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CHARACTERIZATION OF EFFECTS OF ANTI-BETA AND ANTI-BETA' MONOCLONAL ANTIBODIES ON THE ACTIVITY OF THE RNA POLYMERASE FROM ESCHERICHIA COLI

City University of New York PH.D. 1986

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CHARACTERIZATION OF EFFECTS OF ANTI-BETA AND ANTI-BETA' MONOCLONAL ANTIBODIES ON THE ACTIVITY OF THE RNA POLYMERASE FROM ESCHERICHIA COLI

by

PATRICIA ROCKWELL

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

1986

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

June 18, 1986
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Joseph S Krakow
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ABSTRACT

TITLE: Characterization of Effects of Anti-Beta and Anti-Beta' Monoclonal Antibodies on the Activity of the RNA Polymerase from Escherichia coli

BY: Patricia Rockwell

ADVISOR: Professor Joseph S. Krakow

Monoclonal antibodies (mAbs) directed against antigenic determinants on the beta and beta' subunits of the E. coli RNA polymerase were prepared using purified subunits as the immunogens. Anti-beta and anti-beta' mAbs were found which inhibited polymerase activity. Inhibition by the anti-beta mAb 210E8 varied with template sequence and conformation while the anti-beta' mAb 311G2 exhibited a potent inhibition on all templates studied. The abortive initiation reaction was more greatly affected by mAb 210E8 on linear than on supercoiled templates. On the supercoiled TAC16 promoter inhibition by mAb 210E8 was relieved when the spacer length between the -10 and -35 consensus regions was shifted from 16 to 18 base pairs. On supercoiled lac UV5 mAb 210E8 did not change the rate at which polymerase formed the closed promoter complex but decreased the rate of isomerization to form the open complex.

The reactivity of polymerase-lac UV5 (and TAC16) promoter complexes with DNase I and dimethyl sulfate was modified by the inhibitory mAbs. The mAb 210E8-polymerase-lac UV5 promoter complex forms an unstable intermediate that

could not convert to a fully active RP_O. Anti-beta' mAb 311G2-polymerase-promoter complexes are stable but inactive.

The data suggest that the beta and beta' subunits perform different functions. Evidence is provided supporting the role of the beta subunit in catalysis and in the positioning of polymerase on the promoter preceding RP_O formation. The beta' subunit is also implicated in catalysis in addition to its role in template binding. The studies demonstrate that subunit-specific monoclonal antibodies are novel reagents with which to probe the relation of structure and function in RNA polymerase.

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My work has benefited from the efforts of many people. I am gratefully indebted to my supervisor Dr. Joseph Krakow for his ingenious idea which created my project. His continued support and suggestions have helped me attain a greater perspective of science as well as a better understanding of the questions that should be addressed to critically evaluate a scientific problem. I am particularly grateful to the other members of my examining commitee, Drs. R. Rudner, H. Ozer, C. Russell, and E. McFall for their constructive comments on the thesis. I am also indebted to Drs. R. Rudner and H. Ozer for their support throughout my graduate education. I owe a special debt of thanks to E. Beasley for preparing my monoclonal antibodies and to C. Roman, G. Nair, and C. Colbert for their excellent technical assistance. I would also like to express my appreciation to C. Colbert for the arduous task of typing this thesis.

I would also like to acknowledge my parents whose support has been an inspiration towards my goal of obtaining this degree. Finally, I owe a special debt of thanks to my husband, Rocky, who, as my "number 1 fan" has provided me with unfailing support during my graduate education. My work has benefited from his scientific abilities through his preparation of computer programs to analyze a significant portion of my data. I would also like to express my appreciation for his compliance to endure my occassional outburats, preoccupations and frustrations with undaunted love and encouragement.

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Introduction

The regulation of gene expression in prokaryotes is primarily controlled at the level of transcription initiation by RNA polymerase and involves a complex series of interactions between RNA polymerase and specific promoter sites. The rate of RNA chain synthesis occurs with widely varying frequencies and it appears that the DNA sequence, DNA conformation and various regulatory proteins that serve as activators or repressors determine promoter strength, that is, the efficiency at which a promoter initiates the synthesis of RNA transcripts. (see von Hippel et al., 1984 and McClure, 1985 for recent reviews).

The E.coli RNA polymerase is a large complex enzyme consisting of four different subunits alpha, beta, and beta' to form a catalytic core and sigma which associates with the core to form the holoenzyme. The holoenzyme form of polymerase enables the enzyme to bind specifically to promoter sites and initiate transcription. The enzyme engages in a number of sequential steps for the synthesis of RNA (Kumar, 1980). Template binding by polymerase results in a localized denaturation of DNA. Upon strand separation initiation proceeds with the binding by polymerase primarily of a purine nucleoside triphosphate which is complementary to a sequence specific site on the template strand. This is followed by the binding of a second NTP and the formation

of the first phosphodiester bond. The elongation of an RNA chain then proceeds by a processive addition of nucleotides to the dinucleoside tetraphosphate in a 5' to 3' direction. Each addition leads to a translocation event in which the terminal nucleotide is removed from the substrate binding site and the enzyme moves to the next base on the DNA to yield a RNA sequence which is complementary to the DNA template. After the incorporation of approximately 9 nucleotides the sigma subunit dissociates from the enzyme and the complex shifts to an elongation conformation in which polymerization continues with the core enzyme. Elongation ceases when the complex reaches a termination site and both the RNA product and enzyme dissociate from the template.

How RNA synthesis is regulated during these steps is dependent upon specific interactions of polymerase with DNA promoter sites and attendant conformational changes in both the RNA polymerase holoenzyme (Fisher and Blumenthal, 1980) and DNA (Gamper and Hearst, 1982) preceding RNA chain initiation. Thus far, the correlation of enzyme structure with function has not progressed much beyond the stage of assigning functions to particular subunits (Zillig et al., 1976). The enzyme has not yet been crystallized and the three dimensional structure of RNA polymerase derived from X-ray diffraction analysis is not available. Models of the structure of the E.col RNA polymerase derived from neutron small-angle scattering (Stockel et al., 1980), chemical

crosslinking (Hillel and Wu, 1977: Coggins et al., 1977), and immuno-electron microscopy (Tichelaar et al., 1983) have indicated the mode of subunit interaction.

Photochemical crosslinking studies by Hillel and Wu (1978) indicated that the nonspecific interactions of polymerase with T7 DNA resulted in a crosslinked product consisting of sigma, beta and beta' subunits. In contrast, the binding of polymerase to specific promoter sites yielded a crosslinked product containing only the sigma and beta subunit. The model proposed to explain these results states that the beta' subunit may facilitate the binding of RNA polymerase to the template but then moves away from the DNA to permit catalysis to occur at promoter specific sites by the beta subunit. Crosslinking studies with polymerase-lac UV5 promoter complexes have shown that the beta and sigma subunits contact thymine residues near the start site of transcription on the template strand of the DNA (Simpson, 1979).

Various other approaches have been employed to delineate RNA polymerase interactions during transcription: kinetic analysis (Krakow et al. 1976), affinity labeling (Hanna and Meares, 1983a; 1983b) immunological studies (Stender, 1979,1981) and genetic analyses of both polymerase subunit mutants (Yura and Ishihama, 1979) and regulatory mutants affecting the positive control of transcription initiation (Raibaud and Schwartz, 1985). Information regarding subunit involvement during transcription has been

obtained from photoaffinity labeling studies. The results showed that the 5' end of a trinucleotide product synthesized on T7 DNA contacts the DNA, the beta and sigma subunits while the synthesis of a 12 base pair product resulted in contacts with the DNA and the beta and beta' subunits (Hanna and Meares 1983a, 1983b).

The assignment of specific functions to the beta and beta' subunits stems from evidence obtained from mutational analyses, studies with inhibitors of enzyme function and the use of substrate analogs directed to react with functional groups at the active site (Kumar, 1980). The consensus of evidence from these studies has implicated the beta subunit in the catalytic activity of the enzyme and the beta' subunit in the template binding function. The proposed models do not define which regions of each subunit are involved in an interaction with adjacent subunits, the catalytic site, DNA template, RNA products or substrates.

As an alternate approach to the structural analyses of RNA polymerase, information regarding the mechanism of polymerase-DNA interactions has been obtained through structural studies of the DNA. Such studies have focused on two important functional interactions of RNA polymerase during the transcriptional process: first, the specific contacts formed between holoenzyme and promoter sequences during initiation and second, the continued association of the core enzyme with other DNA sites during elongation. The recognition of DNA by the enzyme appears to involve an

electrostatic interaction between polymerase and specific base pair sequences within the duplex helical conformation of the template. It has been proposed that the initial binding step also involves a series of associations and dissociations in which RNA polymerase diffuses or slides along the DNA to specific promoter sites (von Hippel, 1982).

Three major approaches have been employed to define the RNA polymerase recognition signals on the DNA. First, a DNA sequence analysis has shown that certain homologous regions occur with high frequencies among promoters (Rosenberg and Court, 1980). These consensus sequences are known to lie -35 (TTGACA) and -10 (TATAAT) base pairs upstream from the initiation site. Second, a genetic analysis of mutated base pairs by substitution or deletion has shown that mutations which lie within the consensus regions result in a decrease (down mutation) or an increase (up mutation) in promoter activity (Stefano & Gralla, 1982a). Additional evidence that the consensus regions form the structural basis for promoter strength is obtained from the use of enzymatic and chemical reagents to probe the DNA structure during complex formation. In this procedure the location and extent of polymerase binding is indicated by the promoter regions which are protected from attack by the reagent. A DNase 1 footprint of polymerase-promoter complexes show that the protection by polymerase covers an area which stretches from positions -47 thru +22. However, a compilation of the data from these structural studies shows that polymerase appears

to bind to one side of the DNA from approximately position -55 to +20 with major contacts in the major grooves of the -35 and -10 consensus regions (von Hippel et al., 1984).

The kinetics of the RNA polymerase-promoter interaction have been extensively studied by employing the nitrocellulose filter binding assay, the abortive initiation assay and direct transcriptional analysis. In the filter binding technique protein-DNA complexes are trapped on a nitrocellulose filter while the free DNA is not retained. If the DNA is radiolabeled then the RNA polymerase-promoter complexes which form are estimated by the amount of radioactivity retained on the filter. Rate constants for the mechanism of binding for both specific and nonspecific RNA polymerase-promoter interactions have been obtained using the filter binding assay (Hinkle & Chamberlin 1972, Roe et al., 1984). However, this technique has limited use when employed to obtain accurate measurements of kinetic constants for polymerase binding with complexes that rapidly dissociate or DNA fragments possessing more than one promoter.

Studies with T7 DNA have provided evidence that transcription proceeds via a sequential formation of a closed promoter complex (RP_C) between RNA polymerase and the DNA to a stable open promoter complex, RP_O (Stahl and Chamberlin, 1977). The consequence of these events is the formation of a highly stable complex in which the DNA strands are locally melted for the subsequent initiation

step. The formation of a stable RP_o has been found to be affected by ionic strength, temperature, DNA topology and for certain promoters accessory proteins that serve as regulatory factors (McClure, 1985b). In E.coli, cyclic adenosine 3',5' monophosphate (cAMP) and the cAMP receptor protein (CRP) are required to activate RP_o formation for the lac, gal, and other promoters that are sensitive to catabolite repression (de Crombrugghe et al., 1984).

The measurement of an active RP_o has been obtained from the use of the abortive initiation assay (McClure, 1980b) or by a direct analysis of the transcribed RNA product. Although the direct analysis provides information on the size and number of the transcripts formed, the results of in vitro transcription studies have been shown to be distorted by premature termination or polymerase pausing. For example, in the lac UV5 promoter a cycling reaction involving the synthesis of short oligonucleotides appears to contribute to the overall frequency of long chain transcript formation. (Carpousis and Gralla, 1980).

The regulation of transcription in vivo appears to be a function of promoter strength (McClure, 1980). For this reason, the abortive initiation assay has been employed as a means to define promoter strength. The assay is a holoenzyme specific, steady state reaction in which RNA polymerase synthesizes a short transcript that is complementary for the start sequence of transcription. The product aborts from the complex due to the omission of one

(or more) ribonucleoside triphosphates to prevent elongation. Using this assay promoter strength is operationally defined in terms of initiation rates of RNA chain synthesis. The method relies on the assumption that the rates of abortive product formation are directly proportional to RNA polymerase in RP_o formation. In addition, the time required to attain the steady state rate has been shown to be promoter specific. Based on this criterion the abortive initiation reaction is employed as a means to quantify intrinsic promoter strength using a kinetic analysis that partitions RP_o formation into two functional parameters. The analysis yields independent measurements of the binding of polymerase to promoter (K_B) and the rate of isomerization of the closed complex to an open state (k_2).

Intrinsic promoter strength depends upon DNA sequence, DNA conformation, electrostatic interactions within the DNA template and the spacer length between the consensus regions (McClure,1985). Any change in these components will alter promoter strength. Promoter activities can also be changed by the binding of accessory proteins that serve as transcriptional activators or repressors. Promoter strength changes are measured as increases or decreases in either the affinity of polymerase for promoter (K_B) or the rate of RP_o formation (k_2).

The use of the abortive initiation assay in conjunctions with a footprinting analysis has provided

additional information on the number of polymerase-promoter interactions involved in the formation of active initiation complexes on the lac UV5 promoter (Buc and McClure, 1985; Spassky et al., 1985). As a result of these studies the pathway to RP_O formation has been defined as a multistep scheme in which the formation of a stable but inactive intermediate complex is the rate limiting step.

$$R + P \underset{K_1}{\rightleftharpoons} RP_C \underset{\kappa_{-2}}{\overset{\kappa_2}{\rightleftharpoons}} RP_I \underset{\kappa_{-3}}{\overset{\kappa_3}{\rightleftharpoons}} RP_O$$

It should be stressed that the quantification of the scheme as two separate constants is merely a simplified expression of the complexity of the RNA polymerase-promoter interactions encompassing each value. The K_B value comprises the initial binding reaction in which polymerase interactions with nonspecific DNA eventually lead to the formation of a closed complex of polymerase bound to specific promoter targets. Also the final conversion step to RP_O may occur with a number of intermediates along the pathway. Indeed, the formation of more than one intermediate complex has been postulated to explain kinetic estimates of RNA polymerase interactions with the lambda P_R promoter (Roe et al., 1984). The complexity of the final isomerization step to an active RP_O is also inferred from structural analysis of RNA polymerase-lac UV5 promoter complexes (Spassky et al., 1985). The results of these studies suggest that the formation of a preinitiation

complex demands a strict positioning of polymerase with its DNA contacts in the consensus regions followed by a conformational shift of the enzyme to penetrate the grooves of the DNA for specific protein-base pair interactions. It has also been proposed that the mechanism by which polymerase recognizes the consensus involves a direct hydrogen bond interaction between specific base pairs on the DNA and a binding site on the enzyme (von Hippel et al., 1982). This recognition step would also demand a strict orientation between the enzyme and the template for an interaction that would most likely occur in the major or minor grooves of the DNA double helix.

The formation of RP_O ultimately results in a topological unwinding of the DNA and a "melting" of base pairs near the start site of transcription as a prerequisite the subsequent transcription of the single stranded template. Evidence for the melted region comes from studies in which DNA sequencing techniques were employed to demonstrate that the binding of polymerase to a promoter results in a DNA strand separation of approximately 12 base pairs. In one approach (Siebenlist, 1979) protein-DNA complexes are treated with dimethyl sulfate , polymerase is then removed and the enzyme is treated with S1 nuclease, an enzyme specific for single stranded DNA. Melting is detected by a chemical modification of the N1 positions of adenines which are normally engaged in hydrogen bond formation in duplex DNA but cannot renature when methylated.

A digestion of the DNA with the single strand specific nuclease S1 followed by electrophoresis thru DNA sequencing gels reveals the region of unpaired bases. Using this approach a region of 12 base pairs (-9 to +3) on the lac UV5 and T7 A3 promoters was shown to be disrupted by polymerase binding. An alternate approach was employed by Kirkegaard et al. (1983) to detect unpaired cytosine residues downstream from the -10 region of lac UV5-polymerase complexes. In this method the methylation of the N-3 position of cytosines in the single stranded region followed by a subsequent treatment with hydrazine leads to a preferential chain cleavage at N-3 methylcytosine sites on the DNA. Using this approach "melting" was demonstrated by the methylation of the cytosines at positions -1, -2, -4, and -6 of the template strand of lac UV5.

A bipartite organization of the promoter has been proposed by Gilbert (1976) in which the -35 consensus region involves the promoter recognition by polymerase while the -10 consensus region would be responsible for the subsequent "melting" reaction required for RP_O formation. Evidence to support this model comes from kinetic measurements of promoters containing mutations within the consensus region. A change in the homologous sequence of either the -35 or the -10 region of a promoter was found to effect a change in the rate constants for either binding (K_B) or isomerization to RP_O respectively (Hawley and McClure, 1982). The binding of accessory proteins to specific sites on the DNA and changes

in DNA topology can further modulate promoter strength. Proteins which serve as activators or repressors of transcription and DNA supercoiling have been shown to alter the kinetic parameters of binding and isomerization.

Repressors bind to operator sites on the DNA and block transcription by steric hindrance. A kinetic analysis of the effects of the cI repressor on the lambda P_R promoter showed that the blocking of polymerase binding by the protein elicited a decrease in the K_B (McClure, 1985). Activators of transcription initiation are known to bind to sites near or upstream from the -35 region of a promoter. The binding of cAMP-CRP to the lac P^+ promoter activates transcription by a direct enhancement of polymerase binding to promoter. Promoter strength measurements revealed that the activation of lac P^+ by CRP-cAMP increased the rate of RP_O formation by increasing the K_B constant but not the k_2 (Malan et al., 1984). It was postulated that the binding of cAMP-CRP to the lac promoter activated transcription by directing polymerase to bind exclusively to the major lac P1 promoter and by preventing occupancy at the minor lac P2 promoter (Malan and McClure, 1984). However, the exact structural interactions between CRP, RNA polymerase and the promoter DNA during the activation process remains to be elucidated. The DNA binding domain of CRP has been defined as having a helix-turn-helix motif which appears to be important for the recognition and binding to a major groove sequence (TGTGA) in right handed B-DNA (de Crombrugghe et

al., 1984). It has been shown that the binding of CRP alters the structure of DNA (Wu and Crothers, 1984). It is proposed that this binding of the DNA by CRP stabilizes the RNA polymerase-promoter in a conformation that potentiates activation.

Changes in the conformation of DNA would also alter intrinsic promoter strength. Supercoiling has been shown to have the paradoxical effect of activating or inhibiting transcription (Wang, 1982). Studies with the beta-lactamase, tetracycline resistance and RNA 1 promoters have shown that supercoiling stimulates transcription initiation by enhancing RP_O formation (Ehrlich et al, 1985). Thermodynamically the detorsion produced by negative supercoiling should favor the equilibrium of a reaction that results in the unwinding of DNA (Wang, 1982). Supercoiling could enhance promoter strength by facilitating melting of the DNA or by making DNA binding contacts more accessible to their counterparts on the polymerase surface.

Kinetic analyses of the lac promoter have shown that supercoiling can elicit complex effects on both parameters of promoter strength. A simultaneous change in K_B and k_2 was found to occur on supercoiled templates of the lac promoter rather than the single component change observed in other studies (Malan and McClure, 1984). These results also showed that supercoiling may not exert a favorable effect on the individual steps of the transcriptional process. Comparative measurements of RP_O formation on linear and

supercoiled templates of the lac promoter have shown that supercoiling can exert both a positive and a negative effect on the promoter strength measurements of lac UV5. On a supercoiled template the affinity of polymerase for the lac UV5 promoter (K_B) was greatly increased while the rate of isomerization to an active RP_o (k_2) was decreased. In contrast, the k_2 value of the lac P^+ on a supercoiled template was dramatically increased. Similarly, supercoiling decreased the k_2 of the TAC16 (UpG) promoter and increased the k_2 of the TAC16 (ApU), TAC17 and TAC18 promoters (Mulligan et al., 1985). In vivo studies have shown that transcription from the lac UV5 promoter is activated in the presence of gyrase inhibitors (Sanzey, 1979). Studies with the lac P^s promoter on templates ranging from fully relaxed to fully supercoiled have provided additional evidence that changes in DNA topology can induce varying rates of transcription (Borowiec and Gralla, 1985). The results indicated that transcriptional activity was stimulated by supercoiling up to densities relating to form 1 DNA and then inhibited when higher supercoiling tensions were placed in the template.

The DNA sequence in the spacer region between the -10 and -35 regions is poorly conserved and has not been shown to be directly involved in the regulation of promoter activity. Nevertheless, the spacer length between the consensus regions of promoters is highly conserved with the number ranging from 16 to 19 base pairs. Transcriptional

studies of promoters of varying strengths have indicated that optimal activity is achieved with a spacer distance of 17 base pairs. The role of spacer length as a determinant of promoter strength has been demonstrated by mutational studies with the lac P^s promoter (Stefano and Gralla, 1982b) and a kinetic analysis of the TAC 16 promoter (Mulligan et al, 1985). The results of these studies indicate that a change in spacer length by addition or deletion reduces activity by changing the rate of RP_o formation.

It has been postulated that supercoiling and spacer length could perturb polymerase-promoter interactions by altering the critical positioning of polymerase with respect to the DNA prior to RP_o formation (von Hippel et al., 1984, McClure 1984, Spassky et al, 1985). One model (Stefano and Gralla, 1982b) proposes that the initial contact between polymerase and promoter requires a rotation of the -35 region to a position relative to the -10 region for the enzyme to correctly align with the DNA. A change in spacer length by addition or deletion of a base pair and/or an increase in the torsional tension by supercoiling could greatly distort the angular orientation of polymerase contacts within or near the consensus region. Under such conditions a proper binding of polymerase to the promoter would necessitate a realignment that could only be compensated for by an unwinding or winding of the DNA or a distortion in polymerase itself (Wang, 1982). Intrinsic

promoter activity could also be modulated by alternate recognition modes of polymerase binding. The existence of phase-shifted -10 regions within a promoter has been proposed to explain the altered sites of transcription initiation observed in the TAC16 promoter (Brosuis et al., 1985; Mulligan et al.,, 1985) and the ANT promoter of the Salmonella typhimurium phage P22 (Grana et al., 1985).

