indicated by the enhancements at -52 and -54 bp on the template strand, is not seen. The enhancements are reduced to that seen with cAMP-CRP alone (Fig.10, compare lanes d and i with lanes c and e-h). DNase I footprints of polymerase bound to the lac promoters in the absence of cAMP-CRP do not show the enhancements at

-52 and -54 bp (Fig. 10, lane b, Fig. 12, lanes f-l). The effect of mAb 126C6 on the DNA conformation in the CRP binding site of lac P+ is a more direct indication of CRP-polymerase interactions than is the effect of the mAb on polymerase-lac P+ interactions. Effects of mAb 126C6 on the interactions of polymerase with the lac UV5 fragment are also seen (Fig. 11, 12, and 14) and could be more strongly evinced with lac P+ promoter. If the polymerase-lac P+ interactions alter the conformation of the CRP binding domain of polymerase to favor protein-protein contact then the destabilizing affect of the mAb 126C6 on the polymerase-lac P+ could indirectly contribute to the distortion of the CRP-polymerase contacts. Alternatively, binding of mAb 126C6 to polymerase may distort polymerase-CRP interactions by directly affecting conformation of the CRP-binding domain of RNA polymerase. Although polymerase and cAMP-CRP remain bound to the promoter, binding of the mAb 126C6 to the lac P+ open promoter complex renders it transcriptionally inactive (Table I). Polymerase should thus be able to interact with CRP if its putative CRP binding domain were not conformationally or sterically affected by the bound anti-alpha mAb. Whether binding of the mAb 126C6 to the preformed cAMP-CRP-lac P+-polymerase complex affects first the RNP contacts with the lac promoter that might lead to a change in the conformation of the polymerase CRP binding domain, or whether the CRP-polymerase contacts are disturbed

first by the mAb binding is not clear. It is possible that the binding of the mAb to polymerase affects both CRP-polymerase and polymerase-lac promoter interactions. Regardless of the sequence of the events the effects of anti-alpha mAb 126C6 on the lac P+ open promoter complex can be best interpreted as indicative of CRP-polymerase contacts.

When the effects of the mAb 126C6 on polymerase interactions with cAMP-CRP-lac P+ were compared with the results of mAb 126C6-polymerase interaction with lac L8UV5 promoter an additional role of CRP in stimulation of transcription from lac P+ was suggested. Interaction of the preformed mAb 126C6-polymerase complex with lac L8UV5 or binding of mAb 126C6 to the lac L8UV5-polymerase open promoter complex clearly has an overall pronounced effect on polymerase interactions with the both strands of the promoter. Despite these distortions the catalytic activity of the polymerase is not noticeably affected after the lac L8UV5 open promoter complex forms (compare K_m and V_{max} in the presence and absence of the mAb). In contrast, binding of mAb 126C6 to the RP_o formed with cAMP-CRP-lac P+ completely inhibits initiation of transcription even though the DNA strands near the transcription start site appear to remain separated (Table I; Fig. 16, lanes b, c, d). This suggests that the positioning of the lac P+ transcription start site with respect to the catalytic site of polymerase

is affected by binding of the mAb 126C6. Thus it appears that cAMP-CRP not only directs the binding of polymerase to the P1 site of the lac P+ promoter and facilitates the unwinding and melting of the DNA but also helps to keep the catalytic site and the downstream region of the template strand in register for the formation of the initial phosphodiester bond(s). This apparently requires direct contact between CRP and polymerase.

Binding of cAMP-CRP to the lac L8UV5 mutant is polymerase-dependent. Binding of cAMP-CRP to the non-template strand of the lac L8UV5 and lac P+ is comparable (Fig. 9, lanes c and d; Fig. 13, lane b). However, the binding of the cAMP-CRP to the template strand of lac L8UV5 is less efficient than binding to the template strand of the lac P+ promoter (Fig. 10, lanes c and d; Fig. 13a, lane f). Nevertheless the effect of the CRP on polymerase interactions with the template strand of the lac L8UV5 is seen as a partial supression of the enhancement at -38 and -49 bp (Fig. 13a, lanes f, h, i). Malan et al. (1984) showed that abortive transcription from the linear lac promoter containing the L8 mutation and the unchanged -10 region of lac P+ promoter does not occur in the absence of cAMP-CRP. Abortive transcription from the lac P+L8 promoter in the presence of cAMP-CRP was 30% of the transcription from the linear lac P+ promoter and required about a 20-fold excess of CRP (over the amount required for transcription stimulation from the

wild-type lac P+). Taken together the results of the footprinting with DNase I and the results of Malan et al. (1984) suggest that low affinity CRP-lac promoter interactions indirectly affect polymerase-CRP contacts. Incubation of mAb 126C6 with the RP_o formed with cAMP-CRP-lac L8UV5 results in dissociation of cAMP-CRP from its site on this promoter (Fig.13, lane g). Identical results were obtained when F_{ab} fragments of mAb 126C6 were used in place of the intact mAb (Fig. 13, lane j). More than one factor may account for the dissociating affect of mAb 126C6. A direct effect of the mAb 126C6 on the conformation in the polymerase CRP-interacting domain and/or an indirect affect mediated by distorted polymerase-lac promoter contacts may both contribute to the dissociation of the CRP from its site on the lac L8UV5 promoter. Although mAb 126C6 has an overall effect on the interactions of polymerase with lac L8UV5 the relative decrease of protection against the DNase I attack on both strands of the promoter in the region adjacent to the CRP binding domain is more pronounced. These contacts may contribute to the stability of the cAMP-CRP-lac L8UV5-polymerase complex. A steric effect of mAb 126C6 on dissociation of CRP from the promoter is also possible and may occur if one of the alpha subunits in polymerase is proximal to the CRP binding domain. Binding of mAb 126C6 to this alpha subunit may impede contact of CRP with RNA polymerase or bring the mAb and CRP into direct contact. The

latter is also suggested by the decreased protection at -57 and -58 bp in the CRP binding domain of the template strand of the lac P+ fragment (Fig. 10, lanes e-h). The affect of the anti-alpha mAb 126C6 on interactions of cAMP-CRP with lac L8UV5 suggests that at least one of the alpha subunits may be proximal to the CRP in an open promoter complex, although the dissociating affect of the mAb on CRP may not be direct.

An asymetric interaction of polymerase with the template and non-template strands of lac UV5 and lac P+ promoters has been demonstrated by Siebenlist et al. (1980) and Shanblatt and Revzin (1986). The essential contacts between phosphates on the non-template strand of lac UV5 and lac P+ and polymerase were determined by alkylation interference. No contacts were observed on the template strand of lac P+ and only one contact was detected on the template strand of the lac UV5 promoter. No specific contacts between nucleotide bases and polymerase on the template strand of lac P+ were detected (Shanblatt & Revzin, 1986). The time course of incubation of mAb 126C6 with the preformed cAMP-CRP-lac P+-polymerase (Fig. 10, lanes e-g) points to a more pronounced effect of the mAb on interaction of polymerase with the template strand as indicated by a gradual loss of the enhancement at -25 bp on the template strand. The data are consonant with an asymetric interaction of polymerase with the template and non-template strands of the lac promoters.

It is not clear whether binding of mAb 126C6 to polymerase directly affects the interaction of the alpha subunits with the promoter or whether the effect is transmitted to the DNA binding domain of the enzyme. Crosslinking of polymerase with lac UV5 showed no contacts between the DNA and the alpha subunit (Chenchick et al., 1981). The absence of evidence cannot not prove that a direct interaction between the alpha subunit and DNA does not occur. However, it is likely that the observed effects of mAb 126C6 on the interactions of polymerase and cAMP-CRP with the lac promoters are indirect since only a relatively small area of alpha is exposed on the enzyme surface (Tichelaar et al., 1983). Binding studies with [^{125}I]labeled mAbs, crosslinking experiments (Hillel & Wu, 1977) and immunoelectron microscopy studies (Tichelaar et al., 1983) all suggest that RNA polymerase holoenzyme at least one of the alpha subunits is adjacent to the sigma subunit. The affinity of mAb 126C6 for holoenzyme is greater than for core polymerase (Table III). This indicates that interaction of the sigma subunit with core enzyme has some effect on the conformation of the alpha subunit(s). This implies that binding of mAb 126C6 to alpha subunit may induce conformational changes in the sigma subunit. These changes may in part be responsible for the inhibitory effects of the anti-alpha mAb 126C6.

Binding of mAb 126C6 to the alpha subunits in the E. coli RNA polymerase affects polymerase contacts with the lac

promoter that span the DNA region from +9 to -50 bp. It appears that binding of the non-inhibitory anti-alpha mAb 129C4 to polymerase has a slight effect on contacts with the guanine residues at +9 and +11 bp. Interaction of the mAb 129C4-polymerase-mAb 124D1 complex with lac L8UV5 plus cAMP-CRP does not inhibit CRP binding to its site on the promoter. Simultaneous binding of mAb 129C4 and anti-beta mAb 210E8 to polymerase is sterically hindered; binding of the mAb 210E8 to polymerase affects interactions of the enzyme with guanine bases in the downstream region of the linear lac UV5 promoter and inhibits the DNA strand separation near the start site of transcription (Rockwell & Krakow, 1988). Considering the proposed arrangement of the alpha subunits within RNA polymerase it is tempting to speculate that during polymerase interaction with the lac promoter the more exposed alpha is situated in the enzyme domain that contacts the -10 region of the promoter, whereas the shielded alpha is in the polymerase domain that interacts with an upstream promoter sequence.

Table I. Effect of anti-alpha mAbs on *lac* promoter-directed synthesis of ApApUpU and on the $d(A-T)_n$ directed synthesis of $r(U-A)_n$ by RNA polymerase holoenzyme.

mAb	Ig class	*lac* P+	*lac* L8UV5	$d(A-T)_n$
		% residual activity[a].		
129C4	IgG2a,k	87	92	92
124D1	IgG2b,k	99	104	90
126C6	IgG2b,k	7[b]	42	95
121C2	IgG2b,k	106	111	110

RNA polymerase holoenzyme (2 pmol) was preincubated with the indicated monoclonal antibody for 30 minutes at 37°C. The molar ratio of antibody to alpha was 20 to 1 for mAbs 129C4, 124D1, and 121C2 and 5 to 1 (or 10 to 1 for the $d(A-T)_n$-directed reaction) for mAb 126C6. Enzyme activity was assayed as described under Materials and Methods. [a]Residual activity is expressed as the percent of the following control values for incorporation of [^{3}H]UMP: 5 nmol for the synthesis of $r(U-A)_n$; 367 pmol and 421 pmol for the synthesis of ApApUpU with *lac* P+ and *lac* L8UV5, respectively. [b]Polymerase was preincubated with cAMP-CRP-*lac* P+ for 15 minutes at 37°C and then for an additional 20 minutes with the monoclonal antibody.

Table II. Effect of anti-alpha monoclonal antibodies on the reconstitution of RNA polymerase core enzyme.

	effect of antibody when incubated with[a]		
antibody	subunits	premature core	mature core
mAb 129C4	1 (4:1)	62 (16:1)	130 (16:1)
mAb 129C4-F_{ab}	2 (4:1)	81 (4:1)	116 (4:1)
mAb 124D1	107 (10:1)	ND	ND
mAb 124D1[b]	78 (10:1)	ND	ND
mAb 126C6	11 (4:1)	8 (13:1)	90 (13:1)

Core enzyme was denatured by incubation in buffer containing 6 M guanidine hydrochloride for two hours at 4°C. The reconstitution mixtures contained 100 pmol of alpha subunit and 50 pmol each of beta and beta' subunits. Reconstitution to form the premature core was carried out by dialysis for 16 hours at 4°C against reconstitution buffer. Incubation of the antibody with the premature or mature core enzyme was for 3 hours on ice followed by incubation at 30°C for 40 minutes. Aliquots (25 ul) containing 4 pmol of reconstituted core enzyme were assayed for $d(A\text{-}T)_n$-directed synthesis of $r(U\text{-}A)_n$ as described in Materials and Methods. ND indicates that the effect of the antibody was not determined. The ratio of antibody to alpha used is given in parenthesis. [a]Residual activity in the presence of monoclonal antibody is expressed as the percent of the control value for incorporation of [^{3}H]UMP: 100% was 1.6 nmol in 10 min at 37°C. [b]mAb 124D1 was incubated with the isolated alpha subunit for 30 min at 37°C prior to reconstitution.

Table III. Dissociation constants for binding of the anti-alpha monoclonal antibodies to free and RNA polymerase associated alpha subunit.

antibody	alpha	core	holoenzyme
mAb 126C6	0.29 nM (a)	0.16 nM (a)	0.08 nM (a)
	7.25 nM (a)(b)		
mAb 129C4	1.10 nM (a)		
	2.45 nM (c)	2.73 nM (c)	2.77 nM (c)
mAb 124D1	3.75 nM (a)		
	2.06 nM (c)	2.70 nM (c)	3.85 nM (c)

Conditions used are described in Materials and Methods.

(a) The K_d values were determined by the method of Friguet et al. (1985).

(b) Alpha subunit was stored in PBS buffer in the absence of mercaptoethanol or dithiothreitol.

(c) The K_d values were calculated from Scatchard plots obtained with ^{125}I-labeled mAbs; the concentration of the [^{125}I]monoclonal antibody was varied from 0.25 nM to 12 nM. For [^{125}I]mAb 129C4 the immobilized antibody was mAb 124D1, for [^{125}I]mAb 124D1 the immobilized antibody was mAb 129C4.

Table IV. Effect of modification of sulfhydryl groups of the alpha subunit on the reconstitution of core enzyme.

[^{3}H]NEM, nmol incorporated per nmol of alpha	% residual polymerase activity after reconstitution
0	100.0
1	90.2
3.6	96.8
4.1	88.3

Alpha subunit was prepared by chromatography of urea denatured core enzyme on Bio-Rex 70 (Lowe & Malcolm, 1976) or by gel filtration in the presence of 6M guanidine hydrochloride. Modification of SH groups of the alpha subunit was carried out as described in Materials and Methods. Reconstitution mixtures contained 100 pmol of unmodified or NEM-modified alpha subunit and 50 pmol each of beta and beta' subunits prepared by gel filtration in 10 mM potassium phosphate buffer (pH 7.2) containing 6 M guanidine hydrochloride. Reconstitution mixtures were dialyzed against reconstitution buffer overnight at 4^{o}C followed by incubation for 40 min at 30^{o}C. Aliquots (25 ul) of reconstituted core enzyme were removed for determination of d(A-T)$_n$-directed synthesis of r(A-U)$_n$. The control value for incorporation of [^{3}H]UMP was 1.6 nmol.

Table V. Effect of urea treatment on the binding of anti-alpha monoclonal antibodies to immobilized alpha and RNA polymerase.

Immobilized antigen	Urea (M)	[^{125}I]mAb bound (fmol) 129C4	124D1	126C6
alpha	0	150	175	85
"	4	160	170	105
"	6	145	168	110
core	0	55	58	100
"	4	95	87	50
"	6	60	64	55
holoenzyme	0	53	33	75
"	4	77	58	39

(See legend on page 80.)

Table V. Effect of urea treatment on the binding of anti-alpha monoclonal antibodies to immobilized alpha and RNA polymerase.

The antigens were immobilized to the wells of microtiter polystyrene plates overnight at 4°C. Each well received 500 ng of the alpha, or core, or holoenzyme. The remaining protein binding sites were blocked with PBS + 0.2% BSA. Where indicated 100 ul of 4 or 6 M urea in PBS was added and incubated for 1 hour at 37°C. The plates were washed 3 times with PBT (PBS containing 0.2% BSA and 0.04% Tween 80) washing buffer. Immobilized antigens were incubated with 100 ul (3.4 pmol) of the ^{125}I-labeled mAbs for 1 hour at 37°C. After washing 3 times with PBT buffer, binding of the ^{125}I-labeled mAb was determined. The specific radioactivity was: mAb 129C4, 217 cpm/fmol; mAb 124D1, 155 cpm/fmol; mAb 126C6, 155 cpm/fmol.

Table VI. Binding of ^{125}I-labeled anti-alpha monoclonal antibodies to plate-immobilized monoclonal antibody-alpha complexes.

Immobilized anti-alpha mAb	[^{125}I]mAb bound (fmol)		
	129C4	124D1	126C6
129C4	2	36	45
124D1	50	7	56
126C6	50	40	5

The indicated antibody (0.5 ug/ 60 ul of PBS) was adsorbed onto wells of polystyrene plate overnight at 4^{o}C. Remaining protein-binding sites were blocked with PBS +2 mg/ml BSA. Alpha subunit (10 pmol in 100 ul PBS/BSA) was incubated with the immobilized monoclonal antibody for 60 min at 37^{o}C. After washing 3 times with PBT, the indicated ^{125}I-labeled mAb in 100 ul PBS/BSA was added and incubated for 90 min at 37^{o}C. After washing 3 times with PBT, binding of [^{125}I]mAb was determined. Alpha and ^{125}I-labeled mAbs were added at saturating concentrations. The specific radioactivity was: mAb 121C2, 1000 cpm/fmol; mAb 129C4, 1311 cpm/fmol; mAb 124D1 1124 cpm/fmol; mAb 126C6 100 cpm/fmol.

Table VII. Binding of ^{125}I-labeled anti-alpha monoclonal antibodies to plate-immobilized monoclonal antibody-polymerase and alpha$_2$beta complexes.

Immobilized mAb	[^{125}I]mAb bound (fmol)								
	129C4	124D1	126C6	129C4	124D1	126C6	129C4	124D1	126C6
	<--alpha$_2$beta-->			<-----core------>			<-holoenzyme-->		
anti-alpha									
129C4	2	163	198	1	110	187	1	29	73
124D1	182	5	137	105	1	139	30	0	77
126C6	180	133	7	3	7	59	2	4	25
anti-beta									
210E8	5	7	68	1	1	185	0.7	0	173

The indicated antibody (0.5 ug/100 ul of PBS) was adsorbed onto wells of polystyrene plate overnight at 4^{o}C. Alpha$_2$beta (700 ng), core (1 ug) or holoenzyme (1 ug) was incubated with the immobilized antibody for 16 hours at 4^{o}C. After washing with PBT the indicated ^{125}I-labeled mAb (1 pmol in 100 ul PBS/BSA) was added and incubated for 16 hours at 4^{o}C. Bound [^{125}I]mAb was determined after washing with PBT buffer. [^{125}I]mAbs and antigens were added at saturating concentrations. The specific radioactivity was: mAb 129C4, 1170 cpm/fmol; mAb 124D1, 300 cpm/fmol; mAb 126C6, 249 cpm/fmol.

Table VIIa. Binding of ^{125}I-labeled anti-alpha monoclonal antibodies to alpha$_2$beta and polymerase complexed with plate-immobilized anti-alpha monoclonal antibody.

Immobilized mAb	[^{125}I]mAb bound (fmol)					
	121C2				129C4	126C6
	<-alpha->	<-a$_2$B->	<-core->	<-holo->	<-core->	<-core->
121C2	6	3	3	2	58	56
129C4	80	48	40	27	1	63
124D1	51	16	6	5	66	64
126C6	35	17	3	4	3	23

The indicated antibody (0.5 ug/100 ul of PBS) was adsorbed onto wells of polystyrene plate overnight at 4^{o}C. Alpha (0.375 ug), alpha$_2$beta (0.7 ug), core (1 ug) or holoenzyme (1 ug) was incubated with the immobilized antibody for 16 hours at 4^{o}C. After washing, the indicated ^{125}I-labeled mAb (1 pmol in 100 ul PBS/BSA) was added and incubated for 90 min at 37^{o}C. [^{125}I]mAbs and antigens were added at saturating concentrations. Bound [^{125}I]mAb was determined after washing with PBT buffer. The specific radioactivity was: mAb 121C2, 1000 cpm/fmol; mAb 129C4, 1170 cpm/fmol; mAb 126C6, 1869 cpm/fmol.

Table VIII. Binding of ^{125}I-labeled anti-alpha mAb 129C4 complexed with polymerase and $alpha_2beta$ to Protein A-monoclonal antibody complexes.

Immobilized mAb	[^{125}I]mAb 129C4 bound (fmol)		
	<-$alpha_2beta$->	<--core-->	<-holoenzyme->
anti-alpha			
129C4	30	0	0
124D1	563	393	251
126C6	610	418	226
anti-beta			
210E8	96	72	14

[^{125}I]mAb 129C4 (10 pmol, 1500 cpm/fmol) was incubated with $alpha_2beta$ (1.1 ug), core enzyme (2.4 ug) or holoenzyme (3 ug) for 30 minutes at 37^{o}C. The indicated monoclonal antibody adsorbed to Protein A on Staph. aureus cells was mixed with 50 ul of the preformed immune complex containing [^{125}I]mAb 129C4 and incubated for 30 min at 37^{o}C. Bound [^{125}I]mAb was determined after washing with PBT buffer. For details see Materials and Methods.

Table IX. Binding of ^{125}I-labeled anti-beta mAb 210E8 and ^{125}I-labeled anti-beta' mAb 311G2 to polymerase and $alpha_2beta$ complexed with the plate-immobilized mAbs.

Immobilized mAb	$[^{125}I]$mAb bound (fmol)				
	<-anti-beta mAb 210E8->			<-anti-beta' mAb 311G2->	
anti-alpha	a_2B	core	holoenzyme	core	holoenzyme
129C4	15	18	13	7	4
124D1	12	17	14	5	4
126C6	45	79	75	28	32
121C2	9	13	9	3	2
anti-beta					
210E8	5	7	6	26	30
anti-beta'					
311G2	ND	53	50	3	3

The assay was carried out essentially as indicated in legend to Table V. $Alpha_2beta$ (0.7 ug), core enzyme (1 ug) or holoenzyme (1 ug) was incubated with the immobilized monoclonal antibody for 60 min at 37°C. After washing, 1 pmol of the indicated ^{125}I-labeled monoclonal antibody was added and incubated for 90 min at 37°C. Specific radioactivities were 170 cpm/fmol and 684 cpm/fmol for anti-beta mAb 210E8 and anti-beta' mAb 311G2, respectively.

Figure 1. Effect of the increasing concentrations of anti-alpha mAbs on reconstitution of core enzyme.
Core enzyme was reconstituted from alpha and beta + beta' subunits separated by gel-filtration in the presence of 6 M guanidine hydrochloride. Reconstituted mixtures contained 100 pmol (3.65 ug) of alpha and 50 pmol each of beta (7.75 ug) and beta' (8.25 ug) subunits. Monoclonal antibodies were added to reconstitution mixtires as indicated. Reconstitution mixtures were dialyzed against reconstitution buffer (50 mM Tris-HCl, pH 8, 0.2 M KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 20% glycerol, 0.5 mM DTT) overnight at 4°C, followed by incubation at 37°C for 30 minutes. 25 ul (4 pmol) aliquots of reconstituted core enzyme were removed for determination of the enzyme activity. The activity of the reconstituted enzyme was assayed by $d(A-T)_n$- directed synthesis of $r(U-A)_n$ as described in the Materials and Methods. In the absence of antibodies about 400 nmol of UMP was incorporated per nmol of core enzyme per 10 minutes at 37°C.

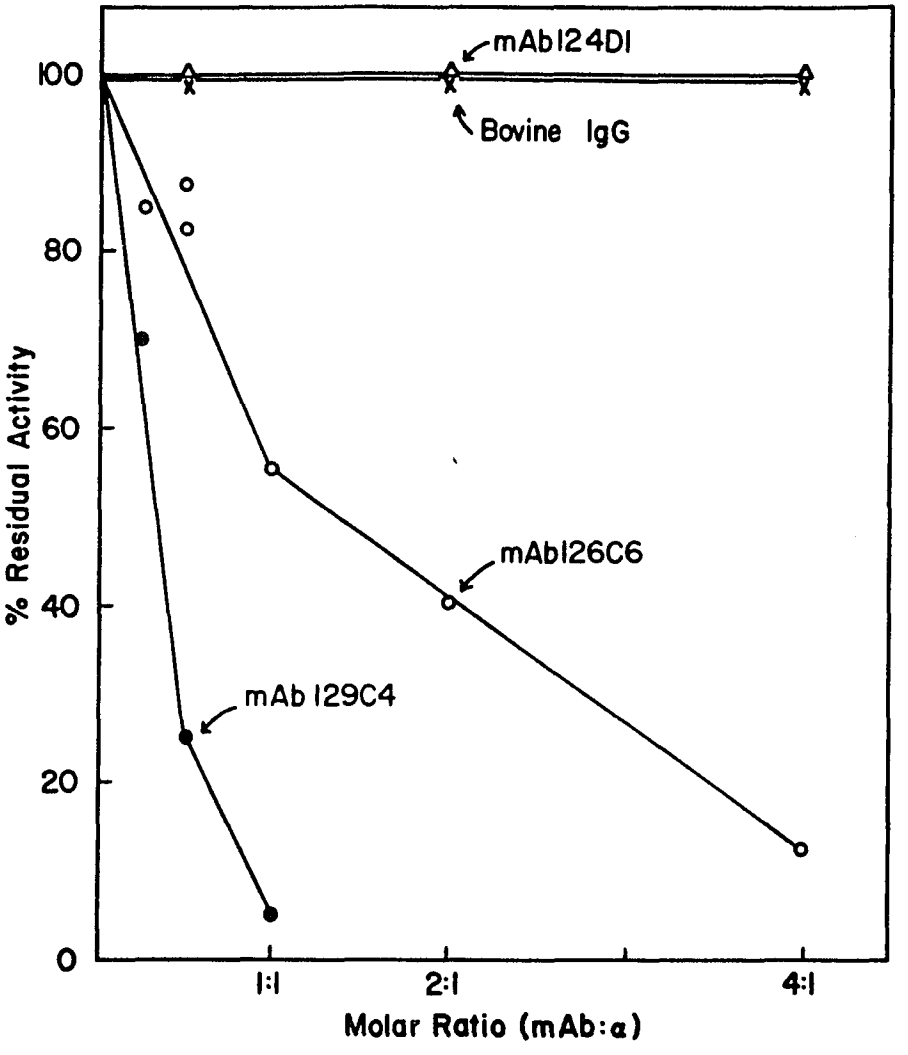

mAb124D1
Bovine IgG
mAb126C6
mAb 129C4
100
80
60
40
20
0
% Residual Activity
1:1
2:1
4:1
Molar Ratio (mAb:α)

Figure 2. 10% SDS polyacrylamide gel electrophoresis of the intact and F_{ab} fragments of mAb 129C4 and mAb 126C6. Panel A, mAb 129C4: a. intact mAb; b. F_{ab} after removal of F_c by treatment with protein-A sepharose; c. F_{ab} plus F_c; d. F_{ab} (electrophoresed in the absence of mercaptoethanol) reconstituted from the light chain and the antibody binding fragment of the heavy chain after overnight dialysis of the papain digested mAb 129C4 against PBS buffer; Panel B, mAb 126C6: a. intact mAb; b. F_{ab} after removal of F_c by treatment with Protein-A Sepharose.