The location of the initiating nucleotide site (+1 position) corresponds to a distance that relates to the position of the -35 and -10 base pair regions. Transcription occurs at a start site which is 6 or 7 base pairs downstream from the -10 region. Kinetic studies have shown that the incorporation of NTP during initiation is an ordered process with the initiating NTP binding to its site at a K_m value which is much higher than successive nucleotides (Krakow et al., 1976; McClure et al., 1978). Studies have shown that catalysis during initiation involves two distinct binding sites: one which binds to the initiating NTP and one which binds the following substrate NTP as a Mg^{++} chelate.

Following the incorporation of NTPs the initiation complex converts to a stable ternary complex comprising core polymerase, the DNA template and a short RNA transcript. The exact nature of this conversion is not clearly defined. A ternary complex is best characterized by a strong resistance to rifampicin and to dissociation by heparin and high salt. In addition, the shift to an elongation mode is

assumed to occur when RNA polymerase relinquishes its initial promoter contacts and the sigma factor is released. The heparin insensitivity of initiation indicates that product formation proceeds without the dissociation of polymerase from the template. The sensitivity of initiation to rifampicin results from a direct inhibition of the translocation step leading to chain elongation (McClure and Cech, 1978).

The stability of a ternary complex has been shown to vary with the initiating nucleotide. On a $d(A-T)_n$ template the use of UpA rather that ApU as the initiating primer leads to the formation of a product (UpApU) which is synthesized with a rapid turnover and which readily dissociates from the complex (Oen & Wu,1978). During chain elongation the phosphodiester bond is formed by a nucleophilic attack of the 3'-OH end of the nascent RNA chain by the incoming NTP, pyrophosphate is released and the RNA product and enzyme are then translocated by one base pair. The template unwinding induced by RNA polymerase has led to a model to explain the movement of RNA polymerase-elongation complexes along the DNA during catalysis (Gamper and Hearst, 1982). In this model transcription from a locally single stranded template is maintained along the DNA by the formation of a transcription bubble which involves a continuous rewinding and melting of an unpaired region of approximately 17 base pairs. The proposed mechanism also assumes that the RNA-DNA hybrid that results during

elongation is rotated to displace the nascent RNA chain. Finally, transcription termination occurs. This step is accompanied by a complex series of events that lead to the release of an RNA chain from the complex and the dissociation of polymerase from the template.

The assignment of specific roles to the beta and beta' subunits of polymerase has been implicated from the use of inhibitors which affect specific steps during the transcription process. These studies attempt to obtain mechanistic information regarding the structural and functional relationship of polymerase interactions during the multistep reaction sequence with template. For this reason antibiotics, dyes, active site directed chemical modification and affinity probes have been employed as a means to correlate substrate utilization during catalysis with subunit specificity (Kumar, 1980; Krakow et al, 1976).

Strong support for the catalytic role of the beta subunit comes from the demonstration that the antibiotics rifampicin and streptolydigin inhibit initiation and elongation, respectively, by binding to the beta subunit. Polymerase mutants which are resistant to these antibiotics have altered beta subunits. Conversely, Zillig et al.(1976) have shown that heparin, a sulfated polysaccharide which inhibits transcription by blocking template binding, binds specifically to the beta' subunit of RNA polymerase. Genetic studies have shown that mutants of polymerase with defective DNA binding properties possess altered beta'

subunits(Yura and Ishihama, 1979).

Generation of monoclonal antibodies (Kohler and Milstein, 1975) against antigenic determinants on the individual subunits could provide a potentially powerful set of site-specific reagents with which to probe the relation of structure with function in RNA polymerase. Previous studies using antibody preparations raised against the individual subunits demonstrated that such preparations could inhibit in vitro transcription by RNA polymerase (Fukuda et al., 1977; Gragerov and Nikiforov, 1980; Stender, 1981). Anti-subunit polyclonal antibodies have been used by Stender (1979) to demonstrate conformational changes in RNA polymerase. Although subunit-specific, polyclonal antibodies consist of a hetergeneous population of immunoglobulins able to cross-react with many of the antigenic determinants present on the subunits.

Using the hybridoma technique it is possible to generate highly specific monoclonal antibodies (mAbs) directed against a single determinant on a protein. In practice, an animal (normally a rat or a mouse) is injected with the specific antigen of interest. The immune response of the animal is the production of an enormous diversity of antibodies directed against different epitopes on the antigen. The resulting antiserum contains a heterogeneous population of monospecific antibodies, each displaying a specific binding affinity for a different binding site on the antigen.

Hybridoma technology takes advantage of the fact that each individual antibody producing cell (lymphocyte) responds to a single antigen in a clonal fashion to produce an antibody which is both monospecific and monoclonal. The fusion of activated lymphocytes from the spleen of the immunized animal with cultured myeloma cells leads to the production, in vitro of a hybrid cell line that is capable of secreting a monospecific antibody. A subsequent selection, screening and then a cloning of appropriate hybridoma leads to the permanent propagation of hybridoma cell lines of interest.

A detailed study of the binding mechanism of a subunit-specific mAb to polymerase is quite advantageous in determining which regions of the enzyme engage in DNA binding and/or catalysis. Such mAbs could be used as effective probes to characterize the subunit arrangements in the quaternary structure of the enzyme or the conformational flexibility of the native form of polymerase during transcription. A characterization of the structural-functional relationship of a RNA polymerase subunit could be possible by generating a library of mAbs against a number of determinants on each subunit. The delineation of a subunit specific site involved in an essential function could be obtained from a localization of the antigenic determinant within the primary sequence. In this way a direct correlation could be established between polymerase function and the amino acid residues comprising the site of

interaction on a specific subunit of the enzyme molecule.

However, the use of mAbs as definitive probes of enzyme structure and function could have its limitations. Attempts to raise antibodies against an essential site on the enzyme would be unsuccessful if these antigenic determinants were hidden or buried in the native protein during the immune response. It may be difficult to assess whether a mAb induced inhibition of polymerase results from direct or indirect mAb-polymerase interactions. A complex inhibitory pattern would be expected if antibody binding affected only a part of the active site or induced a conformational change that hindered substrate binding or the rate of incorporation. An alternate indirect effect due to immunoglobulin size would be a steric hindrance of an enzyme function at a region distant from the actual determinant site.

The present study deals with the characterization of murine mAbs prepared using purified beta and beta' subunits as immunogens. Using the E.coli RNA polymerase core as the immunogen Nikiforov et al. (1983) have raised monoclonal antibodies against determinants present on the beta and beta' subunits which were able to completely inhibit DNA-directed synthesis of RNA.

In this study mAbs were generated against determinants on the individual subunits to directly probe the relation of structure with the functional properties of RNA polymerase. The anti beta and anti beta' mAbs are employed as reagents

to study subunit-specific interactions of RNA polymerase during transcription on different DNA templates. The results of the present studies were obtained mainly from kinetic and DNA structural analyses of RNA polymerase interactions during complex formation on synthetic and promoter templates in the presence and absence of mAbs. These findings demonstrate that an inhibitory anti beta mAb may be useful as an effective probe to distinguish differences in the catalytic activity of the enzyme as well as differences in promoter strength, promoter recognition and the spacer length between the consensus -35 and -10 regions of a promoter. The present results also infer that both an inhibitory and noninhibitory anti beta' mAb may serve as unique reagents to probe the binding of RNA polymerase to promoter sites and the subsequent steps leading to initiation of transcription.

MATERIALS AND METHODS

Materials- Reagents were obtained as follows: [^{3}H]CTP, [^{3}H]UTP and [gamma-^{32}P]ATP, [alpha-^{32}P]ATP, ICN; uridilyl 3'-5' adenosine (UpA), 5' adenosine monophosphate, (AMP), RIA grade bovine serum albumin (BSA), rifampicin and p-nitrophenylphosphate, cyclic AMP, adenylyl (3'-5') adenosine (ApA), adenylyl(3'-5') uridine (ApU), cytidylyl (3'-5') adenosine (CpA), guanylyl (3'-5') uridine (GpU) and uridylyl (3'-5') guanosine (UpG), Sigma Chemical Co.; d(A-T)$_n$, d(I-C)$_n$ and unlabeled ribonucleoside triphosphates, P-L Biochemicals; T4 polynucleotide kinase, alkaline phosphatase, DNA polymerase I Klenow fragment, restriction endonucleases AluI, EcoRI, HpaII, HindIII, HindII, and PvuII, Boehringer Mannheim; DNase 1, Cooper Biomedical; dimethyl sulfate and hydrazine, Aldrich Chemical Co.; formamide, Amresco; urea, Schwarz-Mann; acrylamide, Serva; Elutip, Schleicher and Schuell; Ultragel ACA34, LKB; fetal calf serum, Sterile Systems Inc.; Dulbecco's modified Eagle medium (DMEM), GIBCO; polyethylene glycol 4000, E. Merck Inc.; phosphatase-coupled goat anti-mouse immunoglobulin, Kierkegaard and Perry Laboratories; Freund's complete and incomplete adjuvants, Pel-Freeze; Protein A-Sepharose, Pharmacia; Liquiflor, New England Nuclear and Fluorosil, Isolab. Mice were obtained from Jackson Laboratory.

Buffers- PBS: 10 mM potassium phosphate (pH 7.2), 150 mM

KCl. Storage Buffer: 50 mM potassium phosphate (pH 7.5), 150 mM KCl, 0.05% sodium azide. ELISA Wash Buffer: PBS containing 2 mg/ml BSA, 0.05% Tween 80, 0.02% sodium azide. P60-BSA: 20 mM potassium phosphate (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 60% glycerol, 2 mg/ml BSA. TM: 40 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 10 mM $MgCl_2$, TKD-1000: 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol. TMM: 50 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 10 mM mercaptoethylamine. TGED: 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 EDTA, 5% glycerol. TKM: 40 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 10 mM mercaptoethylamine, 10 ug/ml rifampicin. TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Filtration buffer: 10 mM Tris (pH 8.0) and 50 mM NaCl. TBE 1 contains 89 mM Tris-borate, pH 8.3 and 2 mM EDTA. TBE 2 contains 100 mM Tris-borate, pH 8.3 and 2 mM EDTA..

Media- The formula for each medium was obtained from Miller (1972). LB broth contains 10g tryptone, 5g yeast extract and 10g NaCl per liter. The medium is adjusted to pH 7.5 with 1M NaOH and autoclaved. After cooling to 45°C 25 mg/ml ampicillin is added. The M9 minimal salts medium was supplemented with 0.5% glucose 0.3% casamino acids, 0.01% thiamine and 25 ug/ml ampicillin. The M9 medium is prepared as follows: For a 1X solution 7g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl and 1g NH_4Cl are dissolved in 940 ml H_2O,

adjusted to pH 7.4 with 1M NaOH, divided between two 2-liter flasks and autoclaved. Prior to inoculation 12.5ml 20% glucose, 7.5ml 20% casamino acids, 5ml 1% thiamine, 0.1ml 1M $MgSO_4$. 0.05ml 1M $CaCl_2$ and 0.5ml 25 mg/ml ampicillin are added. The medium used to grow cell lines for the production of monoclonal antibodies was prepared by adding 5ml 10X penicillin-streptomycin stock (10,000 u/ml), 5ml 100X Na pyruvate (100 mM) and 100ml heat inactivated fetal calf serum to 500ml Dulbecco's Modified Eagle Medium (DMEM).

RNA Polymerase Purification-E.coli RNA polymerase was prepared by a modification of the method of Burgess and Jendrisak, (1975). Holoenzyme and core polymerase were resolved by chromatography on denatured calf thymus DNA-agarose as described by Lowe et al. (1979). Protein concentration was determined using the extinction coefficients: core polymerase, $E_{280nm}^{1\%}$ = 5.8, holoenzyme, $E_{280nm}^{1\%}$ = 6.7 (Levine et al., 1980).

RNA polymerase subunits were prepared by chromatography of urea-dissociated core polymerase on Bio-Rex 70 by the method of Yarbrough and Hurwitz, (1974). The subunits were dialyzed against PBS prior to use.

The cAMP receptor protein (CRP) was prepared by the method of Eilen et al., (1978). CRP concentration was determined using the extinction coefficient, $E_{280nm}^{1\%}$=8.8 (Aiba and Krakow, 1981).

Production of Monoclonal Antibodies- SJL/J female mice were injected intraperitoneally with 23 ug beta subunit or 43 ug beta' subunit emulsified in Freund's complete adjuvant. This was followed by three booster shots of similar doses of the subunits in Freund's incomplete adjuvant administered at approximately 15 day intervals. Spleens from two mice (immunized with beta or beta' subunit) were removed three days after the final injection. Fusion of spleen cells and P3x63Ag8.653 myeloma cells (Kearney et al., 1979) was carried out by a modification of the method of Oi and Herzenberg (1980). Upon removal, spleens were placed in 5ml DMEM containing 20% fetal calf serum (FCS), cut longitudinally, and then gently teased with forceps to obtain splenocytes. Splenocytes were then harvested by centrifugation, washed 3 times with 15ml DMEN containing 20% FCS and treated with 0.83% NH_4CL to remove red blood cells by lysis. Splenocytes were then washed twice with serum free DMEM and suspended in the same buffer. Simultaneously logarithmically grown myeloma cells were washed twice with serum free medium. Myeloma cells and splenocytes were mixed in a ratio of 1:3, harvested by centrifugation and then fused in the presence of 35% polyethylene glycol (PEG 4000). Following the addition of DMEM containing 20% FCS fused cells were harvested at room temperature by centrifugation at 800xg for 6 minutes and resuspended in DMEM containing 20% FCS plus hypoxanthine, aminopterin and thymidine (HAT). Fused cells were then

distributed into 96-well Costar culture plates containing 2 x 10^4 mouse macrophage cells per well. The production of antibodies to beta or beta' was determined by ELISA (see below). Positive antibody-producing cultures were subcloned three times by limiting dilution in DMEN containing 20% fetal calf serum and 2 x 10^4 mouse macrophage cells per well.

Antibodies were prepared from spent media of expanded cultures (200-500 ml) grown to stationary phase. Cells were removed by centrifugation at 4000xg for 10 minutes. Immunoglobulin was concentrated by precipitation with 50% saturated ammonium sulfate, pH 7.0. The antibodies were purified by affinity chromatography on Protein A-Sepharose (Ey et al., 1978) or DEAE cellulose (Parham et al., 1982). Immunoglobulin-containing fractions were concentrated to a volume of about 1 ml by negative pressure dialysis (in an apparatus obtained from Bio-Molecular Dynamics) against Storage Buffer. Purified stocks were stored on ice. The immunoglobulin isotype of each monoclonal antibody was determined using the Mouse Immunoglobulin Subtype Identification Kit purchased from Boehringer Mannheim Corp. Immunoglobulin concentration was determined using the extinction coefficient: $E_{280nm}^{1\%} = 14.0$ (Ey et al, 1978).

Storage and Maintenance of mAb Cell Lines- Antibody producing cell lines are stored frozen as 0.5 ml stocks at high cell density in liquid nitrogen. Stocks are prepared for freezing by centrifugation of 10 ml of cultured cells

(2×10^6) and resuspension in 0.5 ml of 90% FCS plus 10% dimethyl sulfoxide (DMSO). Cells are frozen slowly by a two step process of freezing at -70°C followed by transfer to liquid nitrogen. To produce mAbs from frozen cell lines, retrieved cells are thawed quickly and added immediately to 15 ml centrifuge tubes containing 2ml fetal calf serum at room temperature. Following a slow addition of 10ml DMEM containing 20% FCS cells are centrifuged and resuspended in 1.5 ml of the same medium. Using a 96 well Costar dish 50ul aliquots of the resuspended cells are added to each of 300 wells containing a feeder layer of 200 ul of 4×10^3 macrophage cells per well. Cells are incubated for 2-10 days in a 7% CO_2 incubator before screening.

An alternate plating procedure is employed to retrieve cell lines of low viability. Following centrifugation cells are resuspended in 4 ml DMEM containing 20% FCS. The entire suspension is then added to a 125 ml flask containing a layer of 1 ml of 2×10^4 macrophage cells. The flask is placed on its side in a 7% CO_2 incubator for 7-14 days before screening. Cells are then gradually expanded to a 500 ml culture by dilution with fresh medium to achieve levels of antibody that yields up to 30 ug/ml of mAb.

Solid Phase ELISA- Costar 96-well EIA polystyrene plates were coated with subunit by a three hour incubation at 37°C followed by incubation a 4°C overnight. Each well received 600 ng beta or beta' in PBS. Remaining protein-binding sites were blocked by incubation with 200 ul/well of Wash

Buffer for 90 minutes at 37°C. The plates were then washed twice with Wash Buffer. Following the addition of 50ul of culture supernatant per well the plate was incubated for 60 minutes at 37°C. After washing three times with Wash Buffer 50 ul of phosphatase-coupled goat anti-mouse immunoglobulin (1/2000 dilution in PBS + 1 mg/ml bovine serum albumin) was added and incubated for 60 minutes at 37°C. After washing three times with Wash Buffer 100ul of solution containing 1 mg/ml p-nitrophenylphosphate in 0.1M diethanolamine (pH 9.0) + 2.5 um $MgCl_2$ was added and incubated for 30 to 60 minutes at 37°C. After the addition of 100ul 1M NaOH the absorbance at 410 nm was determined for each well using a Dynatech Microelisa Reader.

Assay of $d(A-T)_n$ Directed $r(A-U)_n$ Synthesis- Monoclonal antibody-polymerase complexes were formed by incubating for 60 minutes at 0°C or 30 minutes 37°C of 1 pmol core or holoenzyme in 10 ul P60-BSA with the indicated amount of antibody in 20 ul Storage Buffer. After addition of 2 nmol $d(A-T)_n$ in 10 ul TM buffer the mixture was incubated for 10 minutes at 37°C . Synthesis of $r(A-U)_n$ was carried out in a reaction mixture (90 ul) which contained: 40 mM Tris-HCl (pH 8.0), 13 mM potassium phosphate (pH 7.5), 33 mM KCl, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 1 mM sodium azide, 400 nmol ATP, 100 nmol [^{3}H]UTP (5000cpm/nmol), 44 ug bovine serum albumin and 7% glycerol. After incubation for 20 minutes at 37°C the $r(A-U)_n$ was precipitated with 5% trichloroacetic acid, collected on glass fiber filters

(Whatman GF/A) and counted in Liquiflor-toluene.

Preparation of Ternary complexes- Two procedures were employed to prepare $d(A-T)_n$ complexes. The ternary complex used to screen the effect of the monoclonal antibodies on elongation (Tables 1 and 2) was isolated by a modification of the method of Rhodes and Chamberlin (1974). For the kinetic studies the ternary complex was prepared by the method of Schmidt and Chamberlin (1984). To form the complex by the modified method 50 pmol holoenzyme was incubated for 1 minute at 37°C with 124 nmol $d(A-T)_n$ plus 400 uM UTP in TMM buffer after which ATP was added to a final concentration of 120 uM. The reaction (250 ul) was incubated for 1 minute at 37°C, placed on ice and terminated by the addition of 10ul 0.5M EDTA (pH 7.6). The reaction mixture was immediately applied to a Bio-Gel P-100 column (2 x 25cm) equilibrated with TGED at 4°C. The mixture was eluted with TGED and the ternary complex located by assaying 50 ul aliquots of the fractions for elongation activity. Fractions containing ternary complex were pooled and stored at 4°C. Ternary complexes isolated for kinetic studies were isolated on 1 x 13 cm column of Ultragel ACA34 equilibrated at room temperature. Fractions were collected and stored at room temperature and assayed within 24 hours.

Assay of Ternary Complex Activity- A 50 ul aliquot of the ternary complex (100-200 elongation units; Rhodes and Chamberlin, 1974) was preincubated for 30 minutes at 37°C with a 20:1 molar excess of monoclonal antibody in 20ul of

Storage Buffer containing 1 mg/ml bovine serum albumin. The ratio of antibody to complex assumed a 100% recovery of polymerase during preparation of the ternary complex. Assay for polymerase activity was carried out in a reaction mixture (100 ul) which contained: 50 mM Tris-HCl (pH 8.0), 10 mM mercaptoethylamine, 30 nM EDTA, 2.5% glycerol, 1 mM sodium azide, 400 uM ATP and 400 uM [^{3}H]UTP (60 cpm/pmol) and 1.0 ug rifampicin. After incubation for 10 minutes at 37° C the reaction was terminated by addition of 5% trichloroacetic acid.

The kinetic studies were carried out using the assay conditions described by Schmidt and Chamberlin (1984). The ternary complex (360 elongation units) in 120 ul was preincubated for 30 minutes at 37°C with 20:1 molar excess of monoclonal antibody in 60 ul Storage Buffer containing 1 mg/ml bovine serum albumin. To initiate the reaction 30 ul of the preincubation mixture was added to tubes containing (final volume 50 ul): 44 mM Tris-HCL (pH 8.0), 10 mM $MgCl_2$, 14 mM mercaptoethylamine, 2% glycerol, 1 mM sodium azide, 14 ug bovine serum albumin, 0.50 ug rifampicin plus ATP and [^{3}H]UTP (500 cpm/pmol) at the indicated concentration. Reactions were incubated for 5 minutes at 37°C and then terminated by addition of 5% trichloroacetic acid.

Abortive Initiation Assay- The abortive assay condition for studies with synthetic and promoter templates were performed using slightly different protocols. Both protocols represent a modification of the abortive reaction conditions

described by McClure et al., (1978).