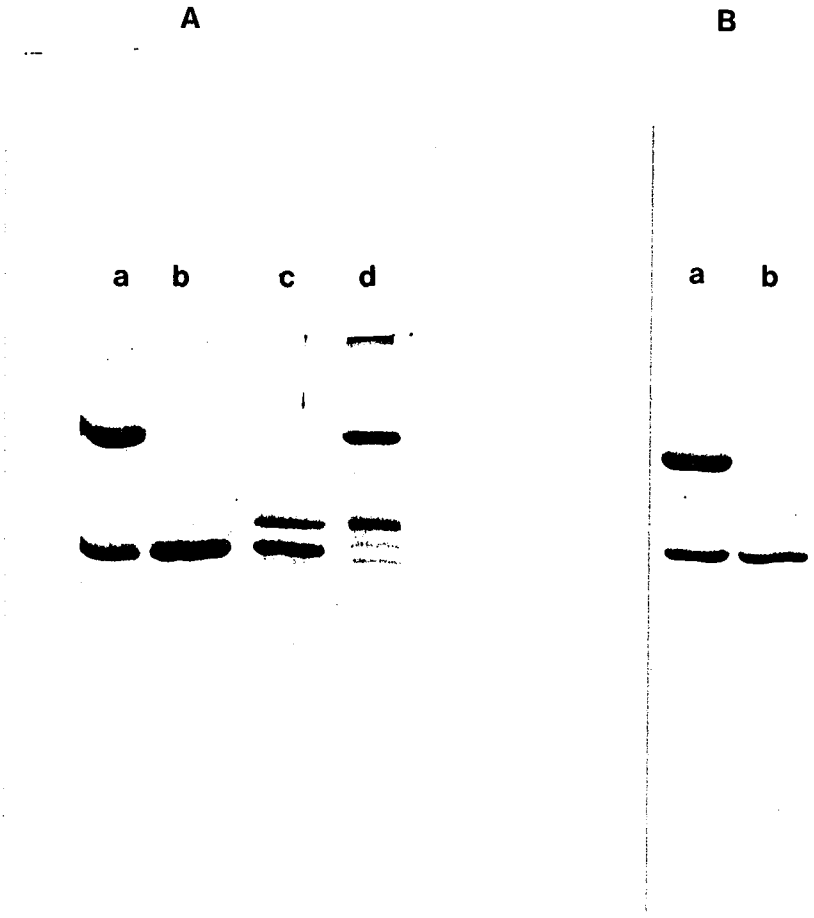

A
B
a
b
c
d
a
b

Figure 3. Determination of K_d for mAb 126C6 and alpha, core and holoenzyme by indirect ELISA (Klotz plot). (o), alpha; (△), core; (▲), holoenzyme. v is the fraction of bound antibody and a is the concentration of free antigen at equilibrium. For details see Materials and Methods.

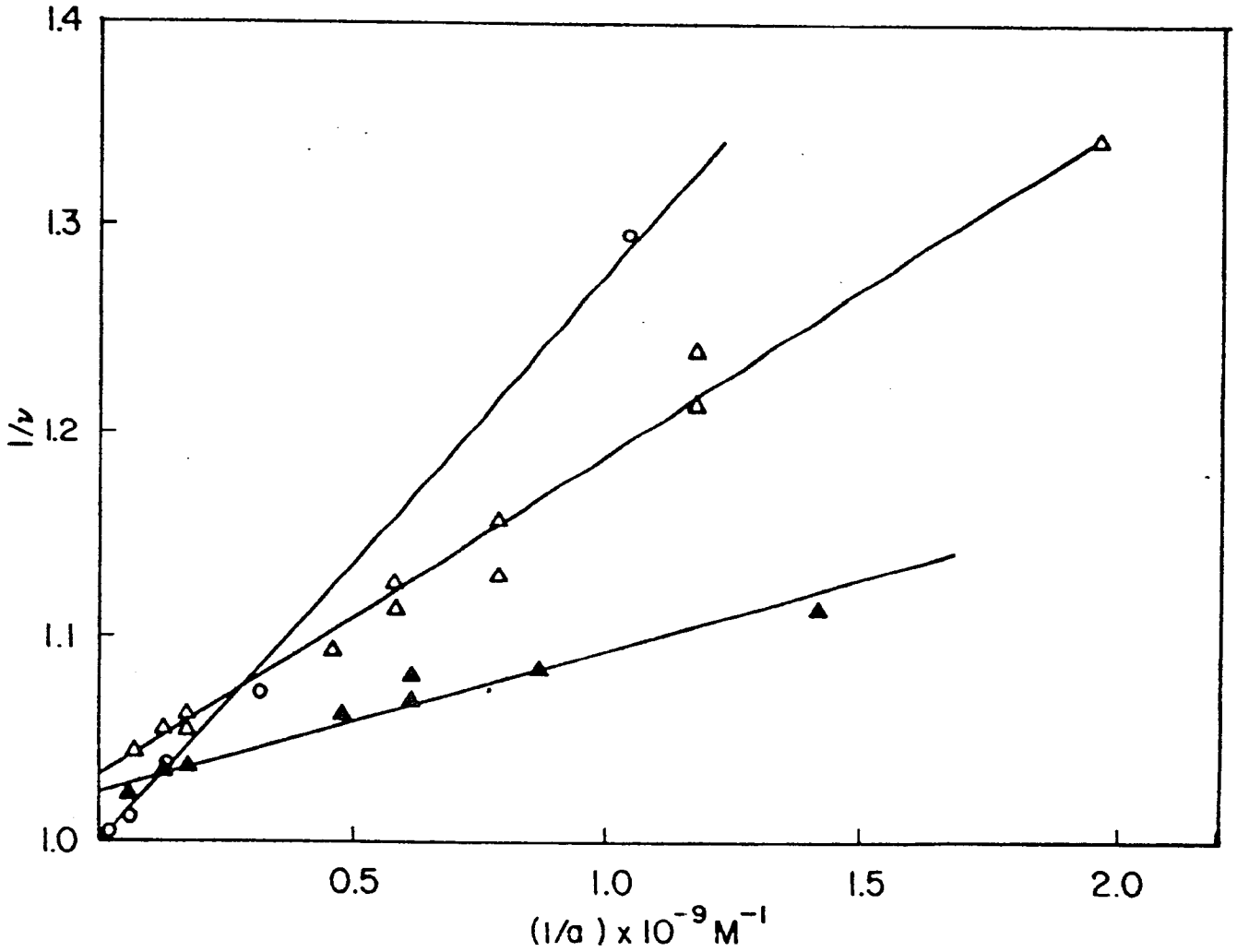

1.4
1.3
1.2
1.1
1.0
1/ν
0.5
1.0
1.5
2.0
(1/a) x 10⁻⁹ M⁻¹

Figure 4. Determination of the K_d for mAb 129C4 and core enzyme in a double antibody and single antibody assays on polystyrene plates (Scatchard plots). Binding of ^{125}I-labeled mAb 129C4 to core enzyme in a single antibody assay (●), K_d = 2.45 nM; and in a double antibody assay (o), K_d = 2.73 nM. The concentration of the [^{125}I]mAb 129C4 was varied from 0.2 nM to 12 nM. In a double antibody assay the immobolized antibody was mAb 124D1. For details see Materials and Methods.

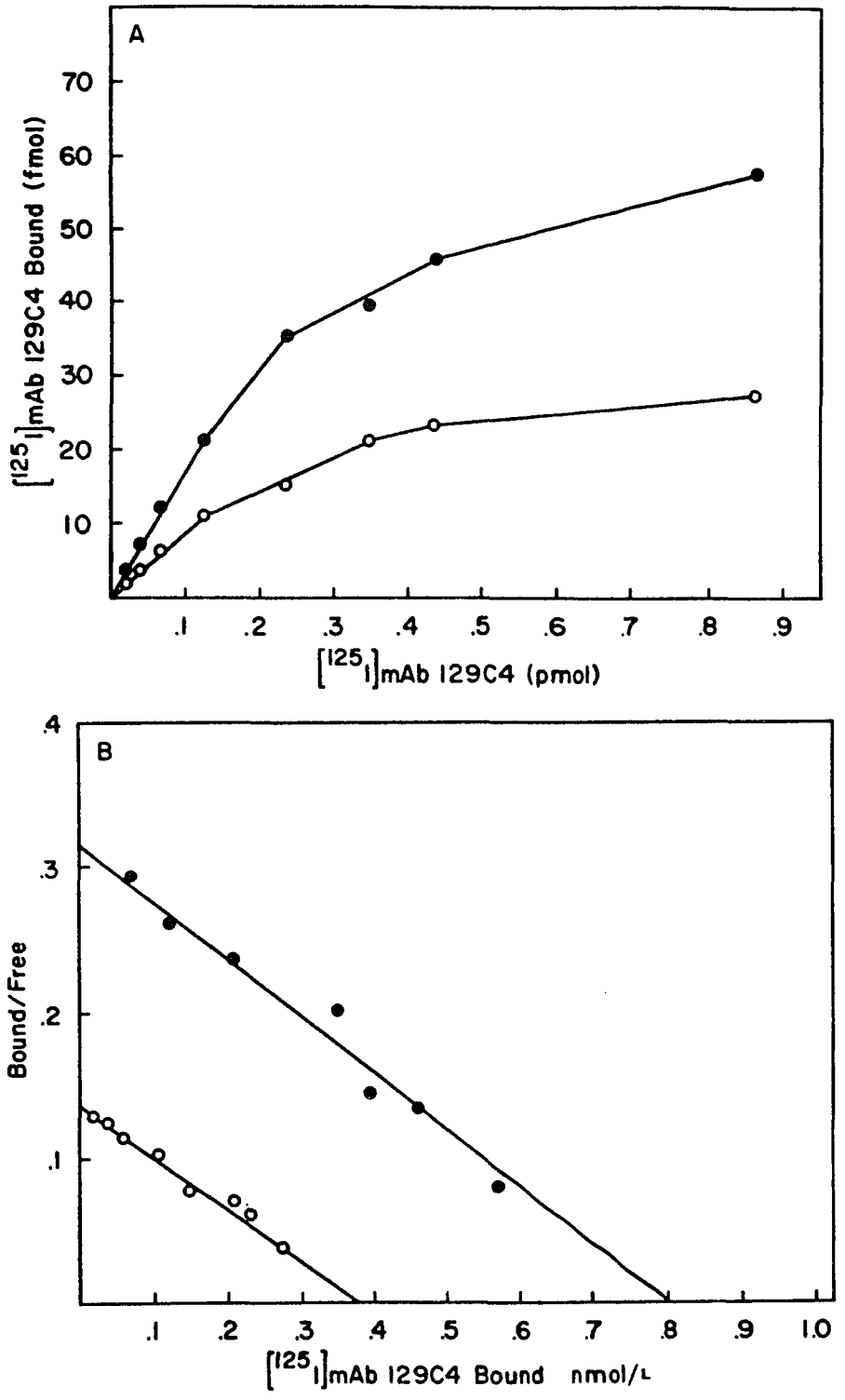

A
70
60
50
40
30
20
10
[125I]mAb 129C4 Bound (fmol)
.1
.2
.3
.4
.5
.6
.7
.8
.9
[125I]mAb 129C4 (pmol)
B
.4
.3
.2
.1
Bound/Free
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
[125I]mAb 129C4 Bound nmol/L

Figure 5. Determination of the K_d for mAb 124D1 in a double antibody assay with S. aureus as the solid support (Scatchard plot).