For the $d(A-T)_n$ or $d(I-C)_n$ directed reaction mAb-polymerase complexes were formed by incubation of 10 pmol holoenzyme in 5 ul P60-BSA buffer plus the indicated amount of antibody in 20 ul Storage Buffer for 30 minutes at 37°C (to assay pApU synthesis) or by incubation of 1 pmol holoenzyme in 5 ul P60-BSA buffer plus the indicated amount of antibody in 10ul Storage Buffer for 60 minutes at 0°C (to assay UpApU or CpGpC synthesis). The immune complex was then incubated for 10 minutes at 37°C in a mixture containing: 40 mM Tris-HCl (pH 8.0), 10 mM or 18 mM potassium phosphate (pH 7.5), 130 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM or 2 mM sodium azide, 20 ug or 30 ug bovine serum albumin, 5% or 6% glycerol and 10 nmol $d(A-T)_n$ or $d(I-C)_n$. The synthesis of pApU was assayed by the addition of 120 nmol 5' AMP and 12 nmol [^{3}H]UTP (15 cpm/pmol) to assay mixtures (60 ul) followed by an incubation for 10 minutes at 37°C. After the addition of 10 ul 0.5M EDTA a 50 ul aliquot of the reaction mixture was applies to a 3 MM Whatman paper strip and chromatographed in the WASP system of Hansen and McClure (1979). Syntheses of UpApU and CpGpC were assayed following the addition of UpA and [^{3}H]UTP (200 cpm/pmol) or CpG and [^{3}H]CTP (200 cpm/pmol) at the indicated concentration. Reaction mixtures (50 ul) were then reincubated for the indicated times at 37°C after which a 10 ul aliquot was chromatographed.

The T7 DNA directed single step addition reactions were

assayed according to the promoter assignments of Oen et al. (1979). Standard reaction mixtures contained (final volume 50ul): 40 mM Tris-HCl (pH 8.0), 10 mM potassium phosphate (pH 7.5), 130 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM NaN_3, 1% glycerol and 20 ug bovine serum albumin. The RNA polymerase concentration used was 20-40 nM (1-2 pmol) and where indicated the anti-beta mAb 210E8 was included at a molar ratio (mAb 210E8: RNA polymerase) of 20:1. The lac and TAC16 promoters were assayed at a final concentration of 2 nM linear fragment and 1 nM supercoiled plasmid. T7 promoter directed reactions contained 0.02 pmol T7 DNA. Unless indicated otherwise the substrate concentrations were 0.5 mM dinucleoside monophosphate and 50 uM [^{3}H]UTP or [^{3}H]CTP (200 cpm/pmol). For the lac P^+ abortive initiation assays 100 nM cAMP receptor protein and 100 uM cAMP were included.

For each promoter assay 2 ul aliquots of RNA polymerase in P60-BSA buffer were preincubated in 10 or 15 ul of storage buffer containing the indicated amount of mAb or in storage buffer alone for 1 hour at 0°C. The indicated template was then added and the incubation were continued for 15 minutes at 37°C. Following the addition of the appropriate substrates incubation were continued for the times indicated. Reaction products were then analyzed by paper chromatography as described above.

To examine the kinetics of substrate utilization during the abortive reaction the K_m and V_{max} values were determined

from lines fitted by linear regression analyses of plots of reciprocal initial velocities (pmol/min) versus the reciprocal of the varied substrate concentration.

Promoter Strength Measurements- These assays were performed by employing the abortive initiation assay in conjunction with a kinetic analysis of promoter strength devised by McClure (1980b). The use of the abortive initiation reaction as a means to measure promoter strength measurements relies upon the characteristic features of the assay as a promoter specific, holoenzyme dependent, steady state reaction (Hawley et al., 1982). The frequency of initiation, as measured by product formation, is assumed to be proportional to promoter occupancy in RP_O. The analysis is based on a derivation of the kinetics of RP_O formation according to the following scheme:

$$R + P \underset{k_B}{\rightleftharpoons} RP_C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_O$$

In this scheme RNA polymerase (R) and a promoter site (P) combine at a rapid equilibrium to form a closed complex (RP_C). The slow transition from RP_C to a functional open complex (RP_O) represents the rate limiting step which is irreversible. Therefore the mechanism of this scheme predicts $k_1 > k_{-1}$, $k_{-1} > k_2$ and $k_{-2} < k_2$.

The determination of <u>in vitro</u> promoter strength

requires a two step process. First, the time required for polymerase to form an active RP_O is measured as a promoter specific lag in an approach to the steady state rate of the abortive reaction. This lag time (tau_{obs}) is measured as a function of the following two experimental protocols. To observe the lag which preceeds the final steady state rate reaction mixtures containing preincubated template and substrates are initiated by the addition of RNA polymerase. A parallel reaction is performed in which mixtures of preincubated enzyme and template are initiated by the addition of substrates. This parallel reaction mixture serves as a steady state control in which no lag is observed. Aliquots from each reaction mixture are then removed at short time intervals to determine the amount of product formed over time. A plot of each curve (radiolabeled substrate incorporated versus time) is then analyzed to yield a tau_{obs} value.

The second step in promoter strength determinations yields the mechanistic information for promoter strength by partitioning polymerase-promoter interaction during RP_O formation into two functional parameters. A measurement of tau_{obs} at various RNA polymerase concentrations can be used to calculate a quantitative separation of the rate of initial binding of polymerase to a promoter (K_B value) to form RP_C and the subsequent rate of isomerization to form a fully active RP_O (k_2 value). By employing the pseudo first order experimental conditions where the concentration of

polymerase is in excess of promoter the relationship of tau_{obs} to the concentrations of RNA polymerase can be expressed as the following simplified equation:

$$tau_{obs} = 1/k_2 + 1/K_B[R]k_2$$

A plot of tau_{obs} vs. $[RNAP]^{-1}$ (tau plot) is linear with an intercept which equals $1/k_2$ and a ratio of intercept to slope that equals K_B.

This approach was applied to the present study to compare promoter lag time measurements in the presence and absence of the anti-beta mAb 210E8. Steady state control values were obtained by incubating an aliquot of preincubated RNA polymerase with template for 10 minutes at 37°C and then initiating the reaction by the addition of substrates. Lag time measurements in the presence and absence of mAb were obtained by preincubating two separate mixtures of the template and substrates for 10 minutes at 37°C and then initiating both of the reactions by adding preincubated mixtures of RNA polymerase to one and RNA polymerase + mAb 210E8 to the other. Aliquots (10 ul) were removed from the three reactions at the times indicated, spotted on Whatman 3MM paper and chromatographed as described in McClure et al. (1978). Effects of mAb 210E8 on the reaction are expressed as the percent residual activity of the control value.

The lag time (tau_{obs}) for the lac UV5, TAC16 (ApU) and TAC16 (UpG) was determined by a least squares analysis of those data points that were at least five times the estimated lag time (McClure, 1980). The binding constant, K_B and the isomerization rate constant, k_2, for the supercoiled lac UV5 promoter complexes formed in the presence and absence of mAb 210E8 was calculated from a TAU plot of $1/tau_{obs}$ versus 1/polymerase concentration. The slope and intercept generated from a least squares fit of the data were used to calculate the constants, K_B and k_2.

The slope values obtained to compare the steady state conditions established for each tau_{obs} reaction were also determined from a regression analysis of those data point assumed to be in steady state (>4 minutes). The slope values for reactions run in the presence and absence of mAb 210E8 were then compared using a student t test to determine whether any significant differences ($p<.05$) were apparent.

The slopes analysis is bases on the a priori decision to sequentially exclude data points over time until the slopes of the three curves of a tau analysis, each estimated thru linear regression, are not significantly different. The last time point excluded is defined as the minimum time required to reach steady state. If the slopes of the three curves, estimated thru linear regression, do not differ significantly from each other it is assumed that all the curves have reached a similar steady state.

Preparation of Plasmid and Purified Promoter Fragment-

Plasmid pMBP lac P^+ and pMB9 lac UV5 containing the lac promoter and cloned into E.coli MM294 were constructed by Dr. F. Fuller and obtained from Dr. A. Revzin. Plasmid pNI171 containing the TAC16 promoter and cloned into E.coli NI200 was provided by Dr. N Irwin. To isolate plasmid, overnight cultures of E.coli MM294 and E.coli NI200 were grown in 50ml M9 and LB medium, respectively, in the presence of 25 ug/ml ampicillin. To amplify cells a 5 ml aliquot of the overnight culture was inoculated into each of 4 flasks containing 500 ml culture medium plus 25 ug/ml ampicillin and then grown with vigorous shaking to an OD_{550} of .5-.7. Chloramphenicol was then added to each flask at a final concentration of 170 ug/ml . Incubation was continued at 37°C for 16-22 hours. Cells were harvested by centrifugation and plasmid was isolated by alkaline extraction followed by a purification on glass powder (Marko et al., 1982). Plasmid stocks were stored in TE buffer and kept on ice at 4°C.

The 203 base pair lac P^+ and lac UV5 promoter fragments were isolated from plasmids digested with EcoR1. The 250 base pair TAC16 promoter fragment was isolated from plasmids digested with EcoR1 and HindIII. The DNA fragments were resolved from restricted vector by electrophoresis on a 7.5% polyacrylamide gel with TBE 1 as the running buffer. The DNA fragment was isolated from the gel using the crush and soak method of Maxam and Gilbert (1980). The eluted DNA fragments were recovered by ethanol precipitation and then

purified using a Elutip according to the Schleicher and Schuell protocol. Purified fragment was ethanol precipitated and stored in 100ul of TE buffer. Purity was assessed by electrophoresis on a 0.8% agarose gel. Concentration was determined by photofluorimetry from a standard curve with calf thymus DNA (LePecq and Paoletti, 1966). The DNA fragment stocks were maintained on ice at 4 ^{o}C.

T7 bacteriophage was provided by F. W. Studier. T7 DNA was purified from phage by three extractions with phenol equilibrated with TE buffer + 50 mM NaCl (Thomas and Abelson, 1966).

[^{3}H]d(A-T)$_{n}$ Binding Assays- Assays were performed using the nitrocellulose binding assay technique. Under the conditions of the assay enzyme-DNA complexes are trapped on a nitrocellulose filter while free DNA is not retained. For these assays preincubation condition of enzyme and mAb were those described for the d(A-T)$_{n}$ directed synthesis of UpApU. Each binding assay contained 2 pmol of preincubated enzyme (or enzyme plus mAb) and 1.5 nmol [^{3}H]d(A-T)$_{n}$ (3700 cpm/nmol) in 20 mM Tris (pH 8.0), 100 mM NaCl and 0.2 mM EDTA at a final volume of 200ul. After a 15 minute incubation at 37^{o}C 2 ml of a filtration buffer was added to each tube. Binding reactions mixtures were then passed thru nitrocellulose filters presoaked in 0.1M KOH. Filters were dried and the [^{3}H]d(A-T)$_{n}$ bound to enzyme was determined by counting in Liquiflor-toluene.

Promoter Binding Assays: Complex Stability and DNA Protection- For these binding assays holoenzyme was preincubated in the presence and absence of the inhibitory mAb anti-beta 210E8 at a molar ratio of 10:1. Binding reactions were performed using the same conditions employed for the abortive initiation assay at the KCl concentrations indicated. The effect of mAb 210E8 on the stability of polymerase-lac UV5 complex formation was examined directly by employing a gel electrophoresis technique devised by Garner and Revzin (1981). To form the DNA-protein complexes 3 pmol preincubated RNA polymerase (or mAb-polymerase complexes) was combined with 1 pmol lac UV5 promoter in abortive assay buffer containing 100M KCL. After a 15 minute incubation at 37°C solution conditions were adjusted as indicated and the reaction mixtures (30ul) were reincubated at either 37° or 0°C. A one fifth volume of a dye mixture containing a 2:1 mixture of 50% glycerol and .1% bromphenol blue was then added and reactions were immediately applied to a nondenaturing 7.5% polyacrylamide gel. The DNA-promoter complexes were analyzed for dissociation after staining gels in a solution of 1 ug/ml ethidium bromide and photographed by ultraviolet light.

For the DNA protection studies (Garner and Revzin, 1982) binding reactions (20ul) contained 2.5 pmol lac UV5 or the TAC16 promoter and 100 nM RNA polymerase preincubated in the presence and absence of the indicated monoclonal antibody in abortive assay buffer containing 100 mM KCl.

After a 15 minute incubation at 37°C reaction mixtures containing lac UV5-promoter complexes were diluted with the same buffer containing no salt to adjust the assay conditions to 32 mM KCl for restriction by HpaII. In binding assays containing TAC16 protein complexes the reaction conditions were adjusted to 50 mM KCl for restriction by HindII. After the addition of $d(A-T)_n$ to 11 uM and 9 units HpaII (or 3 units HindII) reactions were reincubated at 37°C for the times indicated. To stop restrictions, mixtures were adjusted to contain 333 ug/ml heparin, .017M EDTA and .1% sodium dodecyl sulfate. The same dye mixture mentioned above was added and solutions were then applied to a nondenaturing 7.5% polyacrylamide gel. After electrophoresis gels were stained with ethidium bromide (1 ug/ml) to determine DNA protection patterns. In these experiments a 4-fold ratio of polymerase to DNA was sufficient to give full protection. A titration of HpaII or HindII with the appropriate free promoter fragment was performed prior to these studies to determine the amount of restriction endonuclease sufficient to ensure complete digestion of the DNA within the time limits of the assay.

Promoter Binding Assays: DNA Enzymatic and Chemical Modification- The 5′ and 3′ [^{32}P] lac P$^+$ or lac UV5 promoter fragments were labeled with [gamma-^{32}P]ATP by T4 polynucleotide kinase or with [alpha-^{32}P]dATP by DNA polymerase I Klenow fragment. The labeled fragments were then restricted with PvuII which cuts the DNA at -123 and

yields promoter fragments uniquely labeled on either the lower or upper strand (see Figure 9 for restriction site).

The DNase 1 footprinting and guanine dimethyl sulfate treatments were performed according to the procedure of Spassky et al. (1985). The N-3 cytosine methylation reaction was performed using the procedure of Kierkegaard et al., (1983). Experiments were performed using the same conditions employed for the abortive initiation assays at a final KCl concentration of 100 mM. The DNase 1 and chemical modification reactions were performed with binding assays containing 4 nM (0.2 pmol) DNA and 180 nM (5 pmol) RNA polymerase in the presence and absence of the indicated monoclonal antibody at a molar ration of mAb to polymerase of 10:1. After a 15 minute incubation at 37°C DNase 1 or dimethyl sulfate were added. Footprints were determined with both lac promoters by adding DNase 1 to 80 ng/ml while the methylation of guanine and cytosine residues of the lac UV5 promoter was studied by adding dimethyl sulfate to a final concentration of either 50 mM for 1 minute or 200 mM for 2 minutes, respectively. The DNase 1 reaction was stopped by the addition of a 200 ul aliquot of 3M ammonium acetate, 100 ug/ml tRNA and 20 mM EDTA followed by phenol extraction, ethanol precipitation and reprecipitation. The dried DNA pellet was resuspended in 10 ul of formamide loading buffer and then analyzed by electrophoresis on 8% denaturing sequencing gels with TBE 2 as the running buffer (Maxam and Gilbert, 1980). The methylation reactions were

terminated with a 200 ul aliquot of 3M ammonium acetate, 100 ug/ml tRNA and 20 mM EDTA and 1M mercaptoethanol followed by an ethanol precipitation. For the guanine methylation experiments the DNA was then reprecipitated, washed, dried and subjected to a treatment with 1M piperidine before electrophoresis (Maxam and Gilbert 1980). To obtain preferential cleavage at N-3 methylcytosine the dried DNA pellet was subjected to a treatment with hydrazine prior to the piperidine treatment. After electrophoresis gels were autoradiographed at -70°C using KODAK XAR-5 film and Dupont Cronex Hi-Plus intensifying screens.

RESULTS

The effect on polymerase activity of increasing the molar ratio of monoclonal antibody at a fixed holoenzyme concentration is shown in Figure 1. The non-inhibitory mAbs were without effect on $d(A\text{-}T)_n$-directed $r(A\text{-}U)_n$ synthesis even at a high molar excess. The anti-beta' mAb 311G2 showed a 85% inhibition of polymerase activity at a 10 to 1 ratio of antibody to polymerase. The partial inhibition by the anti-beta mAb 210E8 (see Table 1 for other anti-beta mAbs) was retained even at a high antibody concentration. This is consistent with the presence in the beta subunit of a single determinant for each monoclonal antibody. No difference in the extent of inhibition effected by the anti-beta mAb 210E8 was seen on varying the KCl concentration between 30 to 130 mM. The inhibition remained approximately 50% when the antibody-polymerase complex was formed by incubation for 30 minutes at 37°C, 60 minutes at 0°C, or overnight at 0°C. However, the core polymerase was more sensitive to the effect of the inhibitory anti-beta mAb 210E8 than was the holoenzyme when assayed under the conditions used (Figure 2).

Summarized in Tables 1 and 2 are several properties of the seven anti-beta and five anti-beta' mAbs isolated thus far. The monoclonal immunoglobulins contain G1, G2a or G2b heavy chains and kappa light chains. Subunit specificity of

the mAbs was verified by solid-phase ELISA using purified alpha, beta or beta' subunits; the mAbs cross-reacted only with the subunit used initially as the immunogen (data not shown). Five of the anti-beta mAbs (Table 1) and one of the anti-beta' mAbs (Table 2) inhibited the $d(A\text{-}T)_n$-directed synthesis of $r(A\text{-}U)_n$. The core polymerase was more sensitive to the effect of the inhibitory mAbs than was the holoenzyme (see also Figure 2). Comparable results were obtained when the mAbs were added before or after binding of holoenzyme to $d(A\text{-}T)_n$ (data not shown). Polymerase in the ternary elongation complex was inhibited by the mAbs to approximately the same extent as was holoenzyme. The abortive synthesis of pApU was used to determine the effects of the mAbs on initiation by RNA polymerase. This reaction has been shown by Hansen and McClure (1979) to be strongly dependent on sigma and represents a convenient assay for steps involved in initiation. The data presented in Tables 1 and 2 indicate that both initiation and elongation were affected to a similar extent by each of the inhibitory mAbs.

The effects of mAbs 210E8 and 311G2 on the kinetics of initiation and elongation on $d(A\text{-}T)_n$ are summarized in Table 3. The results of abortive initiation experiments in which the activity was determined as a function of varied UpA concentration in the presence and absence of the inhibitory mAbs are shown in Figure 3. Both the V_{max} and K_m^{UPA} were affected by the inhibitory anti-beta' mAb 311G2 indicating a

mixed mode of inhibition. In the presence of anti-beta mAb 210E8 the K_m but not the V_{max} was changed when the UpA concentration was varied. This competitive type of inhibition by mAb 210E8 was also indicated by reversal of the inhibition at UpA concentrations above 150 um (see Figure 4). With UTP as the varied substrate both V_{max} and the K_m were altered in the presence of mAb 210E8 and mAb 311G2 (Table 3). The anti-beta' mAb 311G2 is a potent inhibitor of RNA polymerase and this was reflected in the low V_{max} when either the UTP or UpA concentration was varied. The K_m^{UTP} in the presence of mAb 311G2 was reduced while the K_m^{UPA} was increased compared to the control values.

The effect of the inhibitory anti-beta and anti-beta' mAbs on the apparent K_m for $d(A-T)_n$ was determined in reactions containing a fixed concentration of ATP and UTP (Table 3). As expected each of the mAbs affected the V_{max}. The apparent $K_m^{d(A-T)_n}$ was essentially unchanged in the presence of the anti-beta mAb 210E8 but was greatly reduced in the presence of the anti-beta' mAb 311G2 relative to the control value. None of the mAbs inhibit binding $d(A-T)_n$ by RNA polymerase. Instead those mAbs which inhibit r(A-U)n synthesis have the unexpected property of enhancing [^{3}H] $d(A-T)_n$ binding by holoenzyme (see Table 4).

The effect of the anti-beta mAb 210E8 on the kinetics of ribonucleoside triphosphate incorporation by the ternary elongation complex is shown in Figure 5 and Table 3. Both

the V_{max} and the K_m were slightly altered. The results indicated that even when the enzyme was in the elongation mode the residual activity in the presence of the anti-beta monoclonal antibody was 65% of the control value.

Abortive initiation assays were also performed with $d(I-C)_n$ as the template to determine whether the inhibitory patterns elicited by the inhibitory anti-beta and anti-beta' mAbs were specific for $d(A-T)_n$. The results (see Table 3) show that the anti-beta mAb 210E8 is competitive with regard to CpG concentration during initiation on $d(I-C)_n$. Like the $d(A-T)_n$ directed synthesis of UpApU in the presence of mAb 210E8 inhibition was completely reversed at increased concentrations of primer. However, the loss in inhibition with $d(I-C)_n$ was seen when concentrations of CpG exceeded 4 mM. The anti-beta' mAb 311G2 was a noncompetitive inhibitor of the abortive reactions on the d(I-C)n template. Under these conditions the V_{max} was greatly reduced.

The anti-beta and anti-beta' mAbs were assayed for their effects on the utilization of several promoters. Using specific dinucleoside monophosphate primers abortive initiation reactions were carried out with the lac and TAC16 promoters in both linearized and supercoiled templates and various T7 promoters. The DNA sequences for the lac and TAC16 promoters are given in Figures 6A and 6B, respectively. The L8-UV5 mutations in the lac wild type (P^+) promoter are also indicated in Figure 6A. The UV5

mutation allows transcription to proceed without the requirement for activation by CRP-cAMP. The L8 mutation reduces the affinity of lac promoter for CRP binding. The TAC16 promoter is a hybrid construct of the -35 consensus region of the trp promoter fused the -10 consensus region of the lac UV5 promoter (Ammann et al., 1983) and has been shown to initiate transcription with ApA, ApU or UpG in the presence of UTP (Mulligan et al., 1985).

The effects of the anti-beta and anti-beta' mAbs on abortive initiation by the T7 A1, A3, lac P^+ and lac UV5 promoters are shown in Table 5. The effect of increasing concentrations of the inhibitory anti-beta and anti-beta' mAbs on promoter activity is shown in Figures 7 and 8, respectively. Control titrations were performed with the noninhibitory anti-beta mAb 221C7 and anti-beta' mAb 37106. The results indicate that initiation by RNA polymerase from all 3 promoters was strongly inhibited by the anti-beta' mAb 311G2. In the presence of the anti-beta' mAb initiation from the lac P^+ promoter was virtually abolished. In contrast the anti-beta mAb 210E8 showed a varying degree of inhibition depending on the promoter used. CRP dependent initiation from lac P^+ was the most sensitive showing only 3% residual activity in the presence of the inhibitory anti-beta mAb (see Figure 7). At molar ratios of mAb to enzyme above 10:1 the extent of inhibition by both mAbs was virtually the same. A study of promoter specific reactions on T7 DNA showed that the residual activity in the presence

of the anti-beta mAb appeared to be related to promoter strength (Table 6). These studies were based on promoter strength measurements of Oen et al. (1979). Based on these studies the residual activity with the promoters used in the presences of the anti-beta mAb 210E8 decreased in the following order:T7A1,A3, T7A2, T7D, lac UV5, T7C, T7 unassigned 1 and 2 and lac P^+ promoters. The T7 DNA and lac promoter directed reactions were performed at a molar ratio of enzyme to DNA of 50:1 and 20:1, respectively. Decreasing the enzyme to DNA ratio decreased the inhibition by anti-beta mAb 210E8 for T7A1, A3 directed initiation but had no effect on the inhibition of the lac UV5 promoter (Table 7).

The effect of the inhibitory anti-beta mAb 210E8 on the kinetics of the lac UV5 directed synthesis of ApApUpU and the T7A1,A3 directed synthesis of CpApU at various molar ratios of enzyme to T7 DNA is shown in Table 8. The results indicate that the mAb elicits a complex mode of inhibition on the kinetics of polymerase-promoter interactions during substrate utilization. The anti-beta mAb 210E8 is competitive with regard to primer concentration during initiation on the T7 promoter A1 and A3 at an enzyme to DNA ratio of 10:1. Inhibition is completely reversed at high concentrations of primer. The inhibition by anti-beta mAb 210E8 is noncompetitive with regard to primer incorporation on the lac UV5 promoter and the T7 promoters A1,A3 at enzyme to DNA ratios greater than 10:1. The V_{max} is decreased and except for the T7 promoters A1,A3 promoter at

an enzyme:DNA ratio of 25:1, the K_m is increased.

To further examine the pleiotropic inhibitory pattern elicited by mAb 210E8 the rates of ApApUpU synthesis on the lac P^+ and lac UV5 promoters were compared with the rates of synthesis of ApUpA and UpGpU on the TAC16 promoter in the presence and absence of the antibody. Each promoter was assayed on a linear and supercoiled template.

The data presented in Table 9 show the effects of mAb 210E8 on abortive initiation from the lac and TAC16 promoters in their supercoiled, linearized and purified forms. The following promoters were assayed: lac P^+ in the presence of cAMP-CRP, lac UV5, TAC16(ApU) and TAC16(UpG) The demonstration that the control rates of abortive initiation are independent of supercoiling for the lac UV5 and slightly lowered for linearized lac P^+ is consistent with an earlier finding with the lac promoter (Mulligan et al, 1985). Linearization also lowered the control rates of abortive initiation for the supercoiled TAC16(ApU) and TAC16(UpG) controls. The strong inhibition elicited by mAb 210E8 on linear templates of lac UV5 and TAC16(UpG) promoters was not apparent when these promoters were assayed in their supercoiled forms in the presence of mAb. In contrast the inhibition by mAb of abortive synthesis on the linear TAC16(ApU) was lessened but still evident on a supercoiled template. When the linearized plasmids were assayed in the presence of mAb 210E8 a strong inhibition of abortive synthesis was observed for both lac promoters and

TAC16 promoter independent of the primer used. The TAC16 promoter was most sensitive to the effect of mAb 210E8 when the plasmid was restricted in a double digest with HindIII and EcoR1 to completely remove the fragment from the vector. A mAb induced inhibition of abortive initiation on linearized forms of the lac UV5 and TAC16 promoters was also observed when restrictions were performed at other sites on the plasmid. The linearization of pMBP-lacP$^+$ (or lac UV5) and pNI171-TAC16(UpG) with Pst1, HindIII or PvuII decreased the residual activity to levels comparable to those given in Table 9 for the complete digestion. For pNI171-TAC16 (ApU) digestion with HindIII resulted in a residual activity of 5% while linearizations with either Pst1 or PvuII resulted in residual activity of 27%.

It should by noted that the rates of abortive synthesis for the linearized TAC16 promoter associated plasmid (HindIII or HindIII-EcoR1) and purified fragment were reduced to the same extent in the presence of mAb 210E8. In contrast the residual activities for abortive initiation on the lac promoters in the presence of mAb 210E8 were always relatively lower with the purified fragments that with that obtained with the EcoR1 digest. However. the low residual activities obtained from abortive assays with purified lac promoter fragments in the presence of mAb 210E8 were not changed by the inclusion of the purified restricted pMB9 plasmid in the reaction mixture. The potent inhibition of the linear TAC16 and lac promoters was independent of either

the primer, RNA polymerase or DNA concentrations used. These titrations were performed with concentrations of 0.2 mM to 4 mM primer, 10 nM to 80 nM enzyme and 0.5 nM to 4 nM DNA. The inhibitory mAbs 210E8 and 311G2 elicited the same effect on RNA polymerase when abortive assays were performed at either 100 mM or 130 mM KCl.

The kinetics of ApApUpU synthesis on linear and supercoiled lac UV5 templates are compared in Table 10. As shown previously in Table 8 the inhibition of mAb 210E8 is noncompetitive with regard to primer concentration on the linear lac UV5 promoter. The V_{max} is decreased and the K_m is increased. Under the same conditions the kinetics of ApApUpU synthesis for the supercoiled lac UV5 template in the presence of mAb 210E8 yielded a V_{max} that was similar to that of the control and a slight increase in the K_m. In contrast the anti-beta' mAb 311G2 strongly inhibited abortive synthesis on the lac and TAC16 promoters independent of the template employed.

The effect of anti-beta mAb 210E8 on polymerase binding to the lac UV5 promoter was examined under various solution conditions using a gel electrophoresis technique devised by Garner and Revzin (1981). In this technique enzyme-DNA complexes are directly applied to a polyacrylamide gel. The large size of the undissociated complex prevents migration into the gel. A dissociation of the complex is evidenced by the migration of a free DNA band.

A tabulation of results of these binding studies is

shown in Table 11. The mAb-RNA polymerase-promoter complexes were found to be more sensitive than the control to dissociation by heparin, $d(A-T)_n$ and high salt concentrations at both 37 °C and 0 ° C.

To further examine the effects of mAbs on polymerase-promoter interactions DNA protection studies were performed with RNA polymerase-lac UV5 or TAC16 promoter complexes formed in the presence and absence of the inhibitory anti-beta mAb 210E8 and anti-beta' mAb 311G2. These studies were employed to examine the protection of known promoter sites from digestion by specific restriction enzymes when polymerase is bound to the promoter in the presence and absence of mAb. A complete restriction map of the lac promoter is shown in Figure 9. The restriction site for the TAC16 promoter is indicated in Figure 6B.

In these studies the accessibility of the HpaII site (-19) of the lac UV5 promoter and the HindII (-35) of the TAC16 promoter to digestion was examined with preformed RNA polymerase-promoter complexes after challenge with $d(A-T)_n$. These restriction sites are known to occur within regions which interact with bound polymerase during RP_o formation (Siebenlist et al., 1980; Kierkgaard et al., 1983). Polymerase-promoter complexes were preformed in the presence of an inhibitory mAb and in the absence of mAb as a control. Where indicated, complexes preformed in the presence of a noninhibitory mab also served as a control. The effects of mAbs on the digestion pattern of the Alu1 sites (-120,-59,

and +36) pattern of lac UV5-protein complexes was also examined.

The effect of both inhibitory mAbs on the protection by polymerase over time of the HpaII site of the lac UV5 promoter is shown in Figure 10. A comparison of the protection over time of the HpaII site of lac UV5 in the presence and absence of the inhibitory anti-beta mAb 210E8 versus the noninhibitory anti-beta mAb 221C7 is shown in Figure 11. The HpaII site was completely protected from digestion on lac UV5 promoter complexes formed with polymerase alone and polymerase in the presence of the inhibitory anti-beta' mAb and the noninhibitory anti-beta mAb 221C7. A partial digestion of the HpaII site was always observed with the mAb 210E8 polymerase-lac UV5 complexes. This unstable binding of mAb 210E8-polymerase complexes was found to be less pronounced in the absence of $d(A\text{-}T)_n$. In addition anti-beta' 311G2-mAb-polymerase lac UV5 promoter complexes challenged with heparin resulted in protection patterns which were similar to those obtained with $d(A\text{-}T)_n$. Studies of the protection of the HindII site on mAb 210E8-polymerase-TAC16 promoter complexes yielded protection patterns which paralleled those obtained with the HpaII site on lac UV5 (Figure 12). It is also shown in Figure 12 that the protection patterns of mAb 210E8 polymerase complexes were not changed when abortive substrates ApA and UTP for the lac UV5 directed synthesis of ApApUpU or ApU and UTP for the TAC16 directed synthesis of ApUpU were included in the

binding reactions. The HindIII site of the TAC16 promoter was fully protected over time by the anti-beta' mAb 311G2-polymerase complex (Figure 13). The effect of short chain synthesis from ternary complexes are shown in Figure 14. The inclusion of ATP + UTP + GTP to form a 9 base pair transcript (Munson & Reznikoff, 1981) resulted in a partial digestion of the HpaII site of control polymerase-lac UV5 complexes. These studies were performed in the absence of $d(A\text{-}T)_n$. Protection patterns obtained under abortive conditions of ApApUpU synthesis are included in Figure 14 for comparison. Ternary complexes formed in the presence of the mAb 210E8 showed the greatest sensitivity to cleavage by HpaII. Conversely, the complete protection pattern exhibited by anti-beta' mAb 311G2-polymerase lac UV5 complexes was consonant with its inhibitory properties. Similar protection patterns with the inhibitory mAbs were obtained with ternary complex formation on the TAC16 promoter. The AluI digestion patterns of lac UV5 protein complexes were also included in Figure 14 as a control to show that the binding of either inhibitory mAb-polymerase complex to the lac UV5 promoter is restricted to a similar promoter region as the control.

The direct effect of mAb 210E8 on promoter strength was examined using a quantitative analysis of the abortive initiation assay (McClure, 1980b). As explained in the Materials and Methods, the kinetics of this assay relies on the assumption that RP_O formation proceeds via an initial RP_C following a specific interaction between RNA polymerase

and the promoter. The time required for RNA polymerase to bind to a promoter and isomerize to an active RP_o is measured as a promoter specific lag (tau_{obs}) in an approach to the steady state rate of the abortive reaction. A quantitative separation of the initial binding step, K_B and the final isomerization step to RP_o (k_2) can be calculated from a linear plot (TAU plot) of the tau_{obs} at different concentrations of RNA concentration.

The anti-beta mAb 210E8 affected the lag time required for RP_o formation on linear and supercoiled lac UV5 and TAC16 promoters. Typical tau_{obs} plots are shown in Figures 15,16 and 17. The measurements obtained from these plots (Table 12) show that the mAb 210E8 induced effect on the lag time and the final residual activities varied with the promoter assayed. A pronounced inhibition of the rate of RP_o formation was observed for linear templates in the presence of mAb 210E8. Theoretically all reactions within a given experiment should reach a similar steady state by establishing parallel linear increases in product over time. Thus, the slope values of the reactions initiated by RNA polymerase should eventually approach those values established by the steady state control. The extent of the mAb 210E8 effect was examined by a statistical comparison of the slope values obtained from a least squares analysis of the linear portion of each curve (i.e. values greater than 4 minutes) for each promoter assayed. For each analysis the lag control slope values were compared with those values

obtained for the steady state control and the lag in the presence of mAb 210E8. Using these criteria, only the slope values for the mAb 210E8-RNA polymerase initiated reactions were found to differ significantly ($p \leq .05$) from the control values. Even after three hours incubation the abortive rates of synthesis established by mAb 210E8-RNA polymerase on linear templates were significantly different from the control values. Conversely, abortive synthesis on the supercoiled templates in the presence of mAb 210E8 eventually reached steady state rates which were not significantly different from those of the controls.

The effect of mAb 210E8 was also examined on the lag time of the T7 A1, A3 directed synthesis of CpApU (Figure 18) and the T7 DNA and $d(A\text{-}T)_n$ directed synthesis of pApU (Figures 19 and 20). The lag time required for RP_o formation for each assay condition was altered in the presence of mAb 210E8. The final rate of abortive initiation for each reaction was also less than that obtained for the controls (Table 13). The data for linear lac UV5 in Table 13 is included for comparison. These lag time assays also show that the extent of inhibition is reflected in the tau_{obs} measurements which varied with the template and primer employed.

A comparative slope analysis of those date points assumed to be in steady state within Figure 18-20 showed that the mAb 210E8 exhibited the greatest effect on the T7 DNA directed synthesis of pApU. While the statistical

comparision between the control and the mAb related slope value was significantly different for all reactions it should be noted that the t value associated with the comparison of pApU synthesis on T7 DNA was at least twice that of CpApU synthesis on T7 DNA and pApU synthesis on $d(A-T)_n$.

A tau plot showing the effects of mAb 210E8 on the rate of RP_o formation on the lac UV5 insert is presented in Figure 21. The mAb 210E8 reduced the isomerization rate constant, k_2, by approximately 30% with little effect on the initial binding (K_B) of RNA polymerase to template. The control tau_{obs} values obtained in this study are intermediate to those obtained for a similar study on a fully supercoiled lac UV5 insert (Malan et al., 1984). This finding most likely reflects the fact that the method employed for these studies for plasmid isolation has been shown to yield some open circular forms of plasmid (Marko et al., 1982).

To further investigate the inhibitory pattern elicited by mAb 210E8 and mAb 311G2 on RNA polymerase-promoter interactions, mAb effects were determined on the contacts formed between polymerase-lac promoter complexes during RP_o formation. In these studies RNA polymerase contacts along the DNA were examined by probing both the phosphodiester backbone with DNase 1 and base residues along the DNA with dimethyl sulfate. DNase 1 footprints and guanine methylation patterns were obtained for the lac promoter interactions

with polymerase alone and with mAb-polymerase complexes formed with the inhibitory anti-beta mAb 210E8 and anti-beta' mAb 311G2 and the noninhibitory anti-beta mAb 221C7 and anti-beta' mAb 371D6. A summary of mAb effects on polymerase-lac UV5 promoter complex formation is shown in Table 14.

The results of the DNase 1 footprint analysis showed that RNA polymerase-lac complexes in the presence of the inhibitory mAb 210E8 had patterns which were slightly different than those of the controls. The presence of the enhanced band between -20 and -30 when polymerase binds to the lac promoter was suppressed in the presence of mAb 210E8. This effect was found to be most pronounced on the lac P^+ promoter in the presence of cAMP-CRP (Figure 22). The DNase 1 footprint for the anti-beta' mAb 311G2-polymerase complex did not differ from the control pattern formed in the absence of mAb. The noninhibitory anti-beta' mAb 371D6 showed an enhanced DNase 1 digestion pattern which extended the full length of the fragment. The results presented in Figure 22 also show that the DNase 1 pattern was not appreciably changed when initiation complexes were formed by the addition of ApA + UTP.

Chemical modification studies with dimethyl sulfate revealed altered methylation patterns for RNA polymerase-lac UV5 complexes formed in the presence of mAb 210E8, 311G2 and 317D6 (Figure 23). Significant enhancement or protection patterns for guanine contacts on the control RNA polymerase-

promoter complexes were consistent with previous results (Siebenlist et al., 1980). On the template strand the guanine residues at -32 and -14 positions were found to be protected and enhanced respectively. On the nontemplate strand guanine residues at the -6, -13, and -24 positions were protected against methylation while the guanine residues at the -1, -17, -38 positions were more accessible to methylation. The mAb 210E8-RNA polymerase promoter complexes displayed a reactivity pattern for attack by dimethyl sulfate which was intermediate with those obtained for the control polymerase bound promoter and the unprotected promoter fragment. In the presence of mAb 210E8 the extent of the protection of the guanine at -32 and the enhancement of the guanine at -14 on the template strand was always less than the reactivities obtained for the controls. An overexposure of the gel for this experiment revealed the protection by mAb 210E8-polymerase of an adenine at -37 on the template strand. When compared to the control lanes this protection is only observed on the promoter fragment in the absence of polymerase. In contrast to the effects of the anti-beta mAb 210E8, the binding of the inhibitory anti-beta' mAb 311G2 -polymerase complex to the lac UV5 promoter was accompanied by a greater protection of the guanine at -32 on the template strand as compared to the control. The enhancement of the guanine at -14, however, was always similar to or slightly less than that of the control. A greater protection of guanine -32 was also observed with the

noninhibitory mAb 371D6 polymerase-lac UV5 complexes but this effect was not consistently produced. Like the template strand, the effect of mAb 210E8 on the binding of polymerase to nontemplate strand residues was observed as changes in the methylation pattern of essential guanine contacts. In the presence of mAb 210E8 the binding of polymerase to the nontemplate strand of lac UV5 resulted in a pattern of enhancement or protection which was intermediate to that obtained with polymerase-bound and free DNA.

The binding of polymerase to the lac P^+ promoter in the presence of the noninhibitory anti-beta' mAb 37106 resulted in an enhanced DNase 1 cleavage pattern (see Figure 22) that was paralleled by an enhanced reactivity pattern of guanine contacts to methylation by dimethyl sulfate (Figure 23). This effect of mAb was observed for complex formation on both the nontemplate and template strand of the lac P^+ and lac UV5 promoter fragments. On both DNA strands the effect was most dramatic for contacts within a region extending from the -40 to -20 positions. The enhancements observed within this region are not present on the DNase 1 and dimethyl sulfate reactivity patterns for either the protected or unprotected fragment. Variations in the DNase 1 cleavage patterns were obtained when mAb 371D6-RNA polymerase complexes were bound to the lac P^+ or lac UV5 promoters in the presence and absence of CRP-cAMP. An additional band was observed (at position -80) when mAb 37D16-RNA polymerase complexes were formed with lac P^+ and

lac UV5 in the presence of CRP (Figure 24). The appearance of this band was CRP dependent and was only observed in the presence of mAb 371D6. In addition, the activation of the mAb 371D6-RNA polymerase-lac P^+ promoter by CRP-cAMP was also marked by a change in the intensity of the enhanced bands between the -20 and -30 positions and the -60 to -80 positions of the fragment.

The effects of the anti-beta mAb 210E8 was also examined on the reactivity patterns of the N3 positions of unpaired cytosines at the -6, -4, -2, and -1 positions of the template strand of RNA polymerase-lac UV5 complexes (Figure 25). The results show that the methylation of unpaired cytosines during RP_O formation on lac UV5 could not be detected in the presence of the inhibitory mAb 210E8. Conversely, the chemical modification of the single stranded region induced by RNA polymerase binding is clearly indicated for the complexes formed with polymerase alone and the noninhibitory mAb 221C7-RNA polymerase and the inhibitory ant-beta' mAb 311G2-RNA polymerase. Although not shown, N3 cytosine methylation studies of RNA polymerase-lac UV5 promoter complexes formed in the presence of the noninhibitory anti-beta' mAb 371D6 showed a reactivity pattern similar to the control patterns obtained in Figure 25.

To insure that the patterns obtained were not a reagent induced effect a DNase 1 footprint of complexes treated with dimethylsulfate showed that the conditions for methylation

did not appreciably alter the polymerase-DNA contacts in the presences or absence of mAbs.

A dramatic change in the footprinting pattern of the inhibitory mAb-RNA polymerase-lac UV5 complexes resulted when reactions were performed in the presence of 25% glycerol (Figure 26). The use of 25% glycerol in the binding reaction is known to increase the affinity of the polymerase for the lac P^+ promoter (Reznikoff, 1976) and stabilize the sigma-core interactions of the enzyme (Gonzales et al, 1977). Under these conditions mAb-210E8-polymerase binding resulted in a footprint which showed an enhanced DNase 1 cleavage extending from positions -80 thru -10. The enhanced cutting by DNase 1 was paralleled by an extensive protection from methylation by dimethylsulfate (Figure 27). In both cases mAb 210E8 induced enhancements or protections were greater than that of the control. Protection from methylation of significant guanine contacts was greater for the mAb 210E8-RNA polymerase promoter complexes formed at 2% rather than 25% glycerol. However the enhanced bands at -17 and -1 observed for control complexes were still not obtained in the presence of the inhibitory mAb 210E8. The DNase 1 cutting pattern of mAb 311G2-RNA polymerase-lac UV5 complexes resembled the control patterns obtained at both low and high concentrations of glycerol. However dimethyl sulfate treatments of mAb 311G2-polymerase-promoter complexes formed at 25% glycerol revealed a methylation pattern that showed an extensive protection of

guanine contacts along the DNA. The extent of protection observed with this methylation pattern is similar to but more dramatic than that which appears on patterns of mAb 210E8-polymerase lac UV5 complexes formed under the same conditions.

Similar changes in both the DNase 1 footprint and the guanine methylation patterns were observed with RNA polymerase-TAC16 promoter complexes formed in the presence of the inhibitory mAbs 210E8 and 311G2 at low glycerol concentrations. In the presence of either mAb an enhanced DNase 1 cleavage in the -10 region was paralleled by an extensive protection with regard to guanine methylation along the promoter (Figure 28). A reaction of the mAb-RNA polymerase-TAC16 promoter complexes with dimethyl sulfate revealed that the guanine at position -32 on the template strand was protected by both inhibitory mAb RNA polymerase complexes while the enhancement at position -14 did not occur. Also the reactivity pattern of mAb 371D6-polymerase TAC16 promoter complexes did not show the additional enhancements of contacts observed with the lac promoter complexes. Instead, the binding of mAb 371D6-polymerase to the TAC16 promoter resulted only in a greater enhancement of DNA contacts observed in the footprint and methylation patterns of control complexes formed in the absence of mAb.