S.aureus cell suspension (1mg) was incubated with 2 pmol mAb 129C4 (in 40 ul PBS-BSA) for 15 min at room temperature. After addition of 1 pmol of alpha subunit or 0.5 pmol core enzyme 20 ug of bovine IgG was added (final volume 100 ul PBS-BSA) and the mixture was incubated for 30 min at 37°C. After the unbound antibodies and antigens were removed by washing with PBT, [^{125}I]mAb 124D1 was added and the incubation was continued for 30 min at 37°C. The concentration of [^{125}I]mAb 124D1 was varied from 0.25 nM to 12 nM. After the unbound [^{125}I]mAb 124D1 was removed by washing with PBT buffer (1 ml x 3 times) the bound radioactivity was determined in the LKB Autogamma-counter. Binding of [^{125}I]mAb 124D1 to alpha subunit (●), K_d = 2.06 nM; and to core enzyme (o), K_d = 2.70 nM.

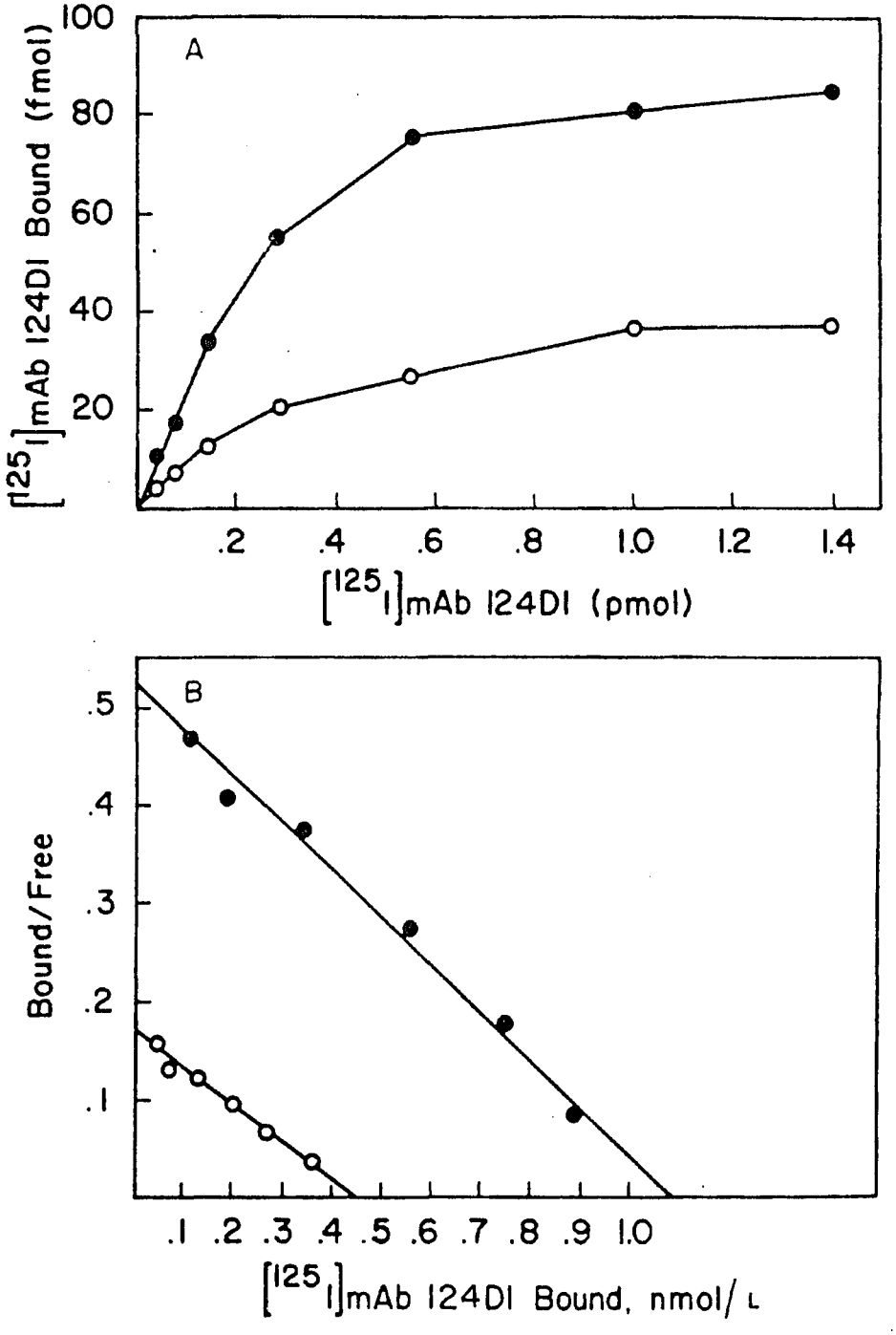

A
100
80
60
40
20
[125I]mAb 124DI Bound (fmol)
.2
.4
.6
.8
1.0
1.2
1.4
[125I]mAb 124DI (pmol)
B
.5
.4
.3
.2
.1
Bound/Free
.1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
[125I]mAb 124DI Bound, nmol/L

Figure 6. Effect of mAb 126C6 on K_m and V_{max} in abortive initiation directed by lac UV5 (Lineweaver-Burk plot). Abortive synthesis of ApApUpU is described under Materials and Methods. RNA polymerase (2 pmol) preincubated in the presence (at a molar ratio of mAb to alpha of 5 to 1) or absence of mAb 126C6 for 30 min at 37°C was then incubated with the promoter (0.1 pmol) for 20 min at 37°C. The substrates were added as follows: the concentration of ApA was varied from 0.05 mM to 2 mM and the fixed concentration of [^{3}H]UTP was 50 uM. The specific radioactivity of [^{3}H]UTP was 230 cpm/pmol UTP. In the absence of antibody (●) the K_m and V_{max} values were 206.9 uM and 20.88 pmol/min, respectively. For mAb 126C6-polymerase complex (o) K_m was 190 uM and V_{max} was 17.46 pmol/min.
[S] = [ApA]; v = rate of nucleotide incorporation, pmol/min.

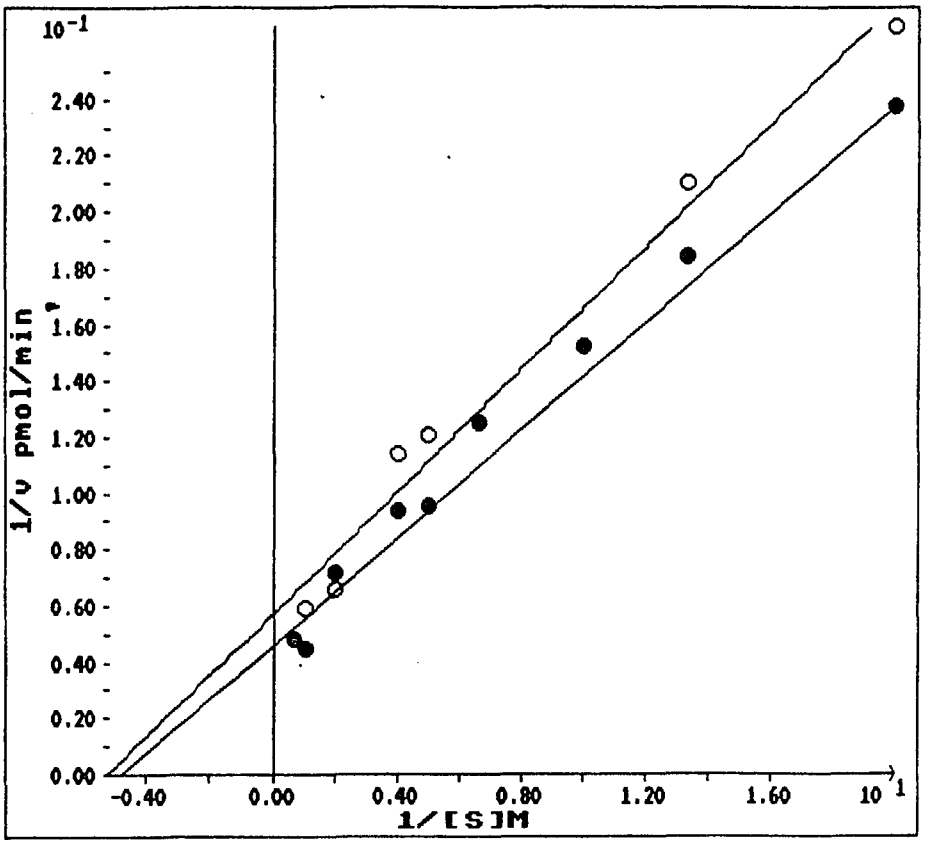

10^{-1}
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1/v pmol/min
-0.40
0.00
0.40
0.80
1.20
1.60
10^{1}
1/[S]M

Figure 7. Effect of the anti alpha mAb 126C6 and mAb 129C4 on the kinetics of open complex formation with lac L8UV5. Abortive synthesis of ApApUpU is described under Materials and Methods. Lag curves were obtained by determining the incorporation of [^{3}H]UTP at the time indicated following initiation of abortive initiation reaction by the addition of 50 nM RNA polymerase holoenzyme or the mAb-polymerase complex to the preincubated at 37^{o}C solution of lac UV5 (0.1 pmole), and ApA (1 mM) and [^{3}H]UTP (50 uM). The molar ratio of antibody to alpha was 5 to 1. (o), the nucleotide mixture was added to the preformed RP_o; (●), RNA polymerase alone (or mAb 129C4-RNA-polymerase complex); (□) mAb 126C6-polymerase complex.

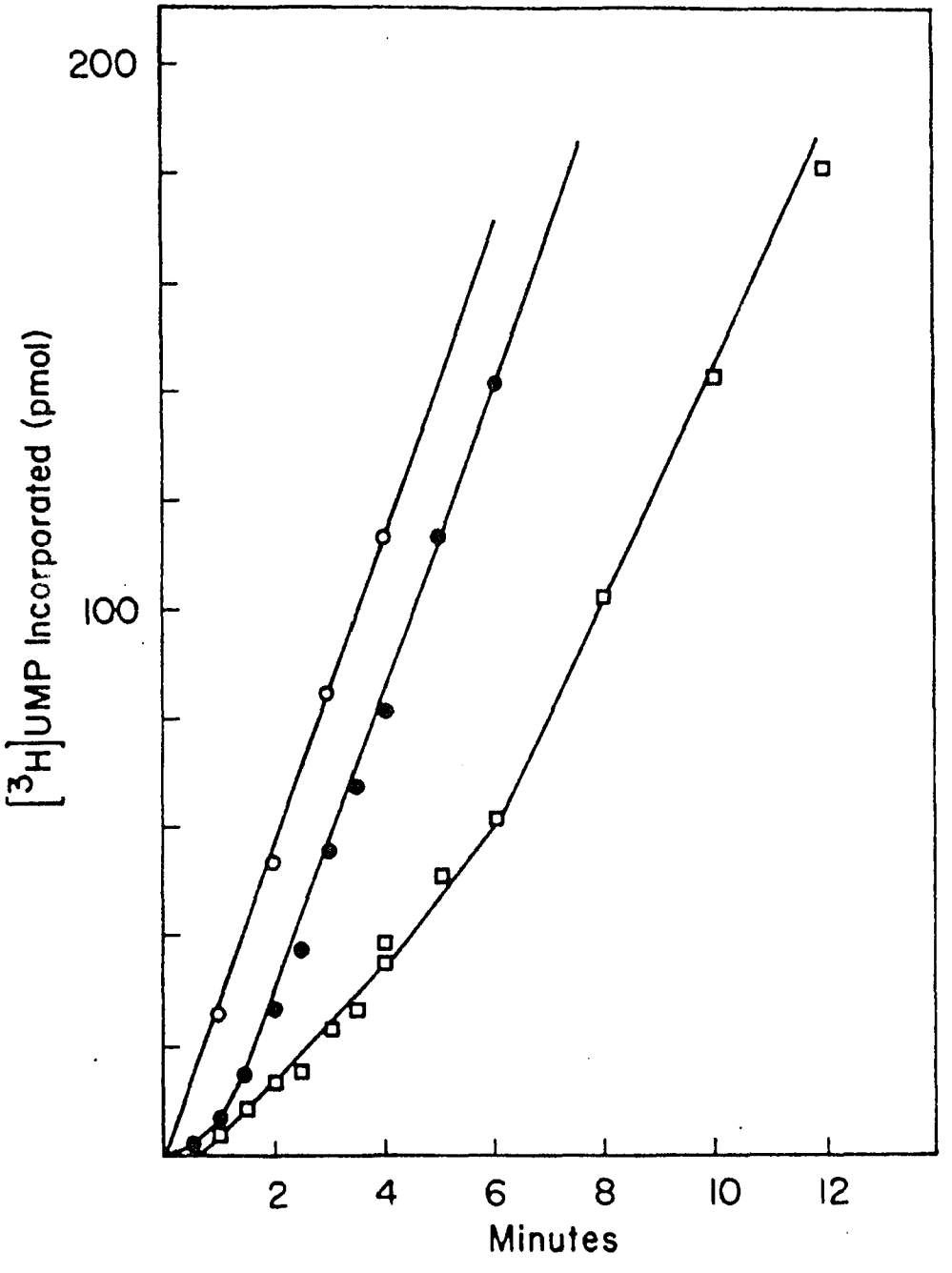

200
100
[3H]UMP Incorporated (pmol)
2
4
6
8
10
12
Minutes

Figure 8. Determination of the binding (K_B) and isomerization (k_2) constants for mAb 126C6-RNA polymerase and lac L8UV5. Abortive synthesis of ApApUpU is described under Materials and Methods. RNA polymerase was preincubated with mAb 126C6 for 30 min at 37°C. The molar ratio of antibody to alpha was 5 to 1. The tau_{obs} measurements were obtained from lag time assays performed at different RNA polymerase concentrations in the presence (●) and absence (o) of mAb 126C6. K_B and k_2 were calculated from the equation: $tau_{obs}=1/k_2 + 1/K_B[RNP]k_2$, (McClure, 1980). The K_B and k_2 values for RNA polymerase (o) are 438 uM^{-1} and 0.03 s^{-1}, respectively, and for mAb 126C6-RNA polymerase (●) 213 uM-^{1} and 0.007 s^{-1}, respectively.