DISCUSSION

In the present study monoclonal antibodies raised against the purified beta and beta' subunits of the E.coli RNA polymerase were used as site specific reagents. This approach is based on the development of the hybridoma technology (Kohler and Milstein, 1975) which has made it possible to obtain antibodies directed against a single determinant present on a protein. An initial characterization of mAb effects on RNA polymerase was performed using the $d(A\text{-}T)_n$-directed reaction as a model system. Certain of the anti-beta and beta' mAbs were shown to inhibit RNA polymerase during the $d(A\text{-}T)_n$ directed synthesis of $r(A\text{-}U)_n$, elongation by preformed ternary complexes and abortive initiation of pApU and UpApU. Although anti-polymerase polyclonal antibodies have been reported to block DNA binding (Gragerov and Nikiforov, 1980, Stender, 1981), the binding of $d(A\text{-}T)_n$ is not inhibited by any of the anti-beta or anti-beta' monoclonal antibodies that have been studied. The apparent $K_m d(A\text{-}T)_n$ is unchanged for the inhibitory anti-beta mAb 210E8 and is markedly decreased for the inhibitory anti-beta' mAb 311G2. The inferred higher affinity for the template in the polymerase-anti-beta' antibody complex may cause a lowered rate of translocation and contribute to the consequent inhibition of the rate of ribonucleotide incorporation. The beta' subunit has been implicated in template binding by RNA

polymerase (Zillig et al., 1976) The effect of the inhibitory anti-beta' monoclonal antibody as a potent inhibitor of both initiation and elongation reactions is indicated by the pronounced effects on the V_{max}. The observed inhibition is not a consequence of blocking template binding, suggesting that the beta' subunit also participates in the catalytic activity of the enzyme.

The preformed ternary elongation complex (Schmidt and Chamberlin, 1984) remains sensitive to the anti-beta mAb 210E8. When the enzyme is in an elongation mode the anti-beta mAb 210E8 exhibits a noncompetitive mechanism of inhibition which results in a decreased rate of catalysis (V_{max}) and a moderate increase in the affinity of the enzyme for substrate (K_m). The abortive synthesis of UpApU on $d(A-T)_n$ and CpGpC on $d(I-C)_n$ by the anti-beta mAb 210E8 appears to be competitive with regard to the UpA or CpG primer as shown by the reversal from inhibition at high concentrations of primer. In the case of UpApU synthesis the mAb 210E8 is noncompetitive with regard to the UTP substrate.

Like the studies on synthetic templates the results of the abortive reactions and binding assays on promoter containing templates showed that the inhibitory anti-beta mAb 210E8 and anti-beta' 311G2 differed in their effects on RNA polymerase. While the binding studies demonstrate that the anti-beta' mAb 311G2 does not interfere with polymerase binding to DNA, its presence imparts a potent inhibition on the abortive initiation with all templates studied. These

findings are consistent with the role of the beta' subunit in catalysis as well as template binding.

Kinetic studies with T7 DNA show that the anti-beta mAb 210E8 is a competitive inhibitor of CpApU synthesis on the T7 A1, A3 promoters only at low molar ratios of enzyme to DNA. Like UpApU and CpGpG synthesis directed by synthetic templates, the mAb induced decrease in the affinity of polymerase for the CpA primer is compensated for by an increased concentration of substrate which allows the reaction to proceed at the same velocity as the control. The competitive mode of inhibition effected by the anti-beta mAb 210E8 on the $d(A\text{-}T)_n$ and T7 A1, A3 directed abortive reactions suggests that the antibody may bind to a region associated with the initiation nucleotide binding site of polymerase. Under these conditions, binding of the antibody could alter the affinity of polymerase for the initiation nucleotide (or dinucleoside monophosphate primer) without affecting the V_{max} of the reaction.

The syntheses of UpApU, CpGpC and CpApU from their respective templates are known to occur as rapid turnover reactions from unstable initiation complexes (Oen and Wu, 1978; Sylvester and Cashel, 1980.) On T7 DNA CpApU formation occurs at a greater frequency on the A1 promoter than on the A3 promoter. It has been proposed that the presence of the 3' terminal pyrimidine on rapid turnover transcripts leads to the formation of an abortive product that readily dissociates, most likely, from an

untranslocated complex (So and Downey, 1970; Cech et al., 1978). The fact that the anti-beta 210E8 mAb only exhibits a competitive mode of action during rapid turnover reactions infers that the rapid synthesis of product occurs via a conformational arrangement of the enzyme with template that is different from those reactions that proceed with a slower turnover of product.

A comparison of the data presented for the various T7 DNA and lac promoters suggests that the extent of inhibition of abortive synthesis in the presence of mAb 210E8 appears to correlate with promoter strength. The greater inhibition of ApApUpU synthesis on the lac promoters relative to the synthesis of CpApU on T7 DNA may reflect the stronger promoter strength demonstrated for the T7 A1 promoter relative to the lac promoters (Hawley et al., 1982). The mAb induced change in the kinetics of the T7 A1, A3 directed synthesis of CpApU at high ratios of polymerase to DNA implies that the initiation reaction under these conditions involves additional enzyme-DNA interactions in which primer is not incorporated. Given the high concentration of primer employed and the fact that abortive initiation is a sequence dependent reaction it seems unlikely that CpA is incorporated at other start sites on T7 DNA.

Binding studies with T7 DNA have shown that at high ratios of enzyme to DNA promoter selectivity is lost as a result of polymerase binding to both promoter and nonpromoter sites on the DNA (Stahl and Chamberlin, 1977;

Hinkle and Chamberlin, 1972). The presence of mAb may impede the binding of polymerase to the T7 A1, A3 promoter by a hindrance of those nonpromoter interactions that have been implicated as an intermediate step in promoter site selection (von Hippel et al., 1982). The inhibition may result from a hindrance of the transfer of polymerase from nonspecific to promoter specific sites and/or the proposed sliding of polymerase along the DNA to a promoter target.

The influence of enzyme to DNA ratios on the mAb induced inhibition of CpApU synthesis on T7 DNA was also apparent by titrating DNA polymerase with increasing concentrations of DNA. The decrease in inhibition of T7 A1, A3 directed synthesis of CpApU which accompanied the increase in DNA concentration may correlate with a greater polymerase occupancy at the T7 A1, A3 promoters, assuming that primer incorporation on T7 DNA is restricted to the A1 promoter at low enzyme to DNA ratios. This was not the case for titrations performed on the linear lac UV5 promoter. Instead, a high level of inhibition was maintained independent of DNA concentration. The potent inhibition of linear lac UV5 promoter by mAb 210E8 was also reflected in the V_{max} and K_m values obtained from kinetic analyses in the presence of the mAb. On the linear template the presence of mAb 210E8 lowers the rate of catalysis and decreases the affinity of polymerase for substrate even at high concentrations of primer.

The lowered V_{max} values on both the T7 A1, A3 promoters

at polymerase excess and the lac UV5 promoter may also involve the effects of bumping phenomena postulated by Shanblatt and Revzin (1984). It is proposed that an excess concentration of enzyme reduces promoter occupancy by creating a situation in which nonspecifically bound enzyme molecules directly displace polymerase from specific promoter sites. The unstable nature and low rate of ApApUpU synthesis elicited by anti-beta mAb 210E8-polymerase lac UV5 complexes could result from such collisions since the presence of mAb could make polymerase more susceptible to dissociation by steric occlusion.

It is difficult to ascertain whether the inhibition of substrate incorporation by polymerase on both synthetic and promoter templates is a consequence of the antibody binding at the initiation site or an indirect effect resulting from an antibody-mediated distortion of the site. A direct inhibition by steric hindrance could result if the monoclonal antibody interacted with amino acid side chains involved in the catalytic domain. Conversely, binding of the antibody could indirectly lower enzymatic activity by freezing a conformation at the determinant with consequent effects transmitted to the catalytic domain. The interaction with the antibody could also hinder any subunit rearrangements or conformational shifts which are necessary for efficient substrate utilization during catalysis. It is interesting to note that the inhibitor streptolydigin, which binds to the beta subunit and inhibits polymerization, shows

a pattern of inhibition which is opposite to that elicited by the anti-beta mAb 210E8. The inhibition of abortive initiation on a $d(A-T)_n$ template by streptolydigin is noncompetitive vs. the initiating nucleotide, AMP, and competitive vs. UTP (McClure, 1980a).

The data presented for the various forms of the lac and TAC16 promoters suggest that inhibition of abortive synthesis in the presence of mAb 210E8 appears to correlate with changes in DNA topology. The potent inhibition of abortive synthesis only on the linear form of these promoters suggests that supercoiling enhances transcription by mAb-210E8-RNA polymerase complexes. Evidence for the mAb 210E8 induced hindrance of promoter activity on linear templates is demonstrated by the results of the lag time assays. In the presence of mAb 210E8 the formation of ApApUpU on linear lac UV5 and ApUpU and UpGpU on linear TAC16 proceeds at exceedingly slow rates with residual activities that were less than 20% of the control values. The results of the tau observed measurements on linear templates also indicate that the mAb 210E8-RNA polymerase complex does not form a fully active open promoter complex capable of yielding steady state rates comparable to those observed with RNA polymerase in the absence of the antibody.

In contrast to the results obtained with the linear promoter fragments the kinetics of the abortive reaction on the supercoiled lac UV5 template in the presence and absence of mAb 210E8 approached steady state rates that paralleled

those of the control. The TAU plot obtained for the lac UV5 insert indicated that the presence of mAb 210E8 decreases the rate at which the closed promoter complex, RP_C, isomerizes to an open promoter complex, RP_O, (k_2 value) but has no effect on the initial rate of polymerase binding to the promoter (K_B). This favorable binding constant may explain the difference in the inhibition of the rates of abortive initiation on linear and supercoiled lac UV5 in the presence of mAb 210E8. Supercoiling may enhance the promoter occupancy for the mAb 210E8-RNA polymerase complex on the lac UV5 insert. Kinetic measurements indicate that stable binding of RNA polymerase to a negatively supercoiled DNA is due to an enhanced rate of association and a reduced rate of dissociation (Wang, 1982). In addition, the unwinding of DNA is known to be more favored on negatively supercoiled templates (Davidson, 1972). It has also been shown that the initial binding constant, K_B, of lac UV5 is greatly increased on a supercoiled template while the rate of isomerization, k_2, is decreased (Malan et al., 1984). The similar K_B value in the presence or absence of mAb 210E8 suggests that the favorable initial association between DNA and RNA polymerase on supercoiled lac UV5 is unaffected by the bound antibody. However the decreased k_2 value in the presence of mAb 210E8 implies that the presence of antibody does have an effect on the final isomerization to an active promoter complex.

The initial model for the formation of open complexes

involved only two intermediates, RP_c and RP_o (Chamberlin, 1974). Based on analyses of the properties of complexes formed between RNA polymerase and a lac UV5 fragment an additional intermediate has been identified. This intermediate species, RP_i has been characterized by kinetic (Buc and McClure, 1985) and footprint (Spassky et al., 1985) analyses. Based on the results obtained these authors have proposed the following multistep scheme in which the formation of RP_i is the rate limiting step:

$$R + P \underset{K_1}{\rightleftharpoons} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} RP_o$$

Spassky et al. (1985) suggest that the formation of RP_i involves the initial positioning of the enzyme with the -35 and -10 regions of the promoter. At 37°C this step is immediately followed by a rapid isomerization to form the RP_o. They also suggest that the transition from RP_i to RP_o involves a cooperative transconformation of enzyme and DNA in the binary complex. The appropriate contacts between amino acid side chains and DNA sites are then established and consequent unwinding of the DNA around position +1 occurs to yield the active RP_o complex.

The effect of mAb 210E8 on the initiation complexes formed on linear and supercoiled templates suggests that RNA polymerase-promoter interactions vary with the topology of the DNA. The data indicate that supercoiling facilitates

the conversion of the mAb 210E8-RNA polymerase-lac UV5 promoter complex to an active open promoter complex. Buc and McClure (1985) indicate that in the case of a superhelical template the system is driven toward formation of the open complex. It would appear that the effect of mAb 210E8 on the inhibition of RNA polymerase with linear promoters is compensated for when the promoter is in a superhelical state.

The results of the DNase footprints of mAb 210E8-RNA polymerase-lac promoter complexes suggest that the positioning of RNA polymerase with respect to the DNA backbone is not greatly altered by the inhibitory mAbs. The suppression of the enhanced band between the -20 and -30 positions does indicate the partial loss of a significant binding contact to the lac promoter. However, the obvious difference in the reactivity of mAb 210E8-RNA polymerase-lac UV5 complexes to methylation by dimethyl sulfate revealed that the presence of the inhibitory mAb induces an alternate mode of RNA polymerase binding to the lac promoter. Under such conditions RNA polymerase interacts with essential guanine contacts on both the template and nontemplate strands of DNA in a form which is intermediate between a fully protected and unprotected fragment. The inability to methylate cytosine residues at positions -6, -4, -3, -1 on the template strand indicates that the mAb 210E8-RNA polymerase-lac UV5 complex more closely resembles a closed rather that an open promoter complex. This failure to

detect single stranded cytosines in the mAb 210E8-RNA polymerase-promoter complex suggests that the antibody hinders the polymerase mediated unpairing of specific base pairs and thus, the transition from a closed to an active open promoter complex. However, the results of the lag time assays show that the mAb-RNA polymerase-lac UV5 complex on a linear template is still active since abortive product slowly accumulates over time.

A further demonstration that the mAb 210E8-polymerase promoter complex does not form a stable RP_O was shown in the inability of mAb 210E8-polymerase to bind to and prevent a cleavage of the HpaII site of the lac UV5 and the HindII site of the TAC16 promoters. The partial protection patterns obtained from these studies suggests that the mAb induced binding of polymerase to the lac UV5 or TAC16 promoters results in the formation of a binary complex with a partial sensitivity to dissociation by $d(A\text{-}T)_n$.

These data suggest that product may be formed from an unstable intermediate complex in which the DNA is partially unwound or from a RP_O, when formed, that has a half life which is too short for detection. The antibody appears to trap RNA polymerase in a conformation during complex formation that prevents the final conversion to the formation of a stable RP_O. The consistent low level of abortive synthesis on the linear templates suggests that the transformation of the mAb-210E8-RNA polymerase-promoter complex to the open complex proceeds very slowly. Under

such conditions polymerase binding may lead to the formation of a single stranded region within the duplex DNA in which the N3 positions of cytosines are not sufficiently exposed for methylation. The inaccessibility of the cytosines to methylation could also indicate that a mAb induced shift in the positioning of polymerase, or the mAb itself, blocks the unwound region of the promoter from methylation by dimethyl sulfate.

The effect of anti-beta mAb 210E8 on polymerase interactions with the linear lac UV5 and TAC16 promoters also suggests that the mAb may inhibit abortive initiation from these promoter templates by a similar mode of action. This parallel in the inhibitory mechanism is inferred by the mAb induced low levels of abortive product, the sensitivity to dissociation by $d(A\text{-}T)_n$ and the loss of protection of guanine -14 on the template strand for both polymerase-promoter complexes. A major distinction between the inhibition elicited by mAb 210E8 on these promoters was the protection of guanine -32 on the template strand of the TAC16 but not the lac UV5 promoter, suggesting that the formation of an active RP_o necessitates a proper interaction with both guanines at positions -32 and -14 on the template strand.

According to the model for RP_o formation cited above the conversion of RP_c to RP_o on the linear lac UV5 promoter assumes a requirement for a strict alignment of the -10 and -35 regions with respect to each other and also with respect

to the positioning of RNA polymerase with DNA (Spassky et al., 1985). The inclusion of the RP_i complex in the model as an intermediate step resulted from kinetic analyses of lac UV5-polymerase complexes which showed that a transient, temperature dependent complex forms prior to RP_o formation. (Buc and McClure, 1985). This RP_i was unable to initiate transcripts but was stable to dissociation by $d(A-T)_n$. The mAb 210E8 mediated RP_i proposed in the present study represents a different form of intermediate promoter complex by exhibiting a permanent low rate of product formation at 37°C and a partial sensitivity to dissociation by $d(A-T)_n$. The latter finding is not unexpected if mAb-polymerase complexes did elicit non-optimal enzyme-DNA interactions. Under these conditions initiation complexes on linear templates could be readily dissociated by $d(A-T)_n$ at any step in the kinetic pathway.

Assuming that the inhibition exhibited by mAb 210E8 is mediated through a conformational effect on RNA polymerase in the RP_i complex it is proposed that nonoptimal contacts are established within the linear promoters. This improper positioning might result in an inhibition of the rate and/or extent of unwinding of the double helix which would be reflected in alterations in the kinetics of initiation. Unlike linear lac UV5, kinetic studies have shown that the formation of the RP_i does not appear to be favored on the supercoiled template and a rapid conversion to an active RP_o is observed (Buc and McClure, 1985).

The demonstration that supercoiling modulates the expression of inhibition by mAb 210E8 was complicated by the mAb induced hindrance of ApApU but not UpGpU synthesis on the TAC16 insert. These results indicate that abortive initiation on the supercoiled TAC16 promoter is also sensitive to changes in the spacer distance between the -10 and -35 regions of the promoter. The data suggest that the interactions of mAb 210E8-polymerase with the TAC16 insert are different when the spacer length is shifted from 16 base pairs for ApUpU synthesis to 18 base pairs for the synthesis of UpGpU. Indeed, it has been postulated (Mulligan et al., 1985; Brosius et al., 1985) that the utilization of UpG by RNA polymerase on the TAC16 promoter results from an alternate mode of interaction with a phase shifted -10 region. Mutational studies with the lac promoter (Stefano and Gralla, 1982) and the P22 phage promoter from S. typhymurium (Grana et al., 1985) have shown that promoter activity is sensitive to changes in spacer length. Based on such findings it has been proposed that changes in spacer length would serve as a determinant of promoter strength and like supercoiling affect the correct alignment of RNA polymerase with promoter consensus regions during formation of an open promoter complex.

Although cloned into different plasmids the data also suggest that the lac UV5 and TAC16 promoters on supercoiled templates interact with mAb 210E8 polymerase in a similar fashion. Complexes of mAb 210E8-polymerase formed with

supercoiled lac UV5 and TAC16 (UpG) promoters appear to undergo the transition to an active promoter complex with fewer constraints than the TAC16 ApU insert. The lack of inhibition for the TAC16 UpG and lac UV5 inserts suggests that a promoter with a spacer length of 18 base pairs facilitates abortive synthesis by the mAb 210E8-polymerase complex. On the TAC16 promoter the presence of 2 additional base pairs between the consensus regions appears to alleviate the mAb 210E8 induced hindrance observed when the spacer length is 16 base pairs.

Since the -10 regions of the lac and TAC promoters are identical in sequence the differential effects of mAb 210E8 for the lac UV5 and the TAC16 ApU inserts may also reflect alternate interactions in the -35 region as shown by the mAb induced difference in the methylation pattern of guanine -32 on linear forms of these promoters. However, these data may also be compatible with the results of a comparative footprint analysis which demonstrated that the lac and TAC promoters display different patterns of interaction with RNA polymerase downstream from the -10 region during RP_o formation (Kirkegaard et al., 1983).

Kinetic analyses have shown that supercoiling can elicit complex effects on the parameters of promoter strength. Comparative measurements of RP_o formation on linear and supercoiled templates of the lac and TAC16 promoter (Malan and McClure, 1984; Mulligan et al., 1985) have shown that supercoiling exerts a positive and negative effect on the

promoter strength of lac UV5 and TAC16(UpG). On a supercoiled template the affinity of polymerase for the lac UV5 and TAC16(UpG) promoters (K_B value) was greatly increased while the rate of isomerization to an active RP_o (k_2 value) was decreased. In contrast, supercoiling increased the k_2 values of the lac P^+ and TAC16(ApU). It is conceivable that the lack of inhibition by mAb 210E8 on supercoiled lac UV5 and TAC16(UpG) templates may correlate with the paradoxical effect of supercoiling on these promoters. The demonstration that mAb 210E8-RNA polymerase may require supercoiling and an 18 base pair spacer to achieve optimal promoter activity may actually reflect those nonoptimal polymerase interactions that effect the decrease in the k_2 value of the lac UV5 and TAC16(UpG) promoters on supercoiled templates. The strict requirement that the enzyme orient correctly with specific promoter sites may necessitate that RNA polymerase form a tighter interaction on a promoter with a 16 base pair spacer region. Such a conformation in the presence of mAb 210E8 could render the formation of a stable RP_o more susceptible to a steric hindrance by mAb of either the simultaneous alignment of RNA polymerase with the consensus regions of a promoter, the untwisting of the DNA and the subsequent melting of the -10 region.

The DNase 1 footprints and guanine methylation pattern obtained in the presence of 25% glycerol show that the binding of mAb 210E8 polymerase to the lac UV5 promoter is

changed dramatically under conditions which favored binding. Unlike studies at low glycerol concentrations the presence of 25% glycerol induced a mAb-polymerase interaction with lac UV5 that resulted in an enhanced DNase 1 cleavage of the phosphodiester backbone of lac UV5 paralleled by strong protection from methylation of guanine contacts along the DNA. The nature of the DNase 1 footprint and guanine methylation pattern suggests that the mAb 210E8 induces a binding of excess polymerase molecules along the promoter, possibly, in a situation which is analogous with the enhanced complex formation observed on $d(A\text{-}T)_n$. Studies by Siebenlist and Gilbert (1980) have shown that the binding of polymerase to a promoter is restricted to one side of DNA. Given this premise it appears that the interaction of excess mAb 210E8-polymerase molecules along the DNA may render the phosphodiester backbone more susceptible to cleavage by DNase 1 and the guanine contacts less reactive with dimethyl sulfate.

The results of the binding assays indicate that the anti-beta' mAb 311G2 does not interfere with polymerase binding to promoter templates. The similarity of the mAb 311G2-RNA polymerase-promoter footprint to that of the control suggests that the mAb does not alter the positioning of polymerase on the DNA. The detection of single stranded cytosines by methylation also shows that mAb 311G2 does not prevent the formation of a stable preinitiation complex. However, the results of the kinetic analyses on promoter

templates suggest that the anti-beta' mAb traps polymerase in a conformation which is unable to undergo initiation.