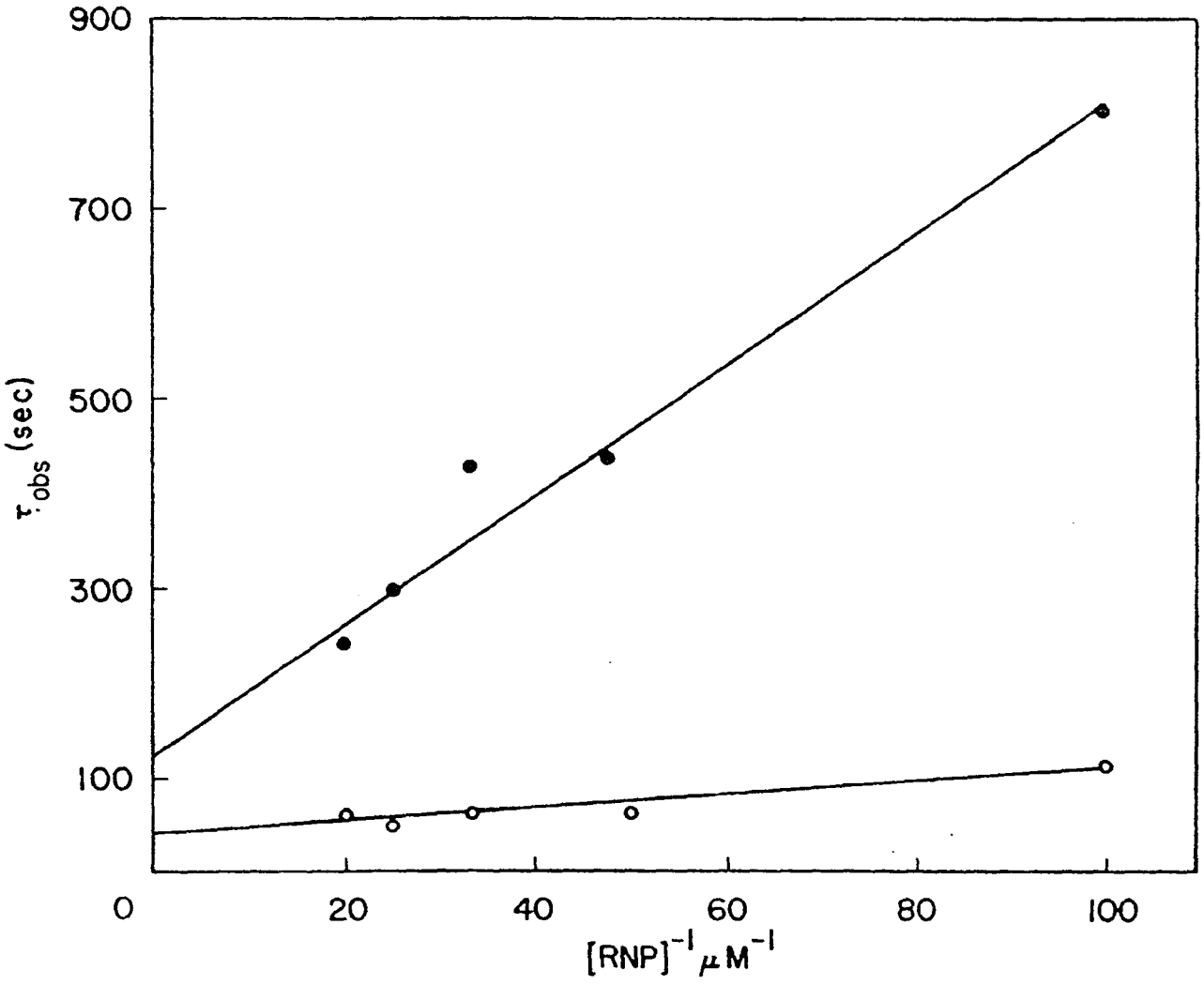
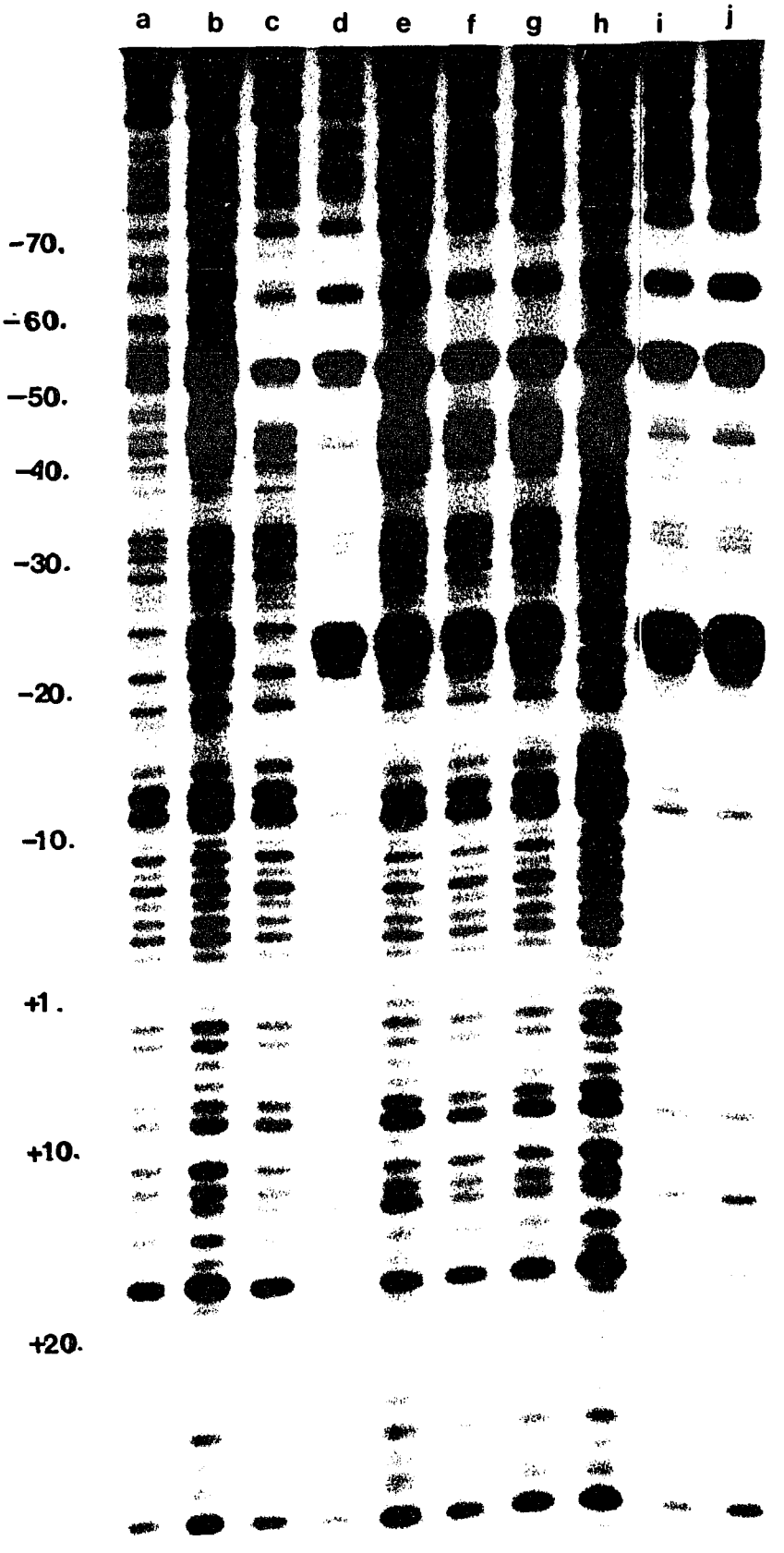

900
700
500
300
100
0
20
40
60
80
100
τ_{obs} (sec)
$[RNP]^{-1}\ \mu M^{-1}$

Figure 9. Effect of mAb 126C6 on interaction of RNA polymerase with the 3'[^{32}P]-labeled non-template strand of lac P+ promoter. Conditions for DNase I footprinting are as described under Materials and Methods using 3 nM 3'[^{32}P]lac P+ fragment and where indicated 40 nM CRP, 100 uM cAMP, and 50 nM RNA polymerase. The molar ratio of antibody to alpha was 5 to 1 for mAb 126C6, and 10 to 1 for mAb 129C4 and mAb 124D1. The immunocomplexes were formed by incubation of RNA polymerase with the indicated antibody at 37^{o}C for 30 minutes. Lane a, [^{32}P]lac P+; lane b, RNA polymerase, [^{32}P]lac P+; lane c, cAMP, CRP, and [^{32}P]lac P+; lane d, preformed open promoter complex (RP_o) = cAMP-CRP-[^{32}P]lac P+-RNA polymerase; lane e, RP_o incubated with mAb 126C6 for 10 min at 37^{o}C; lane f, RP_o incubated with mAb 126C6 for 20 min at 37^{o}C; lane g, RP_o incubated with mAb 126C6 for 40 min at 37^{o}C; lane h, mAb 126C6-polymerase complex incubated with cAMP, CRP, and [^{32}P]lac P+ for 30 min at 37^{o}C; lane i, mAb 129C4-polymerase complex incubated with cAMP, CRP, and [^{32}P]lac P+ for 30 min at 37^{o}C; lane j, mAb 124D1-polymerase complex incubated with cAMP, CRP, and [^{32}P]lac P+ for 30 min at 37^{o}C.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-40.
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+10.
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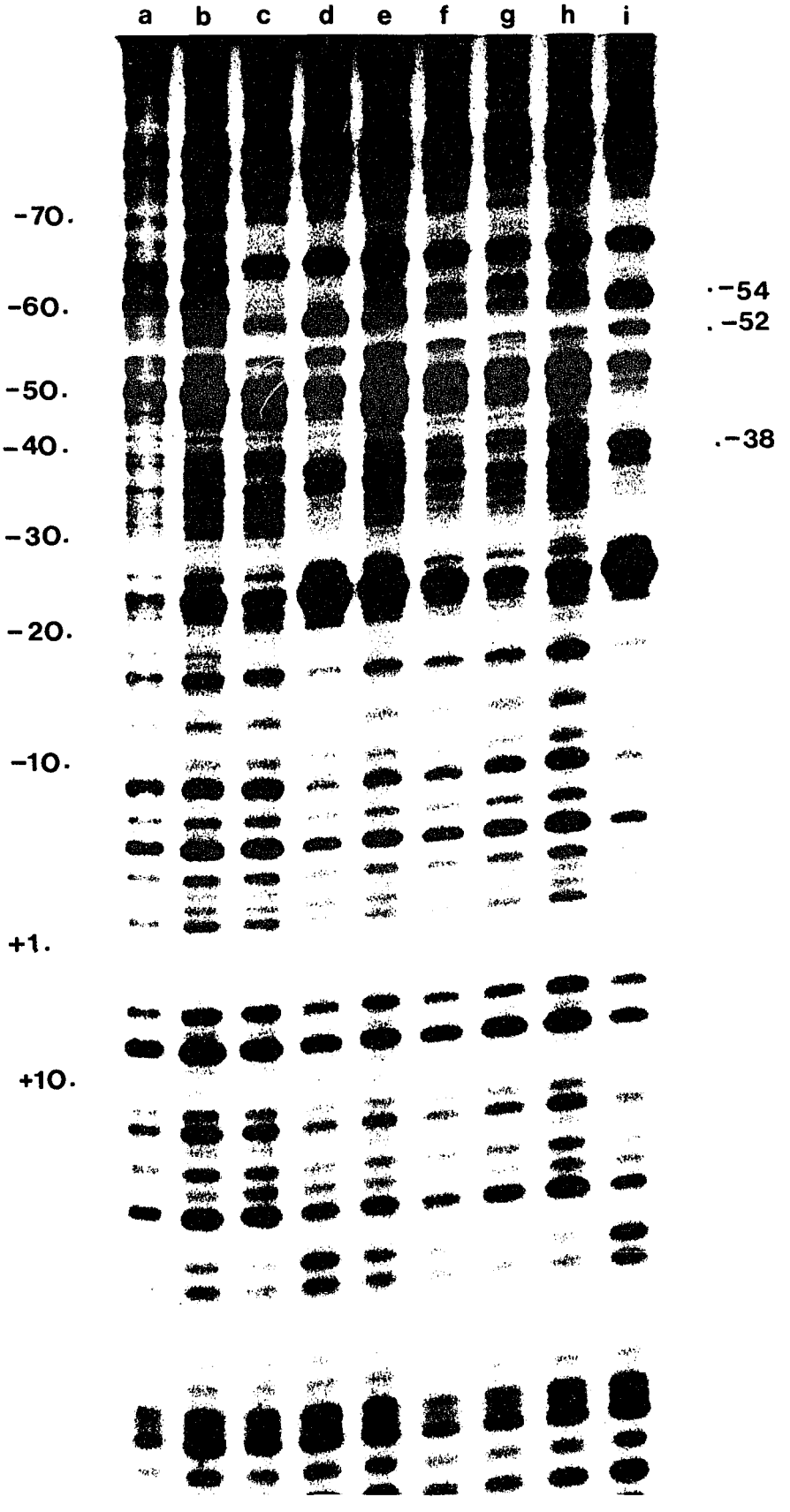
Figure 10. Effect of mAb 126C6 on interaction of RNA polymerase with the 5'[^{32}P]-labeled template strand of lac P+ promoter. Conditions for DNase I footprinting are as described under Materials and Methods using 3 nM 5'[^{32}P]lac P+ fragment and where indicated 40 nM CRP, 100 uM cAMP, and 50 nM RNA polymerase. The molar ratio of antibody to alpha was 5 to 1 for mAb 126C6, and 10 to 1 for mAb 129C4. The immunocomplexes were formed by incubation of RNA polymerase with the indicated antibody at 37^{o}C for 30 minutes. Lane a, [^{32}P]lac P+; lane b, RNA polymerase, [^{32}P]lac P+; lane c, cAMP, CRP, and [^{32}P]lac P+; lane d, preformed open promoter complex (RP_o) = cAMP-CRP-[^{32}P]lac P+-RNA polymerase; lane e, RP_o incubated with mAb 126C6 for 10 min at 37^{o}C; lane f, RP_o incubated with mAb 126C6 for 20 min at 37^{o}C; lane g, RP_o incubated with mAb 126C6 for 40 min at 37^{o}C; lane h, mAb 126C6-polymerase complex incubated with cAMP, CRP, and [^{32}P]lac P+ for 30 min at 37^{o}C; lane i, mAb 129C4-polymerase complex incubated with cAMP, CRP, and [^{32}P]lac P+ for 30 min at 37^{o}C.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-50.
-40.
-30.
-20.
-10.
+1.
+10.
-54
-52
-38

Figure 11. Effect of mAb 126C6 on interaction of RNA polymerase with the 3'[^{32}P]-labeled non-template strand of lac L8UV5 promoter. Conditions for DNase I footprinting are as described under Materials and Methods using 3 nM 3'[^{32}P]lac UV5 fragment and where indicated 50 nM RNA polymerase. The molar ratios of antibody to alpha was 5 to 1 for mAb 126C6, and 10 to 1 for mAbs 129C4 and 124D1. The immunocomplexes were formed by incubation of RNA polymerase with the indicated antibody at 37^{o}C for 30 minutes. Lane a, [^{32}P]lac UV5; lane b, RNA polymerase, and [^{32}P]lac UV5; lane c, mAb 126C6-polymerase and [^{32}P]-lac UV5 incubated for 15 min at 37^{o}C; lane d, mAb 129C4-polymerase and [^{32}P]lac UV5 incubated for 15 min at 37^{o}C; lane e, mAb 124D1-polymerase and [^{32}P]lac UV5 incubated for 15 min at 37^{o}C; lane f, polymerase and [^{32}P]lac UV5 preincubated for 10 min at 37^{o}C followed by incubation with mAb 126C6 for 30 min at 37^{o}C; lane g, polymerase and [^{32}P]lac UV5 preincubated for 10 min at 37^{o}C followed by incubation with F_{ab}-126C6 for 30 min at 37^{o}C.

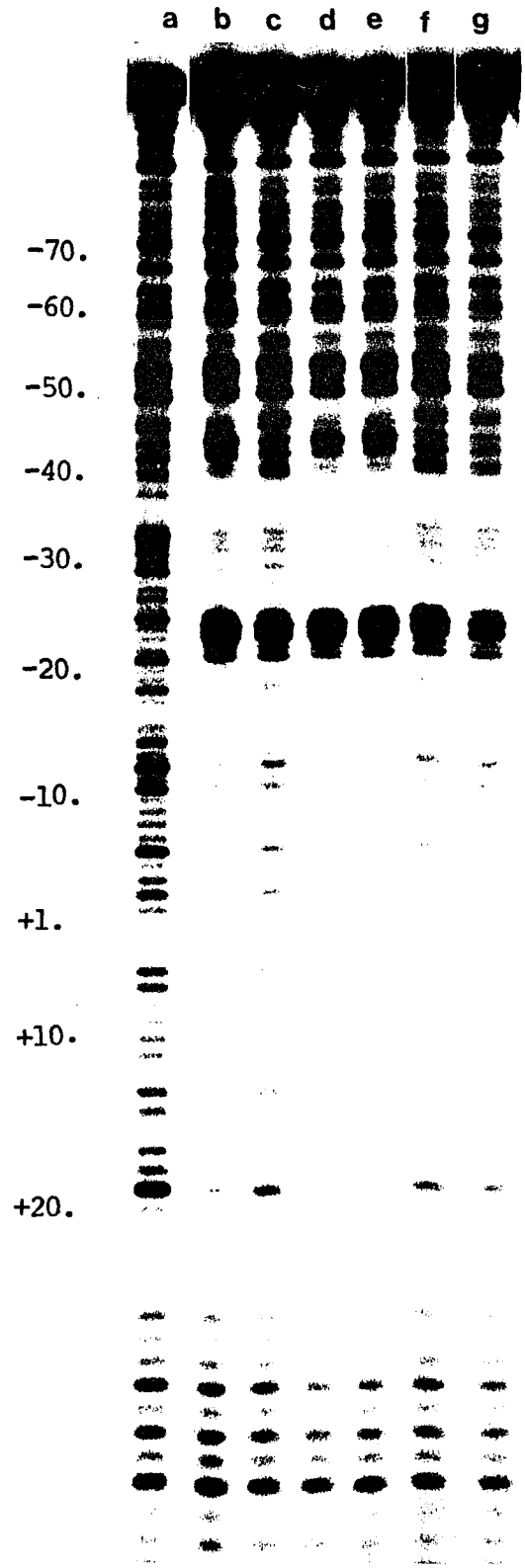
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Figure 12. Effect of mAb 126C6 on interaction of RNA polymerase with the 5'[^{32}P]-labeled template strand of lac L8UV5 promoter: time course for formation of the open promoter complex. Conditions for DNase I footprinting are as described under Materials and Methods using 3 nM 5'[^{32}P]lac UV5 fragment and where indicated 50 nM RNA polymerase. The molar ratio of antibody to alpha was 5 to 1 for mAb 126C6 and 10 to 1 for mAb 129C4. The immunocomplexes were formed by incubation of RNA polymerase with the indicated antibody at 37^{o}C for 30 minutes. Prior to digestion with DNase I the polymerase and indicated mAb-polymerase complex were incubated with the [^{32}P]lac UV5 at 37^{o}C for the times indicated. Lane a, [^{32}P]lac UV5 only; lanes b-e: mAb 126C6-RNA polymerase complex and [^{32}P]lac UV5 incubated at 37^{o}C for 1, 3, 20, and 60 minutes, respectively; lanes f-h: polymerase, and [^{32}P]lac UV5 incubated for 10 seconds, 3, and 60 minutes, respectively; lanes i-l: mAb 129C4-polymerase, and [^{32}P]lac UV5 incubated for 1, 3, 20, and 60 minutes, respectively.

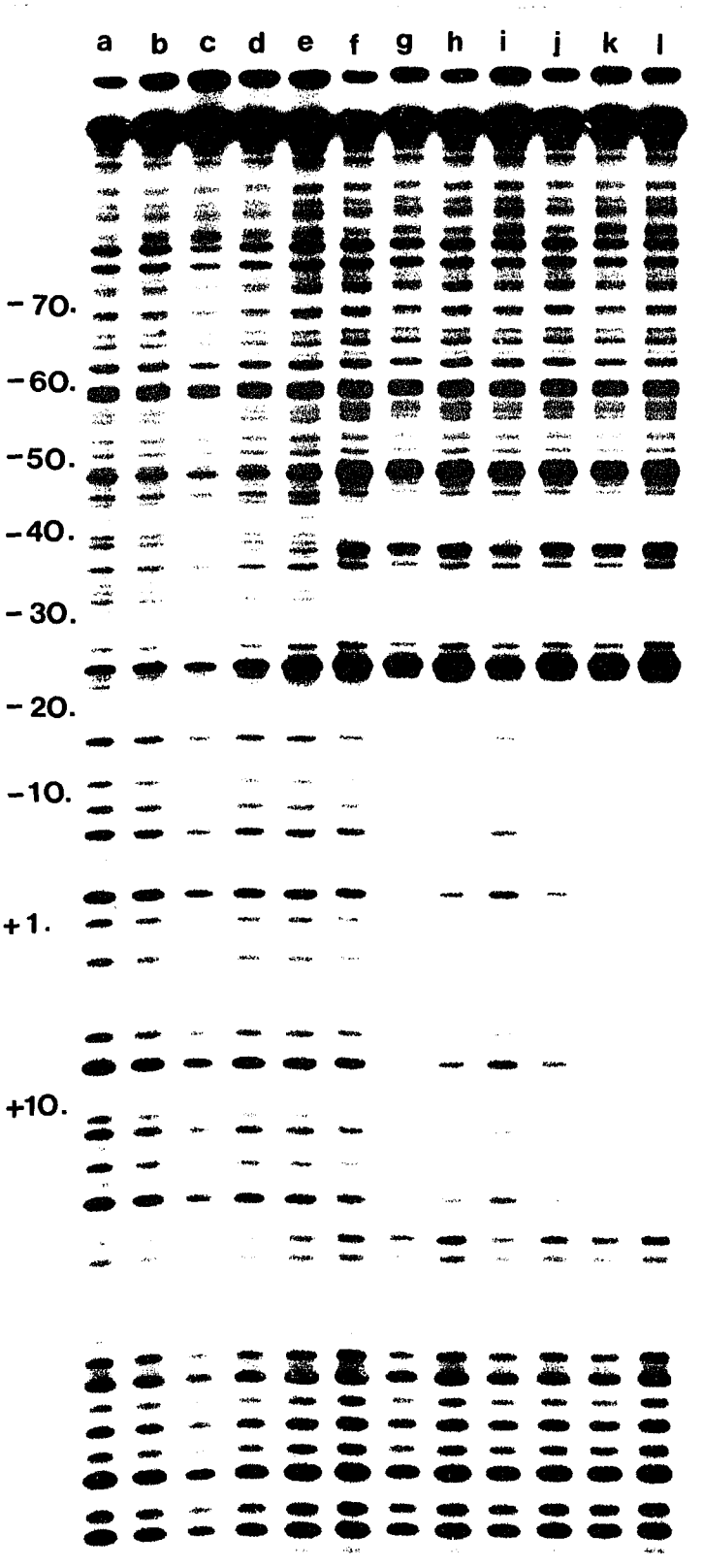
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Figure 13. Effect of mAb 126C6 on the polymerase-dependent interaction of cAMP-CRP with the 3'[^{32}P]-labeled non-template strand of *lac* L8UV5 promoter. Conditions for DNase I footprinting are as described under Materials and Methods using 3 nM 3' [^{32}P]*lac* UV5 fragment and where indicated 40 nM CRP, 100 uM cAMP, and 50 nM RNA polymerase. The molar ratio of antibody to alpha was 5 to 1 for mAb 126C6, and 10 to 1 for mAbs 129C4 and 124D1. The immunocomplexes were formed by incubation of RNA polymerase with the indicated antibody at 37^{o}C for 30 minutes. Lane a, [^{32}P]*lac* UV5 only; lane b, cAMP-CRP, RNA polymerase, and [^{32}P]*lac* UV5 incubated for 45 min at 37^{o}C; lane c, preformed mAb 129C4-polymerase-mAb 124D1 complex, cAMP-CRP, and [^{32}P]*lac* UV5 incubated for 15 min at 37^{o}C; lane d, mAb 126C6-polymerase, cAMP, CRP, and [^{32}P]*lac* UV5 incubated for 30 min at 37^{o}C; lane e, cAMP-CRP-[^{32}P]*lac* UV5-polymerase complex formed by incubation for 15 min at 37^{o}C followed by incubation with mAb 126C6 for 30 min at 37^{o}C; lane f, F_{ab} 126C6-polymerase, cAMP-CRP, and [^{32}P]*lac* UV5 incubated for 30 min at 37^{o}C; lane g, cAMP-CRP-[^{32}P]*lac* UV5-polymerase complex formed by incubation for 15 min at 37^{o}C followed by incubation with F_{ab} 126C6 for 30 min at 37^{o}C; lane h, cAMP-CRP and [^{32}P]*lac* UV5 incubated for 30 min at 37^{o}C.