It has been postulated from crosslinking studies of T7 DNA (Hillel and Wu, 1977) that polymerase interactions with DNA involve an initial contact by the beta' subunit followed by a shift in protein conformation to bring the beta subunit close to the template for catalysis. mAb 311G2 may hinder the necessary conformational shift for catalysis by inducing a tighter binding of polymerase to the promoter. This is inferred by the tight interaction of mAb 311G2 polymerase with the guanine contact at position -32 on the template strand of lac UV5, the mAb induced decrease in K_m values obtained from kinetic studies of UTP incorporation on $d(A-T)_n$, CpG incorporation on $d(I-C)_n$ and $d(A-T)_n$ titrations of $r(A-U)_n$ synthesis. These findings suggest that polymerase has a greater affinity for these templates in the presence of mAb 311G2. The partial digestion of the HpaII site of control RNA polymerase-lac UV5 ternary complexes infers the step at which RNA polymerase relinquishes promoter specific contacts to shift from an initiation conformation to an elongation mode for the production of longer transcripts (von Hippel et al., 1984). The unaltered protection pattern of mAb 311G2-RNA polymerase ternary complexes suggests that the inhibitory anti-beta' mAb hinders this step.

The behavior of the anti-beta' mAb 311G2 mAb-polymerase complexes with promoter templates appears to mimic the effects elicited by L157 mutation of the lac promoter

(Hopkins, 1974). This mutation is a base pair change of the C-G at -32 to A-T. The mutant binds with RNA polymerase to form stable preinitiation complexes which are inactive (Maquat and Reznikoff, 1978). The activity of mAb 311G2 during abortive initiation and binding reactions with the lac UV5 promoter appears to mimic the salient features of the lac L157 mutant. Those characteristics of the mAb which infer a parallel relationship with the L157 mutant are as follows: the ability of mAb 311G2-polymerase-DNA complexes to show a tight interaction with guanine -32, a resistance to challenge with $d(A-T)_n$ or heparin, a melted region of DNA and an inability to initiate transcription. These data imply that the mode of inhibition effected by mAb 311G2 resembles that of the L157 mutant by acting at a step which is subsequent to RP_o formation.

An even stronger protection of the guanine residues of the lac UV5 promoter was elicited by the inhibitory anti-beta' mAb 311G2-RNA polymerase binding in the presence of 25% glycerol. These data suggest that the beta' mAb induces polymerase to form a tighter interaction with guanine residues that is more extensive than that induced by the inhibitory anti-beta mAb 210E8.

It is difficult to assess the dramatic parallel change elicited by the noninhibitory anti-beta' mAb 371D6 on the DNase 1 footprints and guanine methylation patterns of the RNA polymerase-lac promoter complexes. A comparison of these data with previous studies of RNA polymerase lac

promoter interactions (Spassky et al., 1984, and 1985) suggests that the enhancements appear to correlate with normal RNA polymerase contacts along the DNA. The regions of greatest enhancement (between position -20 to -40) coincide with areas of greater intensity on control reactivity patterns. The shift in enhancements of mAb 371D6-RNA polymerase-lac promoter complexes in the presence of CRP-cAMP may parallel the previously documented change in RNA polymerase interactions resulting from CRP binding (Spassky et al., 1984). It was postulated that the CRP induced shift in polymerase binding represents a transfer from the weak P_2 promoter to occupancy at the major P_1 promoter (Malan and McClure, 1984). Furthermore the presence of the CRP dependent band in DNase 1 footprints of mAb 371D6-RNA polymerase lac P^+ (and lac UV5) promoter complexes appears to provide evidence for the reported changes in DNA structure caused by CRP binding (de Crombrugghe et al., 1984) and the existence of a direct protein-protein interaction between CRP and polymerase on the promoter. It should be noted that the effect of mAb 371D6 on polymerase binding appears to be unique to the lac promoter since the unusual binding pattern is not observed when mAb-polymerase complexes bind to the TAC16 promoter. Perhaps this reflects an influence of spacer length on polymerase binding. It should be emphasized, however, that the methylation pattern of significant guanine contacts on anti-beta' mAb 371D6-polymerase complexes with the lac UV5

and TAC16 promoters resembled those of the control in the absence of mAb.

Interpretation of the mechanism of antibody-mediated inhibition is complicated by the complex nature of the events required for transcription to proceed. The RNA polymerase catalytic domain includes in close proximity the sites for initiation and elongation nucleotide binding, template binding and helix unwinding (von Hippel et al., 1984). The present results indicate that the inhibitory anti-beta and anti-beta' monoclonal antibodies bind to antigenic determinants whose availability and conformation are conserved in both the free subunit and the assembled enzyme. The sensitivity of the $d(A\text{-}T)_n$ ternary elongation complex to inhibition by anti-beta mAb 210E8 indicates that the antigenic determinants are not directly involved in the template or product binding site. An explanation of the mechanism of inhibition must also take into account the ordered nature of the polymerase reaction in which incorporation of the substrate ribonucleoside triphosphate is dependent on the prior binding of the nucleotide occupying the initiation/product terminus site (McClure et al., 1978).

In the present study the pleiotropic effects of the anti-beta mAb suggests that the antibody binding with the beta subunit does not affect the active site directly. Instead, the binding of the anti-beta mAb to polymerase most likely is accompanied by a conformational shift within the

beta subunit that allows the active site to remain accessible for catalysis but under conditions which decrease the rate of the reaction. In contrast, the interaction of the anti-beta' mAb with polymerase does not affect DNA binding but does bring about an effective inhibition of catalysis independent of template. It seems unlikely that the inhibitory antibodies act by directly binding to amino acid side chains in the nucleotide binding sites. Such sites are generally found to occupy clefts formed by folding or interaction of subunits and would be inaccessible to molecules as large as immunoglobulins. Protein antigenicity strongly correlates with the accessibility of exposed regions on the protein surface to contact large probes equivalent in size to antibody domains (Novotny et al., 1986).

An antigenic determinant consisting of six to seven amino acid residues (Medgyesi et al., 1978; Lindstrom et al., 1978) covers a relatively small area. The actual area occluded by the arm of a bound antibody is about 35 A (Tzartos et al., 1981). Tzartos et al., (1981) have pointed out that this area is about 5 times that of the antigenic determinant and is referred to as a "region". Because of the large area of the immunogenic region, it is not surprising that the nature of the inhibition effected by the binding of a monoclonal antibody is more complex that exhibited by classical low molecular weight inhibitors. The interaction of sigma with the core unit is accompanied by complex

effects on the kinetic properties of RNA polymerase (Hansen and McClure, 1980). A differential inhibition as a function of the template employed has been reported for RNA polymerase in the presence of the nusA gene product (Schmidt and Chamberlin, 1984).

Considering the large size of an immunoglobulin the presence of mAb 210E8 could possibly hinder the correct alignment of RNA polymerase with promoter contacts in the -35 and -10 consensus regions by impeding the topological unwinding of DNA and/or render certain amino acid residues inaccessible to base contacts during the transition to the tight interaction required for RP_o. The binding of the large antibody to polymerase may impede RP_o formation directly by steric hindrance or indirectly by altering an essential promoter interaction at polymerase regions away from the determinant site. Regardless of the mode of inhibition, the severity of the inhibition elicited by mAb 210E8 on the lac UV5 promoter and TAC16 promoters appears to vary as as function of DNA topology. The added constraint by mAb 210E8 on the spacer length of the supercoiled TAC16 promoter further demonstrates the ability of the mAb to probe the complex nature of polymerase-promoter interactions that are elicited by those factors that serve as determinants of promoter strength.

According to the results obtained in the present study the pleiotropic effects of mAb 210E8 on promoter activity imply that mAb is effectively probing different polymerase

interactions with template sequence, the initiating nucleotide (or dinucleoside monophosphate), DNA topology and spacer length. These results suggest a close involvement of the beta subunit with promoter strength determinants that serve as modulators of the catalytic activity of RNA polymerase during transcription. Conversely, the inhibitory anti-beta' mAb 311G2 appears to be probing a role of the beta' subunit in an essential catalytic step that functions independently of template binding and accessory factors that regulate promoter activity. The anti-beta' mAb 311G2 probably elicits its effect on polymerase activity by a potent inhibition of an essential conformational rearrangement of the beta' subunit required for initiation to proceed at a step which is subsequent to RP_o formation.

Several of the mAbs do not apparently inhibit any of the reactions directed by synthetic or promoter templates. The failure to inhibit can be a consequence of binding to determinants which are not present in critical enzyme domains. Conformational changes are involved in the assembly of RNA polymerase (Ishihama et al., 1979), and it is thought that antibodies are specific for a particular conformation of the antigenic determinant (Todd et al., 1982). It is also possible that the noninhibitory monoclonal antibodies may not bind to polymerase because their determinants are buried as as consequence of subunit-subunit interactions or because the determinants are in a nonreactive conformation.

TABLE 1.
Properties of Anti-Beta Monoclonal Antibodies

Effect of Monoclonal Antibody on $d(A\text{-}T)_n$-directed reactions

mAb	Ig Class	core	holoenzyme		
		A	A	B	C
		%	residual	activity	
210E8	IgG1, K	15	55	51	54
221C7	IgG2b, K	100	93	91	89
222B10	IgG2a, K	9	53	60	66
240D4	IgG1, K	11	53	45	ND
241E6	IgG1, K	11	54	62	ND
260E2	IgG1, K	100	93	93	89
261D5	IgG2a, K	14	55	58	ND

RNA polymerase core (1pmol) or holoenzyme (1pmol) was preincubated with 20pmol of the indicated monoclonal antibody for 30 minutes at 37°C. The mAb-polymerase complex was assayed for the indicated reactions as indicated in Materials and Methods. ND indicates that the effect of the antibody was not determined. Residual activity in the presence of the indicated monoclonal antibody is expressed as the percent of the following control values for incorporation of [^{3}H]UMP: r(A-U)n synthesis by core, 0.8nmol; r(A-U)n synthesis by holoenzyme , 2.5nmol; initiation by holoenzyme, 2.1nmol; elongation by ternary complex, 0.13nmol. Reactions are as follows: A: $r(A\text{-}U)_n$ synthesis; B: initiation; C: elongation.

TABLE 2.
Properties of Anti-Beta' Monoclonal Antibodies

Effect of Monoclonal Antibody on d(A-T)n-directed reactions

mAb	Ig Class	core	holoenzyme		
		A %	A residual	B activity	C
311G2	IgG1, K	15	12	25	18
340C9	IgG2a, K	103	106	133	no
340F11	IgG2a, K	99	101	79	96
370F3	IgG1, K	80	97	100	136
371D6	IgG1, K	104	85	96	132

RNA polymerase core (1 pmol) or holoenzyme (1 pmol) was preincubated with 20 pmol of the indicated monoclonal antibody for 30 minutes at 37°C. The mAb-polymerase complex was assayed for the indicated reactions as indicated in Materials and Methods. See legend to Table 1 for 100% control values used. Reactions are as follows: A: $r(A\text{-}U)_n$; B: initiation; C: elongation.

Legend to Table 3

Standard preincubation and abortive reactions conditions were employed. The K_m and V_{max} values of assays in which UpA and ATP were the variable substrates were determined from a kinetic anaylsis of the abortive initiation and elongation reactions shown in Figures 3 and 5, respectively. For experiments in which UTP was the variable substrate titrations were performed with (^{3}H)UTP at concentrations ranging from .025 to .2 mM in the presence of 25 uM UpA. For the $d(I-C)_n$ directed synthesis of CpGpC the primer (CpG) was varied at concentrations ranging from .2 to 4 mM in the presence of 50 uM (^{3}H)CTP. For the $r(A-U)_n$ directed synthesis of $d(A-T)_n$ concentrations ranged from .001 mM to .2 mM.
The RNA polymerase concentration was 1 or 2 pmol and with the exception of the DNA titrations the $d(A-T)_n$ and $d(I-C)_n$ concentrations were .1 mM RNA polymerase preincubated in the presence and absence of mAb 210E8 and mAb 311G2 were combined with DNA and incubated for 15 minutes at 37°. Subatrates were added and the incubation was continued for 10 minutes. The K_m and V_{max} values were obtained by least squares analysis of double reciprocal plots of1/velocity (pmol/min) versus 1/primer concentration.

TABLE 3
Effect of Monoclonal Antibodies
on the Kinetic Constants
of Substrate Incorporation $d(A-T)_n$ and $d(I-C)_n$.

mAb	Product	Template	Varied substrate	K_m (uM)	V_{max} pmol/min	%RA[a]
Control	UpApU	$d(A-T)_n$	UpA	34	13	100
210E8				83	13	91
311G2				53	5	45
Control				53	19	100
210E8				63	14	78
311G2				32	3	16
Control	CpGpc	d(I-C)n	CpG	472	190	100
210E8				952	190	100
311G2				422	16	7
			Elongation			
Control	r(A-U)n	$d(A-T)_n$	ATP	12	7	100
210E8				14	5	64
Control			$d(A-T)_n$	14	174	100
210E8				13	75	40
311G2				4	22	12

The preincubation and abortive reaction conditions are as described in "Materials and Methods" and the Legends to Figures 3 and 5.
[a]The per cent residual activity at highest titration point.

TABLE 4

Effect of Monoclonal Antibodies
On [^{3}H]d(A-T)$_n$ Binding by RNA Polymerase

Antibody	A [^{3}H]d(A-T)$_n$ Bound	B [^{3}H]d(A-T)$_n$ Bound
	(nmol)	(nmol)
210E8	1.32	ND
222B10	1.30	2.29
260E2	0.45	0.45
311B2	0.78	ND
311G2	0.76	1.10
371D6	0.45	0.50
-	0.50	0.41

Assay conditions were as described in "Materials and Methods". Holoenzyme (2 pmol) was preincubated in the presence and absence of 20pmol mAb 210E8 or mAb 311G2 for 1 hour at 0°C and then combined with (A) 1.5 nmol or (B) 3.0 nmol [^{3}H]d(A-T)$_n$ in binding buffer. After an incubation for 10 minutes at 37°C reaction mixtures were diluted and passed thru nitrocellulose filters. Bound complexes were determined as the amount of cpm retained on the filter. ND refer to assays not performed.

TABLE 5

Effect of Anti-Beta and Anti-Beta' mAbs on the promoter activity of the lac P^+ and lac UV5 promoter fragments and T7 DNA

	PROMOTER		
mAb	lac P^+	lac UV5	T7 A1, A3
	% residual activity		
ANTI-BETA			
210E8	6	19	58
222B10	8	25	55
261D5	5	16	44
241E6	11	16	56
221C7	104	109	101
ANTI-BETA'			
311G2	2	4	7
371D6	85	85	112
372B2	73	118	99

The reactions were assayed as indicated in "Materials and Methods". The abortive initiation reactions measured were T7 A1, A3 directed synthesis of CpApU and the lac P^+ (or lac UV5) directed synthesis of ApApUpU. Preincubated mixtures containing 1 pmol polymerase (or mAb 210E8-polymerase) were combined with 0.02 pmol T7 DNA or 0.1 pmol lac UV5. After 15 minutes at 37°C substrates were added and the reactions mixtures were reincubated for an additional 10 minutes.

TABLE 6
Effect of Anti-Beta mAb 210E8
on Single Step Addition
Reactions by Various T7 Promoters

PROMOTERS	REACTION	%RESIDUAL ACTIVITY
A1, A3	C-A + UTP--> G-A-U	59
C	U-A + CTP--> U-A-C	22
D	U-G + UTP--> U-G-U	33
D	G-U + UTP--> G-U-U	35
A2	C-G + CTP--> C-G-C	43
unassigned 1	C-A + CTP--> C-A-C	17
unassigned 2	G-A + UTP--> G-A-U	17

Standard preincubation and reaction conditions were employed as described in "Materials and Methods". Promoters were assayed with 0.02 pmol T7 DNA. The DNA was combined with enzyme preincubation mixtures and incubated for 15 min at 37° C. After addition of 2mM dinucleoside monophosphate and 50 uM (^{3}H)UTP or (^{3}H)CTP the mixtures were incubated for 10 min at 37°.

TABLE 7
Titration of the lac UV5 Promoter and T7 DNA with Holoenzyme in the Presence and Absence of Anti-Beta mAb 210E8.

mAb 210E8	DNA concentration	Enzyme:DNA ratio	lac UV5		T7 A1, A3	
	pmol		pmol inc.	% RA	pmol inc.	%RA
+	0.10	100:1	3	16	26	60
-			19		43	
+	0.02	50:1	4	12	44	54
-			33		82	
+	0.04	25:1	6	8	115	68
-			73		170	
+	0.10	10:1	13	7	247	76
-			192		326	
+	0.20	5:1	68	18	455	81
-			388		563	

The assay conditions employed are as described in the legend to Tables 5 and 6. The DNA concentration was varied as indicated. The measurements obtained are pmol ^{3}H-UMP incorporated per 15 minute assay in conjunction with the respective per cent residual activity of product formation in the presence of mAb versus the control. RA: residual activity.

TABLE 8
Effect of the Anti-Beta mAb 210E8 on the Kinetic Constants of Abortive Initiation on the *lac* UV5 and T7 A1, A3 Promoters.

Template and Product	E:DNA ratio	Varied substrate	K_m (um)	V_{max} pmol/min	%RA[a] at highest titration point
lac UV5 ApApU		ApA			
Control	20:1		242	20	100
mAb			424	2	10
T7 A1,A3 CpApU		CpA			
Control	100:1		200	12	100
mAb			333	6	41
Control	50:1		130	11	100
mAb			200	7	55
Control	25:1		250	9	100
mAb			250	7	75
Control	10:1		200	20	100
mAb			333	20	88

Standard preincubation and abortive reaction conditions were employed. Primer concentrations were varied in the presence of 50 uM (^{3}H)UTP as follows: *lac* UV5 directed synthesis of ApApUpU, 0.2 to 2 mM ApA and the T7 A1, A3 directed synthesis of CpApU, 0.2 to 4 mM CpA. RNA polymerase (2 pmol) preincubated in the presence and absence of mAb 210E8 was combined with either 0.02 pmol T7 DNA or .1 pmol *lac* UV5 and incubated for 15 minutes at 37° C. Substrates were added and the incubation was continued for 10 minutes. The K_m and V_{max} measurements were obtained as described in the Legend to Table 3

[a]The percent residual activity of mAb related assays relative to the control.

TABLE 9
Effect of mAb 210E8 on Supercoiled, Linearized and Purified Fragments of *lac* UV5 and TAC16 Promoters

Promoter	mAb	s/c (pmol)	%RA	s/c+ HindIII or EcoR1 (pmol)	%RA	Purified Fragment (pmol)	%RA
lac P^+	-	59		33		28	
	+	48	81	13	39	2	7
lac UV5	-	55		53		48	
	+	47	85	18	34	8	17
TAC16(AUU)	-	96		51		36	
	+	46	48	3	5	2	6
TAC16(UGU)	-	37		15		14	
	+	30	81	5	33	2	14

Standard preincubation and reaction conditions were employed. Restriction of pMB9-*lac* P^+, pMB9-*lac* UV5 or pNI 171 was carried out in abortive assay buffer for 60 min. at 37°C. Complete restriction was confirmed by electrophoresis on a 0.8% agarose gel. RNA polymerase (2 pmol) preincubated in the presence and absence of mAb 210E8 was incubated with 1 pmol linear and 0.05 pmol supercoiled (s/c) promoter DNA for 15 min at 37°C. The substrates were added to give a final concentration of 0.5 mM ApA and 50 uM [^{3}H]UTP. The measurements indicated are pmol [^{3}H]-UMP incorporated/nM promoter fragment/minute together with the respective percent residual activities. The data presented were obtained after subtraction of a plasmid control. Plasmid controls employed were purified restricted plasmid isolated by separation from fragment on PAGE and pBR332 with the *tet* region inactiviated by restriction with HindIII. In both plasmids the *tet* marker is destroyed either by insertional activation (pMB9) or removal (pNI 171). These controls were employed since plasmids lacking the respective insert but otherwise indentical in sequence were not available for these studies.

TABLE 10
Effect of mAb 210E8 on the Kinetic Constants of Abortive Initiation on Linear and Supercoiled Forms of the *lac* UV5 Promoters

Promoter	mAb 210E8	K_m (uM)	V_{max} pmol/min	%Residual activity[a]
lac UV5 (s/c)	-	85	100	100
	+	125	91	85
lac UV5(linear)	-	242	20	100
	+	424	2	10

The abortive assay conditions employed were as described in the Legend to Table 8. The K_m and V_{max} values of primer (ApA) incorporation on linear *lac* UV5 is as shown in Table 8 and is shown above for comparison. For the kinetic studies on the supercoiled template 2 pmol RNA polymerase preincubated in the presence and absence of mAb 210E8 was combined with 0.05 pmol *lac* UV5 insert and incubated for 15 minutes at 37°C. Substrates were added and the incubation was continued for an additional 5 minutes. The K_m and V_{max} values were obtained as described in the Legend to Table 3.

TABLE 11
Effect of Varying Solution Conditions on the Stability of RNP-lacUV5 Complexes in the Presence and Absence of Anti-Beta mAb 210E8

Solution Condition	Dissociation[b]	
	Control RNP[a] complexes	antiB-RNP complexes
200mM KCl; 37 C	---	---
200mM KCl; 0 C	---	+
300mM KCl; 37 C	+	+ +
300mM KCl; 0 C	+ +	+ +
150mM KCl; 80 ug/ml heparin	+	+ +
150mM KCl; 1 x 10^{-6}M $d(A-T)_n$	---	---
150mM KCl; 1 x 10^{-5}M $d(A-T)_n$	---	+
150mM KCl; 1 x 10^{-5}M $d(A-T)_n$	---	+ +

The preincubation and assay conditions employed were as described under "Materials and Methods". Binding was carried out with the abortive assay conditions at varied KCl concentration. Assay reactions contained polymerase (2-4 pmol) preincubated in the presence and absence of mAb 210E8 and combined with the lac UV5 promoter at an enzyme to DNA ratio of 3:1. After 15 minutes at 37° C solution conditions were adjusted as indicated and incubation was continued for 10 minutes at 37°C. The DNA-protein complexes were then analyzed directly by polyacrylamide gel electrophoresis. [b]Dissociation of protein-DNA complexes was detected by the migration of a free (uncomplexed) DNA band into the gel on ethidium bromide stained gels.

TABLE 12
Effect of mAb 210E8 on tau_{obs} on Linear and Supercoiled *lac* UV5 and TAC16 Promoters

Promoter	mAb 210E8	RNA Polymerase (nM)	tau_{obs} (sec)	%RA[a]
lac UV5 (s/c)	-	20	58	
	+	20	76	96
lac UV5 (linear)	-	40	22	
	+	40	ND[b]	17
TAC16(AUU) (s/c)	-	13	9	
	+	13	44	75
TAC16(AUU) (linear)	-	40	28	
	+	40	ND	7
TAC16(UGU) (s/c)	-	20	26	
	+	20	36	90
TAC16(UGU) (linear)	-	40	18	
	+	40	ND	14

The protocol employed is as described in the legends to Figures 15-17.
[a] Calculated from 2 hour data points.
[b] ND, not determined.

TABLE 13
Summary of tau_{obs} Assay Conditions for Complex Formation on $d(A-T)_n$, T7 DNA and *lac* UV5 in the Presence and Absence of Anti-Beta mAb 210E8

Template	Product	mAb:RNP[a] ratio	DNA pmol	RNP conc	%RA[b]
$d(A-T)_n$	pApU	20:1	10	10	45
T7 DNA	pApU	20:1	0.04	2	25
T7 DNA (A1, A3)	CpApU	20:1	0.04	2	62
lac UV5	ApApUpU	2.5:1	0.10	1	21
				2	16
				5	25

[a] RNA polymerase
[b] percent residual activity at 16 min. data points.

TABLE 14. Summary of mAb Effects on Polymerase Contacts
During Complex Formation on the lac UV5 Promoter[a].

The abbreviations indicated in the table are used to designate the following reactivities:

E-enhanced
T-template strand
P-protected
NT-nontemplate strand
I-intensity of pattern intermediate to control
+-intensity of pattern greater than control
ND-not determined

M-methylated
NM-not methylated
RNP-RNA polymerase
[a]mAb induced reactivity patterns are based on a comparison between the DNase 1 footprints and methylation patterns obtained for the unprotected fragment and control polymerase-DNA complexes formed in the absence of mAb.

Reaction	Promoter	DNA strand	Position on promoter	Pattern of Binding Occupancy RNP	RNP anti beta 210E8	RNP anti beta 311G2	RNP anti beta' 371D6
DNase 1	lac P+ + cAMP-CRP	T	between -20 to -30	E	P	E	E
		T + NT	-80	NM	NM	NM	M
DNase 1	lac UV5	NT	-20 to -80				
2% glycerol				E	I	I	E
25% glycerol				E	E+	I	E
DNase 1	TAC16	T	-10 region	P	E	E	P
"G"	lac UV5	T	-32	P	I	P+	P
			-14	E	I	E	E
"G"	lac UV5	NT	-38	E	I	E	E
			-24	P	I	P	P
			-17	E	I	E	E
			-13	P	I	P	P
			-6	P	I	P	P
			-1	E	I	E	E
"G" 25% glycerol	lac UV5	NT	-38	E	I	P	E
			-24	P	P	P	P
			-17	E	I	P	E
			-13	P	P+	P+	P
			-6	P	P+	P+	P
			-1	E	I	P	E
"G"	TAC16	T	-32	P	P	P	P
			-14	E	I	I	E
"C"	lac UV5	T	-32	P	I	I	P
			-14	E	I	E	E
			-6,-4,-2,-1	M	NM	M	M

Figure 1. Effect of anti-beta and anti-beta' monoclonal antibodies on RNA polymerase. The $d(A\text{-}T)_n$-directed synthesis of $r(A\text{-}U)_n$ was assayed as described in "Materials and Methods". Preincubation of 1 pmol holoenzyme with and without the indicated amounts of antibody was for 60 minutes at 0°C. mAb 221C7, o--o; mAb 210E8 ●--●; mAb 371D6, △—△; mAb 311G2, ▲—▲. Activity is expressed as the percent of the incorporation obtained with the holoenzyme control: 100% was 3.2 nmol [^{3}H]UMP incorporated in 20 minutes at 37°C.

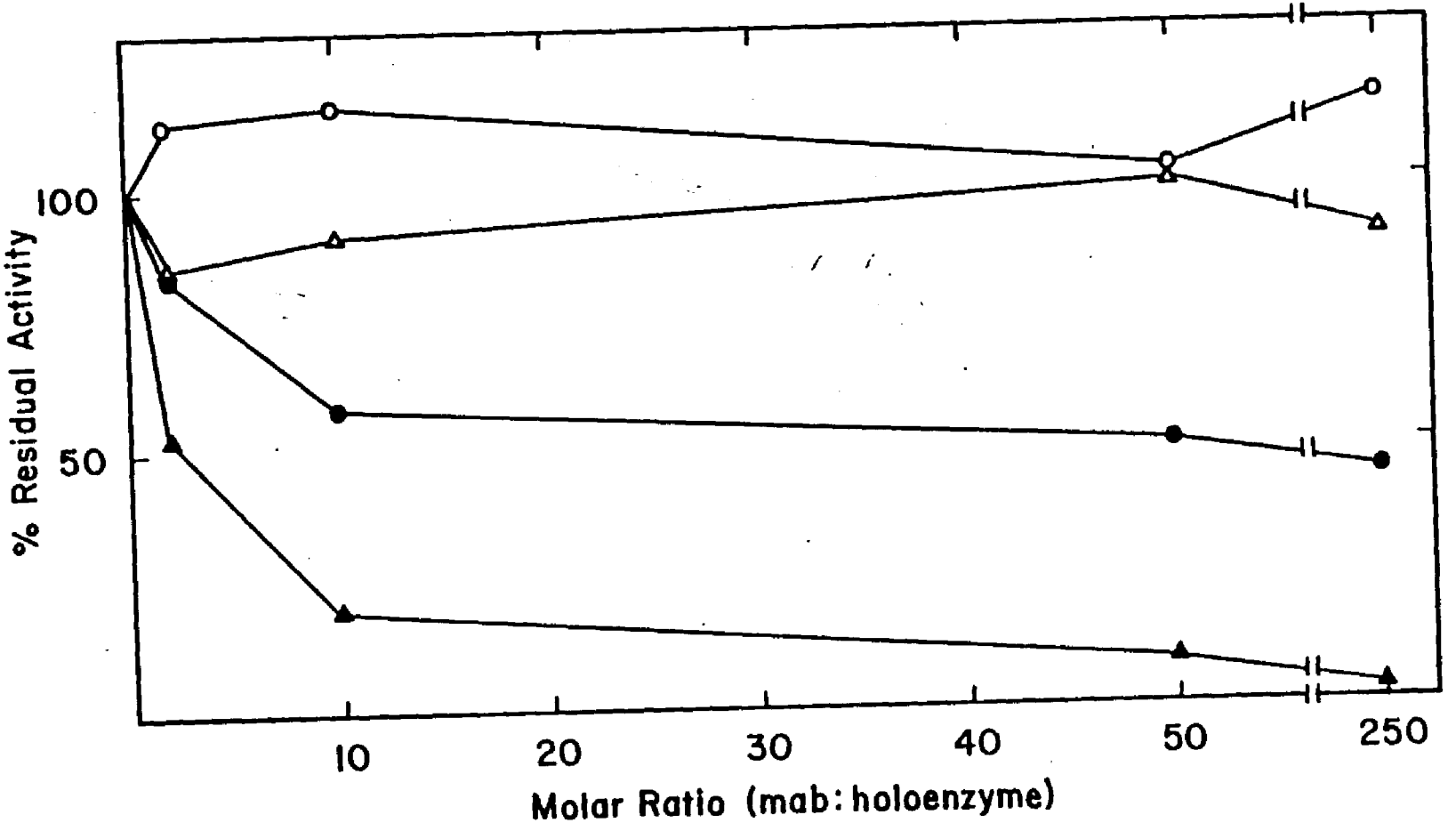

% Residual Activity
100
50
10
20
30
40
50
250
Molar Ratio (mab: holoenzyme)

Figure 2. Effect of anti-beta mAb 210E8 on holoenzyme and core enzyme. The $d(A\text{-}T)_n$-directed synthesis of $r(A\text{-}U)_n$ was assayed as described in "Materials and Methods". Preincubation of RNA polymerase holoenzyme (1 pmol) or core enzyme (1 pmol) in the presence and absence of mAb 210E8 (20 pmol) was for 60 minutes at 0°C. Holoenzyme, o--o, holoenzyme + mAb 210E8, ●--●, core enzyme, □ –□; core enzyme + mAb 210E8, ■ –■ .

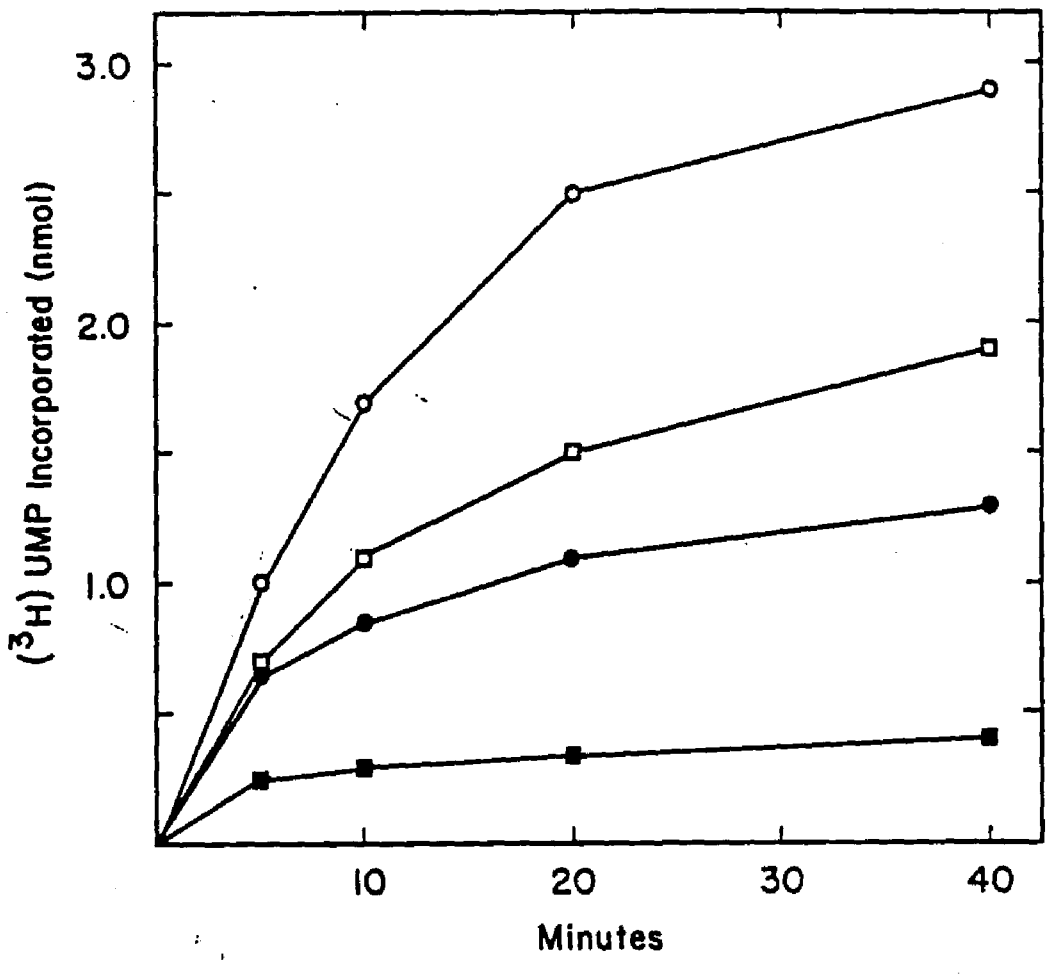

3.0
2.0
1.0
(^{3}H) UMP Incorporated (nmol)
10
20
30
40
Minutes

Figure 3. Effect of anti-beta and anti-beta' mAbs on abortive synthesis of UpApU. Holoenzyme (1 pmol) was preincubated in the presence and absence of mAb 210E8 (20 pmol) or mAb 311G2 (20 pmol) for 60 minutes at 0°C. Abortive synthesis of UpApU was assayed as described in "Materials and Methods" with 50 uM [^{3}H]UTP (200 cpm/pmol) and the concentration of UpA varied as indicated. Reciprocal initial velocities (pmol/min) are plotted versus the reciprocal of the UpA concentrations. Holoenzyme, x--x; holoenzyme + mAb 210E8, ●--●; holoenzyme + mAb 311G2, ▲--▲.

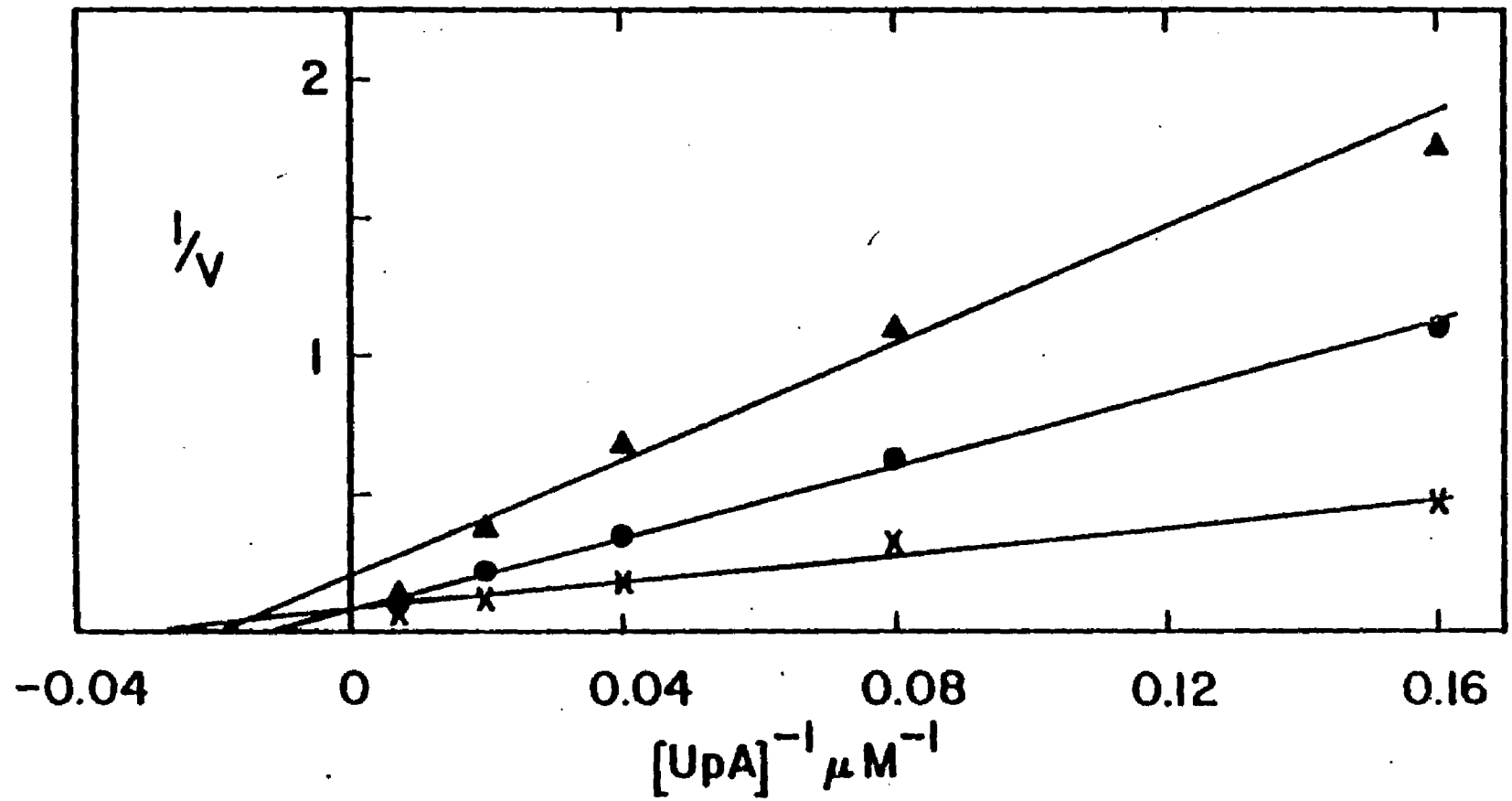

2
1
1/v
-0.04
0
0.04
0.08
0.12
0.16
[UpA]⁻¹ μM⁻¹

Figure 4. Effect of anti-beta mAb 210E8 on the d(A-T)$_n$-directed synthesis of UpApU at various UpA concentrations. Assay conditions were as described in the legend to Figure 3. Holoenzyme (1 pmol) was preincubated in the presence and absence of a 20:1 molar ratio of mAb to enzyme. The abortive reactions contained 50 uM [^{3}H]UTP and UpA at (A) 6.25 uM, (B) 12.5 uM, (C) 25 uM, (D) 50 uM, (E) 150 uM, (F) 300 uM, (G) 600 uM and (H) 1.2 mM; (o) holoenzyme control; (●) holoenzyme + mAb 210E8.

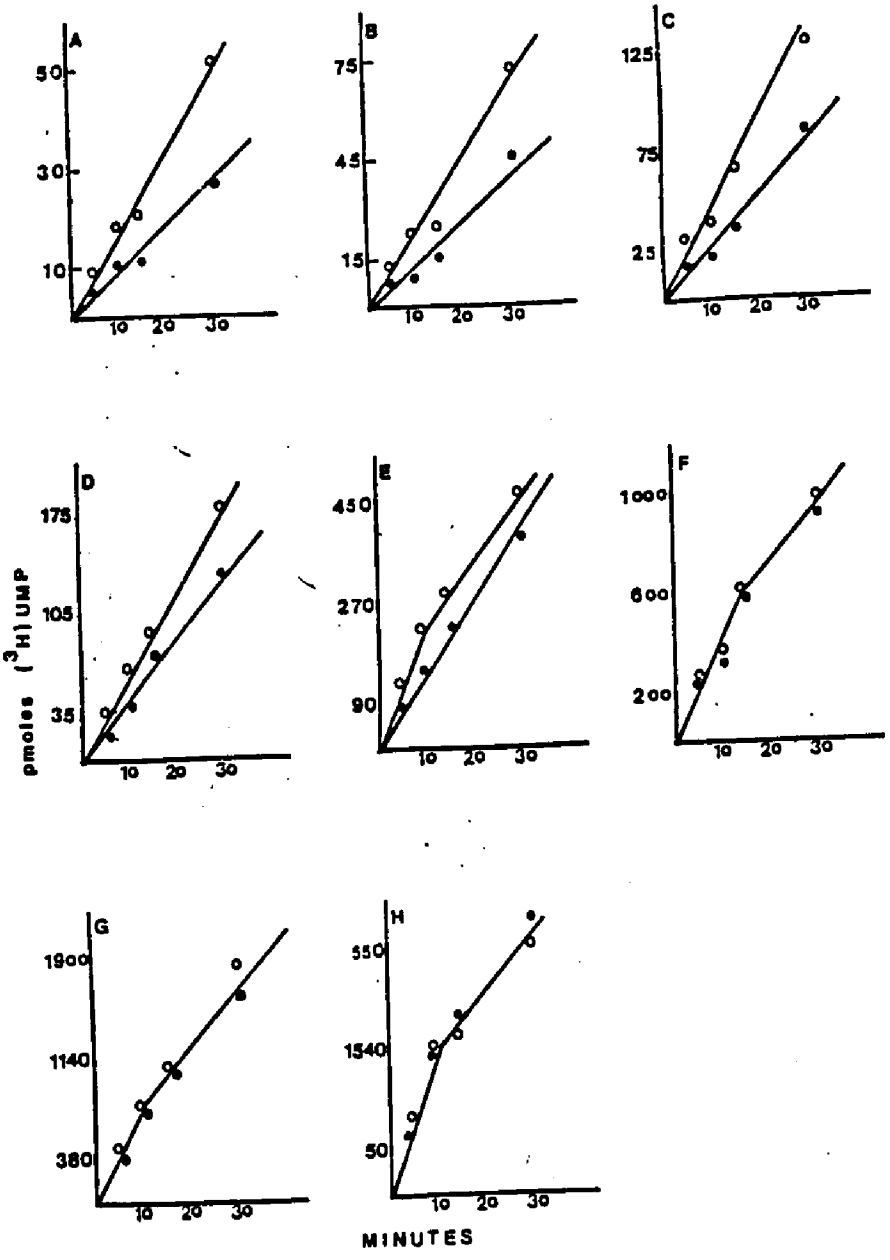

A
50
30
10
10 20 30
B
75
45
15
10 20 30
C
125
75
25
10 20 30
D
175
105
35
10 20 30
pmoles (^{3}H)UMP
E
450
270
90
10 20 30
F
1000
600
200
10 20 30
G
1900
1140
380
10 20 30
H
550
1540
50
10 20 30
MINUTES

Figure 5. Effect of anti-beta mAb 210E8 on the kinetics of elongation. The reactions using the ternary complex prepared by the method of Schmidt and Chamberlin (1984) were carried out as described in "Materials and Methods". The preformed ternary complexes were assayed in the presence and absence of mAb 210E8. Reciprocal initial velocities (pmol/min) are plotted versus the reciprocal of the ATP concentrations. The concentration of [^{3}H]UTP (50 cpm/pmol) was 100 uM. Ternary complex, x--x; ternary complex + mAb 210E8, ●--●.