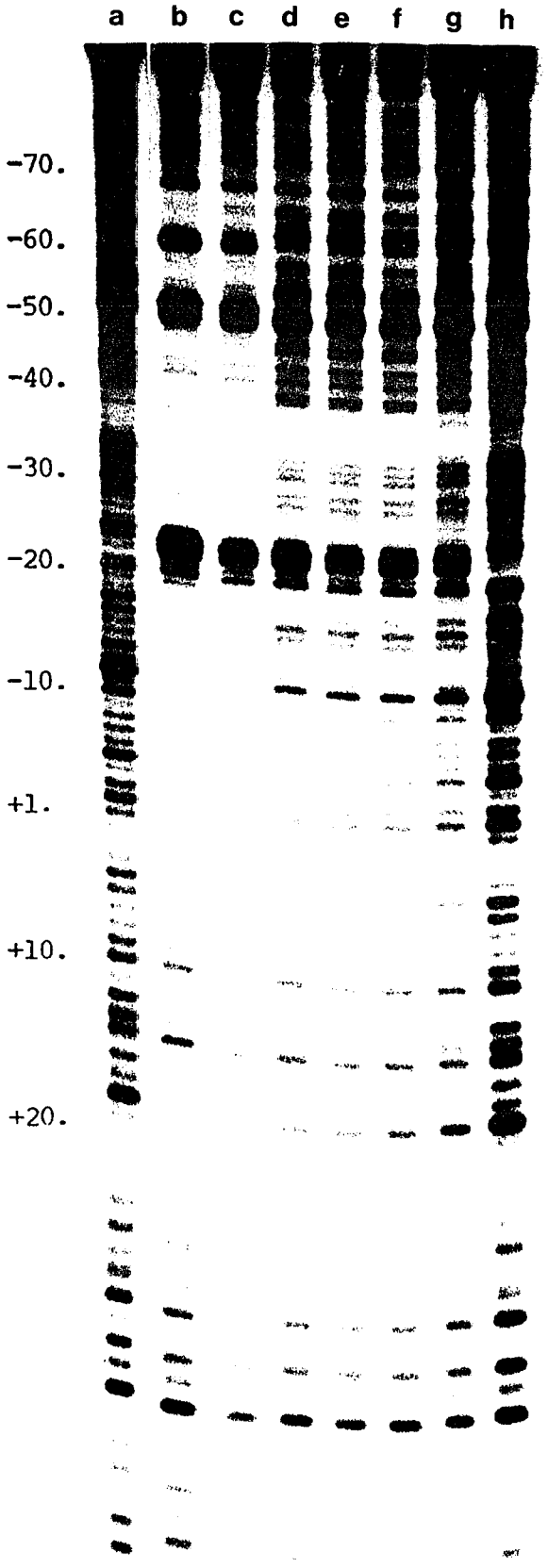
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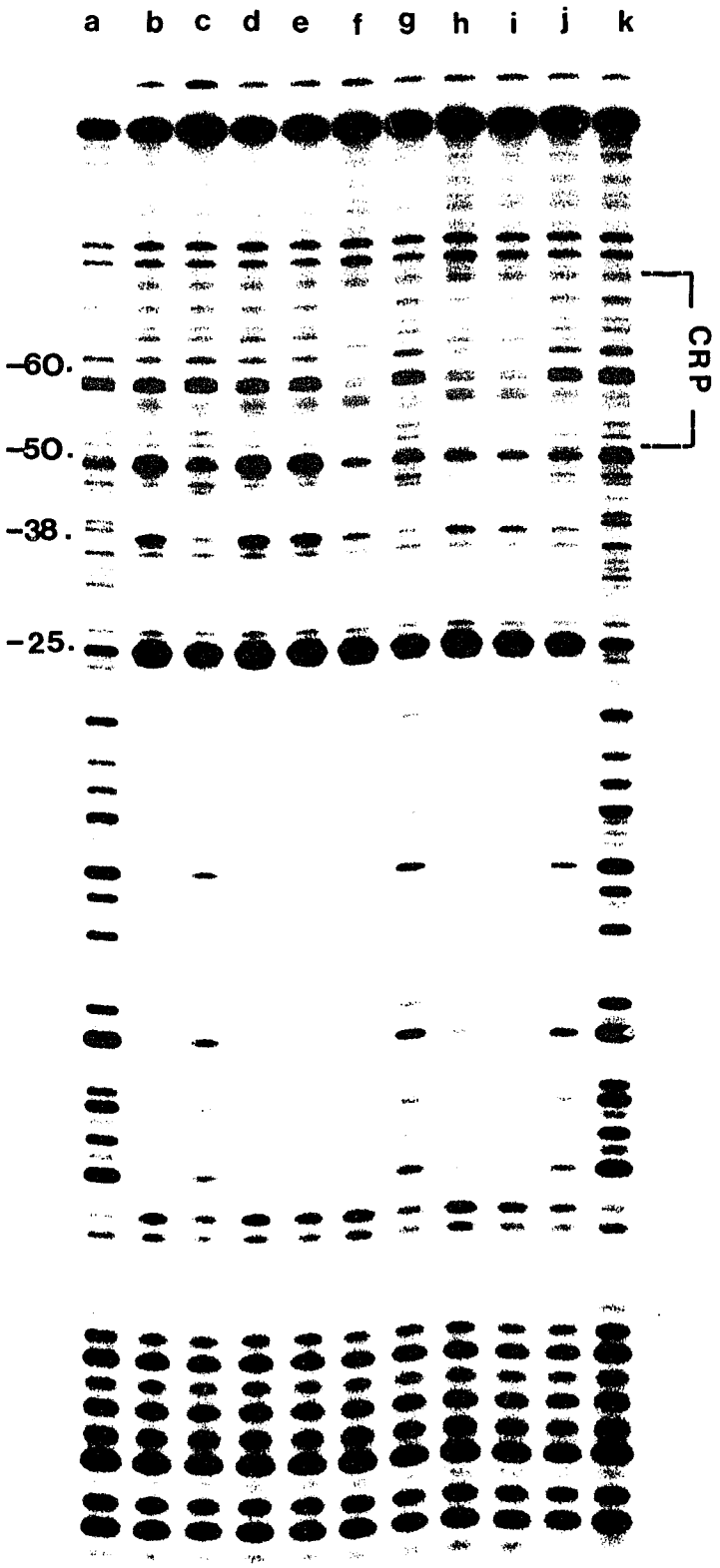
Figure 13a. Effect of mAb 126C6 on the polymerase-dependent interaction of cAMP-CRP with the 5'[^{32}P]-labeled template strand of lac L8UV5 promoter. Conditions for DNase I footprinting are as described under Materials and Methods using 3 nM 5' [^{32}P]lac L8UV5 fragment and where indicated 40 nM CRP, 100 uM cAMP, and 50 nM RNA polymerase. The molar ratio of antibody to alpha was 5 to 1 for mAb 126C6, and 10 to 1 for mAbs 129C4 and 124D1. The immunocomplexes were formed by incubation of RNA polymerase with the indicated antibody at 37^{o}C for 30 minutes. Lane a, [^{32}P]lac UV5 only; lane b, RNA polymerase, and [^{32}P]lac UV5; lane c, mAb 126C6-polymerase and [^{32}P]lac UV5 incubated for 15 min at 37^{o}C; lane d, mAb 129C4-polymerase and [^{32}P]lac UV5 incubated for 15 min at 37^{o}C; lane e, mAb 124D1-polymerase and [^{32}P]lac UV5 incubated for 15 min at 37^{o}C; lane f, cAMP, CRP, RNA polymerase, and [^{32}P]lac UV5 incubated for 45 min at 37^{o}C; lane g, cAMP-CRP-[^{32}P]lac UV5-polymerase complex formed by incubation for 15 min at 37^{o}C followed by incubation with mAb 126C6 for 30 min at 37^{o}C; lane h, RP_o incubated with mAb 129C4 for 30 min at 37^{o}C; lane i, RP_o incubated with mAb 124D1 for 30 min at 37^{o}C; lane j, cAMP-CRP-[^{32}P]lac UV5-polymerase complex formed by incubation for 15 min at 37^{o}C followed by incubation with F_{ab} 126C6 for 30 min at 37^{o}C; lane k, cAMP, CRP and [^{32}P]lac UV5 incubated for 30 min at 37^{o}C.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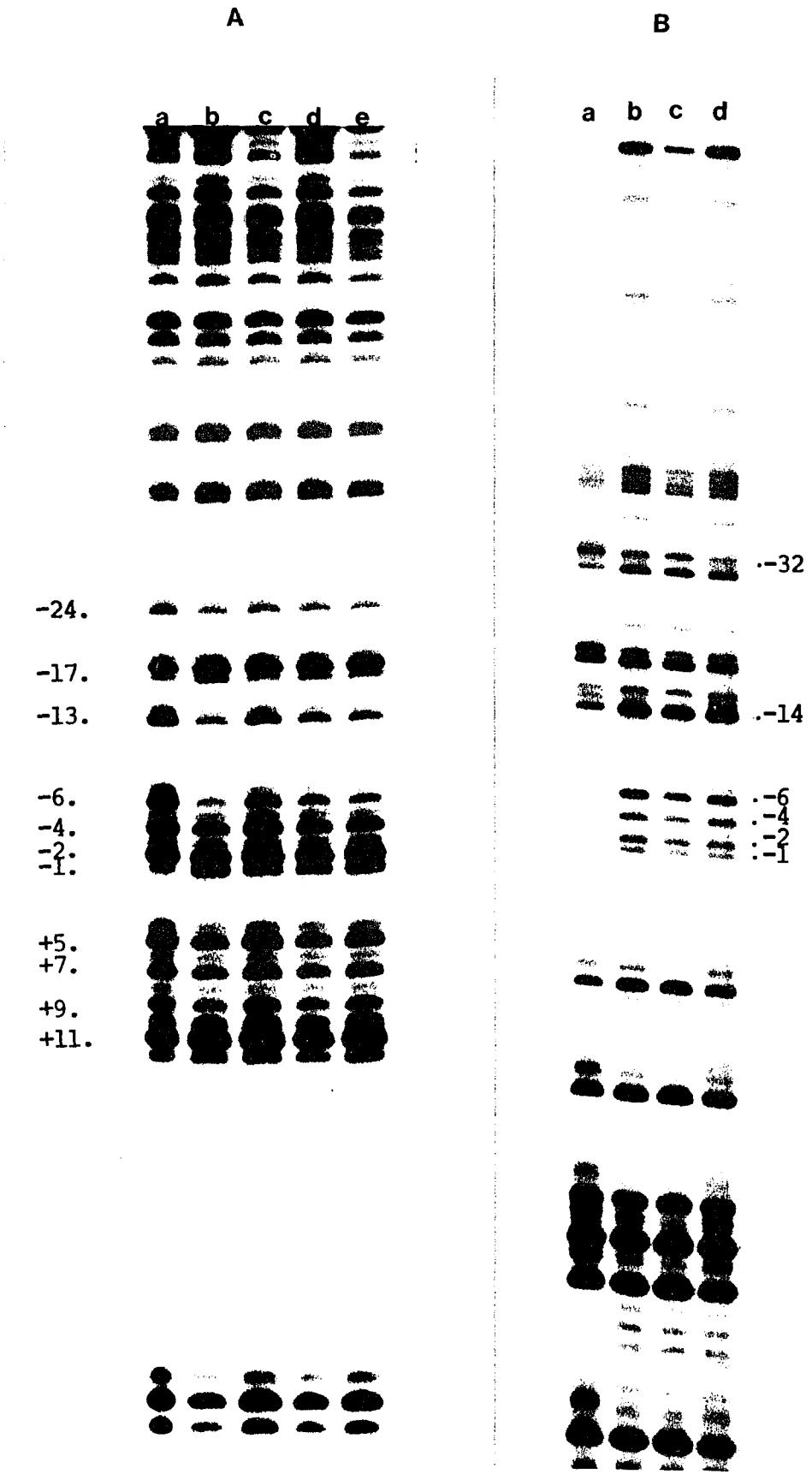
-60.
-50.
-38.
-25.
CRP

Figure 14. Effect of mAb 126C6 on reactivity of guanine and cytosine residues in the RNA polymerase-lac UV5 complex to methylation by dimethyl sulfate. Reaction conditions are described under Materials amd Methods using 3 nM of 3'(non-template) or 5'(template) [^{32}P]lac UV5 promoter fragments and 50 nM RNA polymerase. The molar ratio of antibody to alpha was 5 to 1 for mAb 126C6, and 10 to 1 for mAb 129C4. The immunocomplexes were formed by incubation of RNA polymerase with the indicated antibody at 37^{o}C for 30 minutes. Panel A, non-template strand, guanine methylation pattern ("G" reaction); lane a, 3'[^{32}P]lac UV5 only; lane b, polymerase and [^{32}P]lac UV5; lane c, mAb 126C6-polymerase incubated with [^{32}P]lac UV5 for 20 min at 37^{o}C; lane d, polymerase and [^{32}P]lac UV5 preincubated for 10 min at 37^{o}C followed by incubation with mAb 126C6 for 30 min at 37^{o}C; lane e, mAb 129C4-polymerase incubated with [^{32}P]lac UV5 for 20 min at 37^{o}C. Panel B, template strand, "G" plus "C" reactions; lane a, 5'[^{32}P]lac UV5 only; lane b, mAb 126C6-polymerase incubated with 5'[^{32}P]lac UV5 for 20 min at 37^{o}C; lane c, polymerase and [^{32}P]lac UV5 preincubated for 10 min at 37^{o}C followed by incubation with mAb 126C6 for 30 min at 37^{o}C; lane d, polymerase and [^{32}P]lac UV5.


A
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a b c d e
a b c d
-24.
-17.
-13.
-6.
-4.
-2.
-1.
+5.
+7.
+9.
+11.
-32
-14
-6
-4
-2
-1

Figure 15. Effect of mAb 126C6 on the reactivity of the cytosine residues to dimethyl sulfate during the course of formation of the RNA polymerase-*lac* UV5 open promoter complex. Conditions for methylation with dimethyl sulfate are as described under Materials and Methods using 3 nM 5'[^{32}P]*lac* UV5 fragment and where indicated 50 nM RNA polymerase. The molar ratio of mAb 126C6 to alpha was 5 to 1. The immunocomplex was formed by preincubation of RNA polymerase with mAb 126C6 for 30 min at 37°C. Prior to treatment with dimethyl sulfate the polymerase or mAb 126C6-polymerase complex were incubated at 37°C with the [^{32}P]*lac* UV5 for the time indicated. Lane a, [^{32}P]*lac* UV5 only; lanes b-f: mAb 126C6-RNA polymerase complex and [^{32}P]*lac* UV5 incubated at 37°C for 1, 2.5, 5, 10, and 20 minutes, respectively; lanes g-j: polymerase, and [^{32}P]*lac* UV5 incubated for 0.5, 1, 2.5, and 20 minutes, respectively.

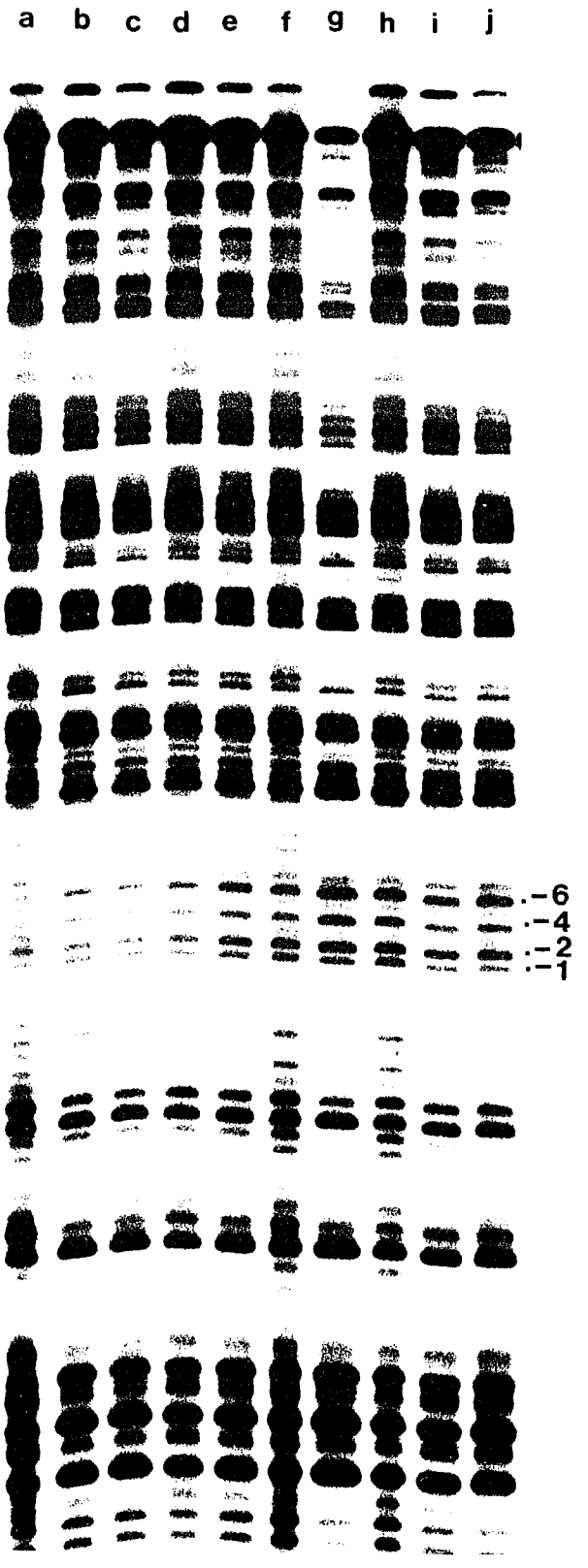
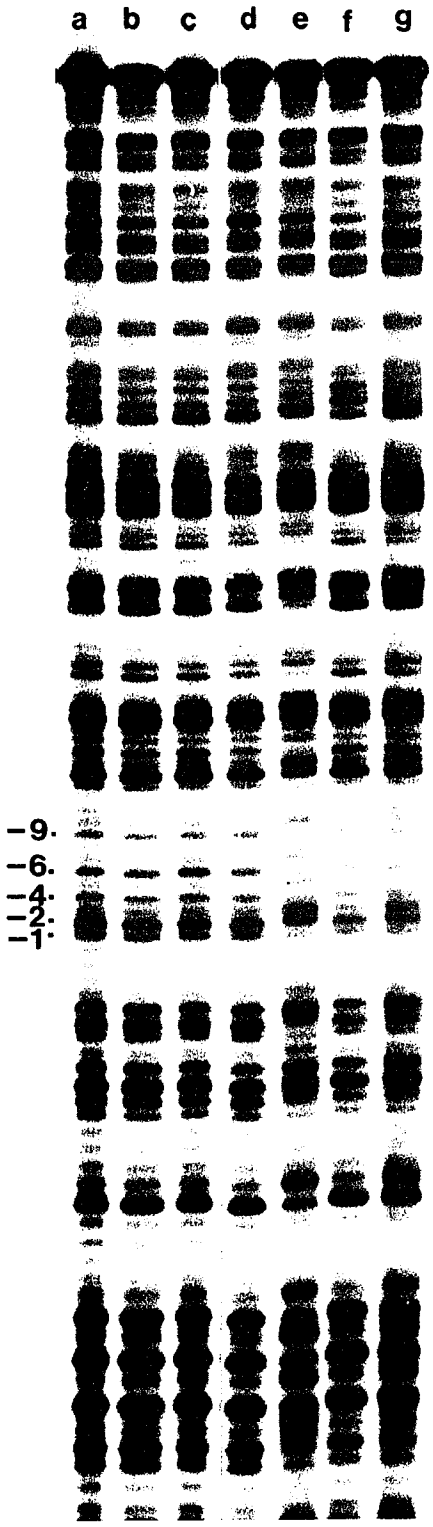

a b c d e f g h i j
-6
-4
-2
-1

Figure 16. Effect of mAb 126C6 on reactivity of guanine and cytosine residues in the RNA polymerase-lac P+ complex to methylation by dimethyl sulfate. Reaction conditions are described under Materials amd Methods using 3 nM of 5'(template) [^{32}P]lac P+ promoter fragment, and where indicated 40 nM CRP, 100 uM cAMP, and 50 nM RNA polymerase. The molar ratio of mAb 126C6 to alpha was 5 to 1. The immunocomplex was formed by incubation of RNA polymerase with mAb 126C6 for 30 min at 37^{o}C. Lane a, RP_o formed by incubation of cAMP, CRP, polymerase, and [^{32}P]lac P+ for 20 min at 37^{o}C; lane b, RP_o incubated with mAb 126C6 for 10 min at 37^{o}C; lane c, RP_o incubated with mAb 126C6 for 20 min at 37^{o}C; lane d, RP_o incubated with mAb 126C6 for 40 min at 37^{o}C; lane e, mAb 126C6-polymerase complex incubated with cAMP, CRP, and [^{32}P]lac P+ for 30 min at 37^{o}C; lane f, polymerase and [^{32}P]lac P+ incubated for 30 min at 37^{o}C; lane g, cAMP, CRP, and [^{32}P]lac P+ incubated for 30 min at 37^{o}C.


a b c d e f g
-9.
-6.
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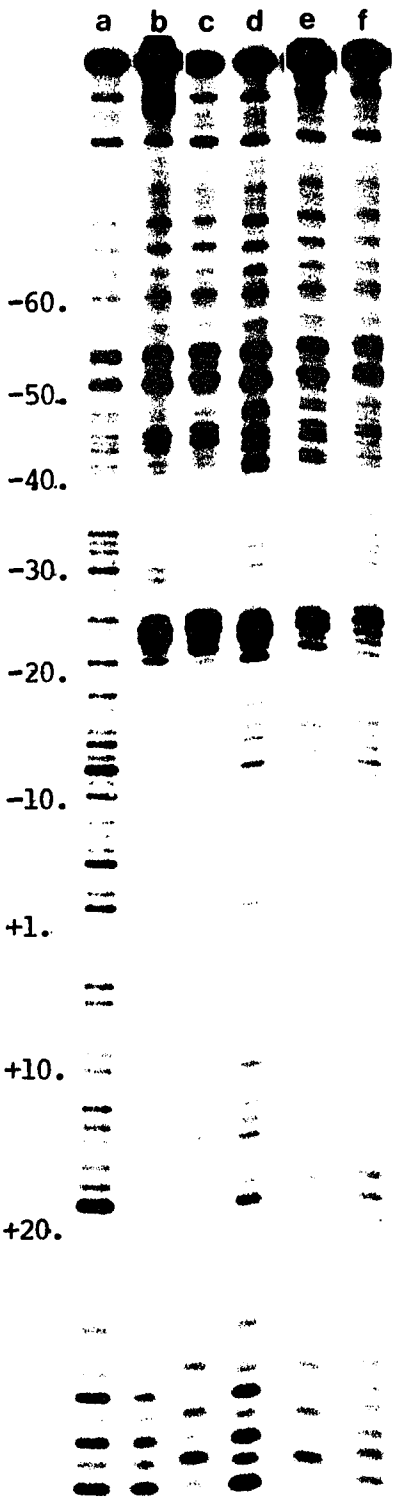
-2.
-1.

Figure 17. Effect of mAb 126C6 on protection of lac UV5 against DNase I attack during abortive transcription. Conditions for DNase I footprinting are as described under Materials and Methods using 3 nM 3'[^{32}P]lac UV5 fragment (non-template strand) and where indicated 50 nM RNA polymerase. The molar ratio of mAb 126C6 to alpha was 5 to 1. The preformed immunocomplex was formed by incubation of RNA polymerase with the mAb for 30 min at 37^{o}C. RNA polymerase or mAb 126C6-polymerase complex were incubated with [^{32}P]lac UV5 for 20 min at 37^{o}C followed by addition of 1 mM ApA plus 50 uM UTP and incubated for the time indicated. Lane a, [^{32}P]lac UV5 only; lane b, RNA polymerase, and [^{32}P]lac UV5; lane c, [^{32}P]lac UV5-polymerase complex incubated with ApA and UTP for 5 min at 37^{o}C; lane d, mAb 126C6-polymerase, and [^{32}P]lac UV5; lane e, mAb 129C4-polymerase-[^{32}P]lac UV5 incubated with ApA and UTP for 5 min at 37^{o}C; lane f, mAb 126C6-polymerase-[^{32}P]lac UV5 incubated for 30 min at 37^{o}C.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+20.

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