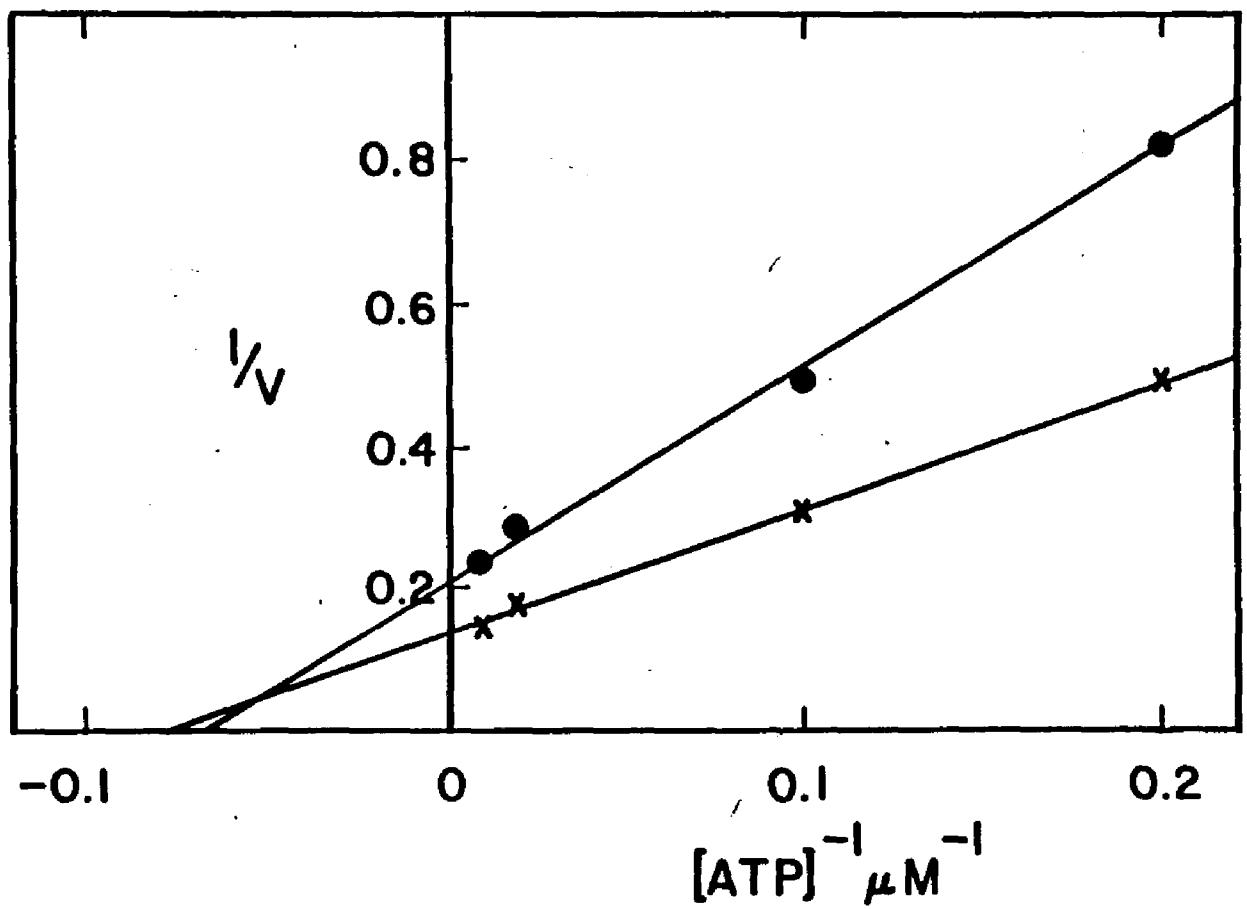
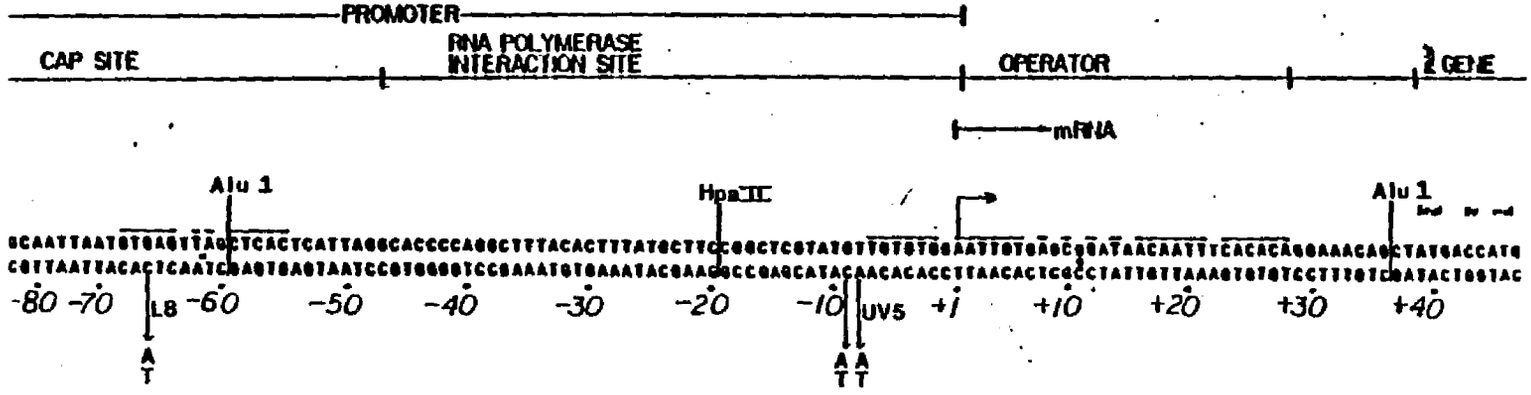

0.8
0.6
1/V
0.4
0.2
-0.1
0
0.1
0.2
[ATP]$^{-1}$ μM^{-1}

FIGURE 6

A The Lac Promoter – Operator Sequence



B Comparison of the Lac UV5 and TAC16 Promoter Sequences

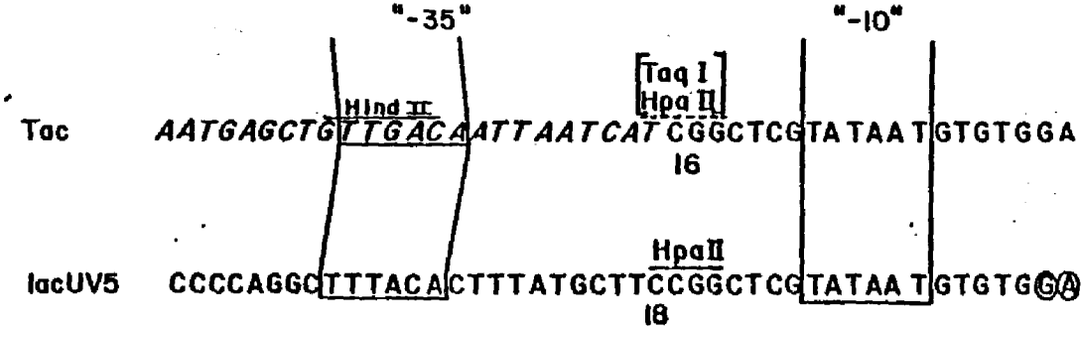


Figure 7. Effect of anti-beta mAb 210E8 on abortive initiation on the lac P^+, lac UV5 and the T7 A1,A3 promoters. The abortive assay conditions for each promoter are as described in "Materials and Methods". The effects of mAb on abortive initiation from each promoter were determined from assays carried out in the presence of increasing concentrations of an inhibitory mAb and a noninhibitory mAb as a control. The data point for each curve represents the percent residual activity obtained from abortive reactions for the T7 A1,A3 (squares) directed synthesis of CpApU and the lac P^+ (circles) and lac UV5 (triangles) directed synthesis of ApApUpU from a series of titrations of 1 pmol holoenzyme with increasing concentrations of the noninhibitory mAb 221C7 (open symbols) and the inhibitory mAb 210E8 (closed symbols).

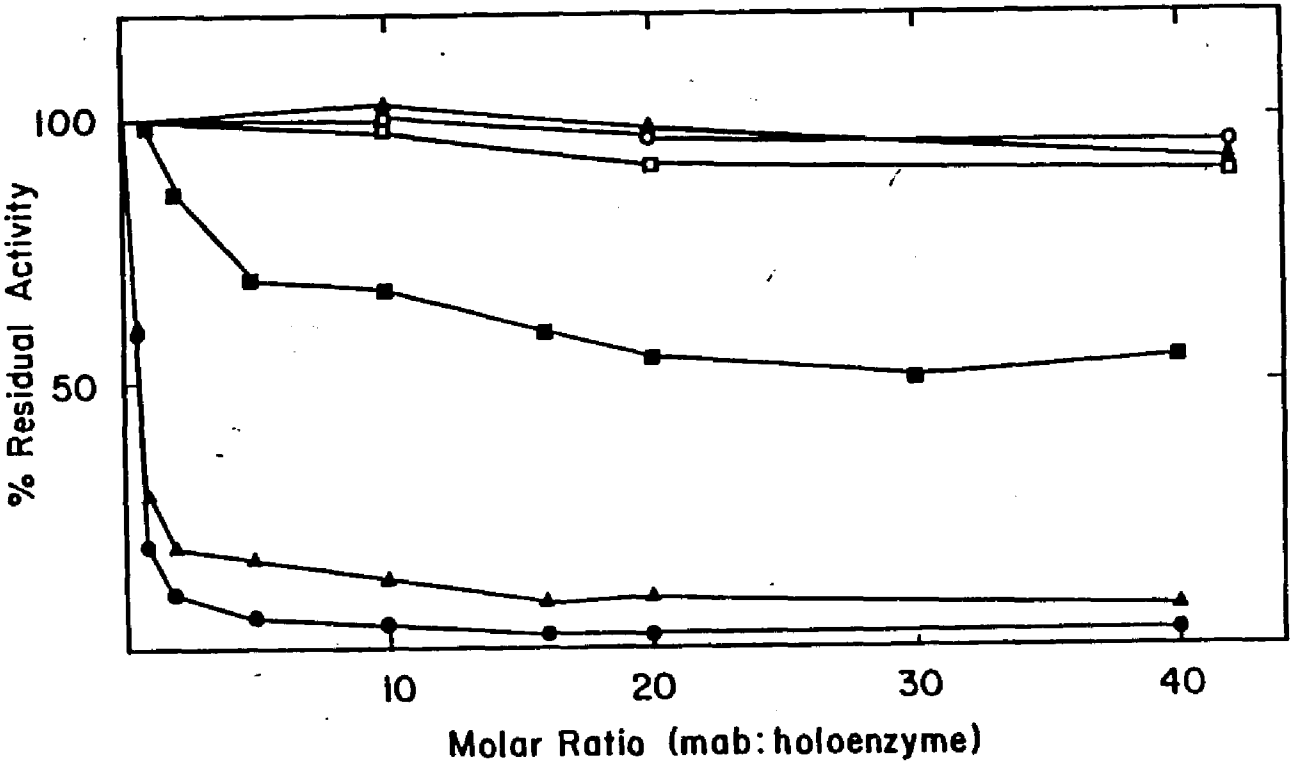

100
50
% Residual Activity
10
20
30
40
Molar Ratio (mab: holoenzyme)

Figure 8. The effect of anti-beta' mAb 311G2 on abortive initiation on the lac UV5 and T7 A1, A3 promoters. The protocal employed is as described in the Legend to Figure 7. Titrations with mAb were carried out on the T7 A1, A3 (squares) and lac UV5 (triangles) promoters in the presence of the noninhibitory mAb 371D6 (open symbols) and the inhibitory mAb 311G2 (closed symbols). No residual activity was observed for abortive reactions on the lac P^+ promoter in the presence of mAb 311G2.

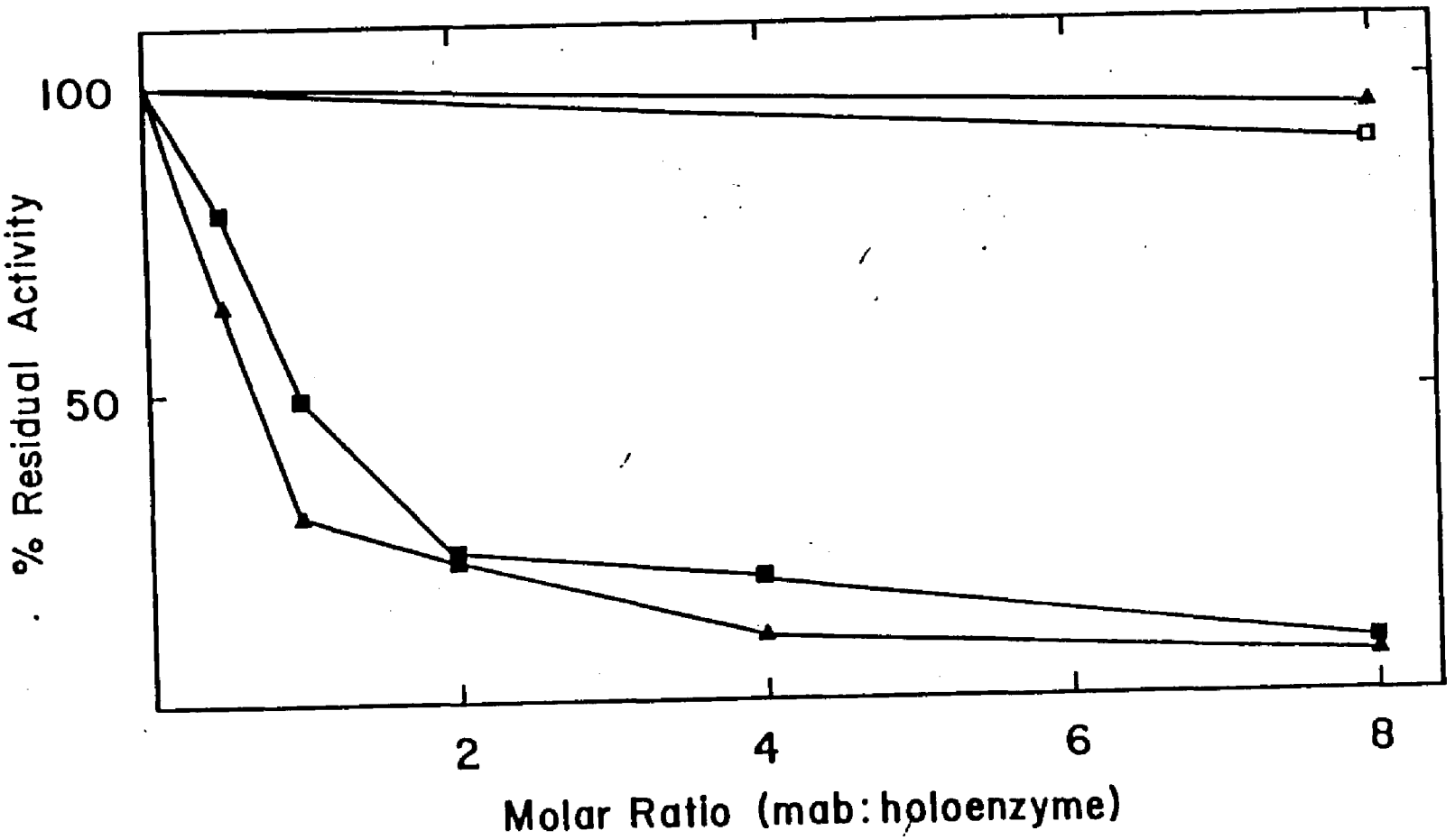

100
50
% Residual Activity
2
4
6
8
Molar Ratio (mab:holoenzyme)

FIGURE 9

Restriction Map of 203 bp lac Promoter Fragment

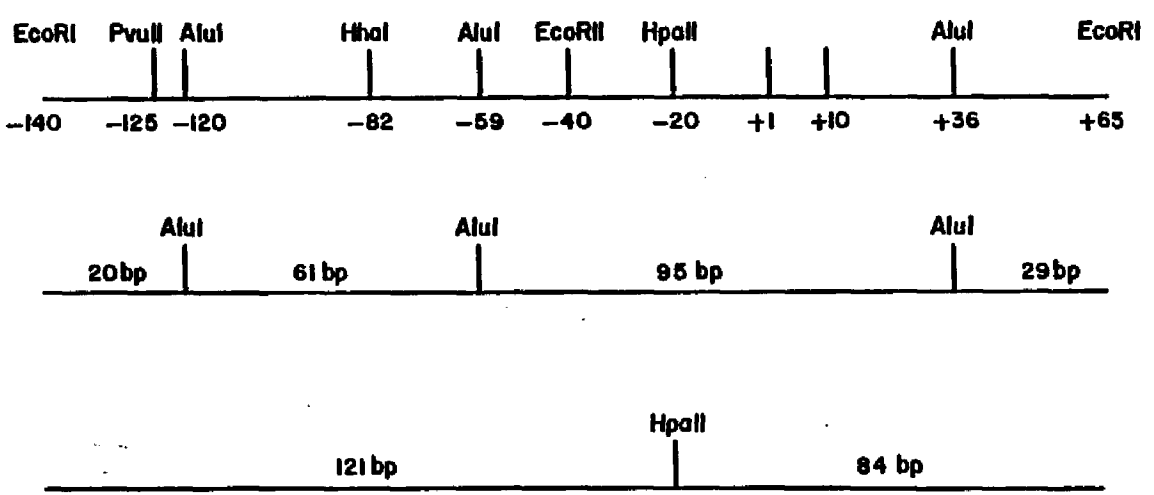


Figure 10. Protection by RNA polymerase over time of the HpaII site of the lac UV5 promoter in the presence and absence of the inhibitory anti-beta mAb 210E8 and anti-beta' mAb 311G2. Binding conditions are as described in "Materials and Methods". Binding reactions (100 mM KCl) contained preincubated mAb-polymerase complexes (or polymerase alone) and the lac UV5 promoter fragment at an enzyme to DNA ratio of 4:1. After complex formation at 37°C reaction conditions were adjusted for restriction by HpaII and challenged with $d(A-T)_n$. After the addition of the restriction endonuclease reactions were reincubated at 37°C, terminated at the times indicated and then analyzed by polyacrylamide gel electrophoresis. DNA protection patterns were determined from gels stained with ethidium bromide. The reaction and their times are as indicated above each lane. The unrestricted fragment is not shown. The results of initial studies indicated that the unrestricted fragment remains intact under the experimental conditions employed.

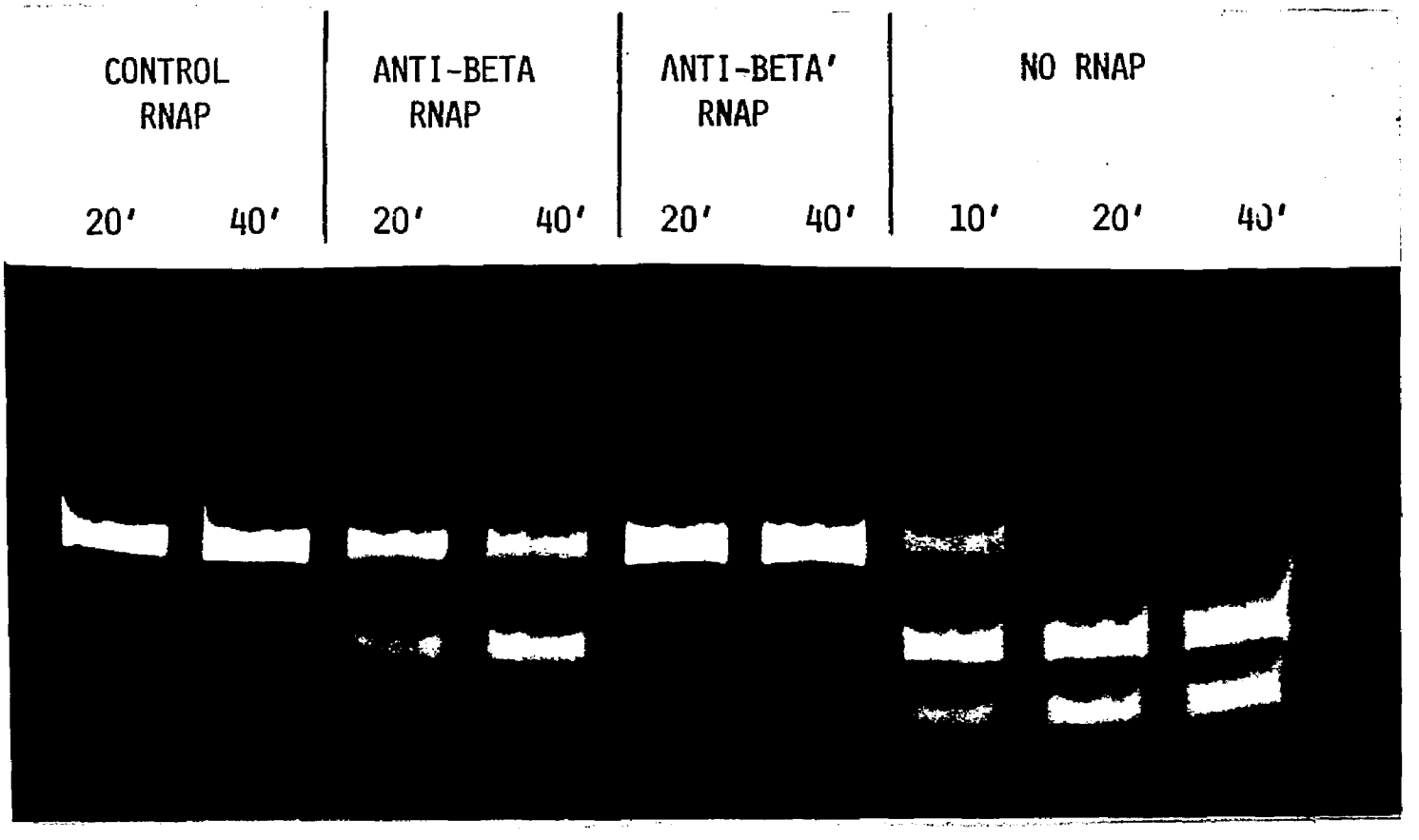

CONTROL RNAP
ANTI-BETA RNAP
ANTI-BETA' RNAP
NO RNAP
20'
40'
20'
40'
20'
40'
10'
20'
40'

Figure 11. Protection by RNA polymerase over time of the HpaII site of the lac UV5 promoter in the presence and absence of anti-beta mAb 210E8. The protocol employed is as described in the Legend to Figure 10. (A) control polymerase, (B) noninhibitory anti-beta mAb 221C7-polymerase, (C) anti-beta mAb 210E8-polymerase, (D) unprotected lac UV5.

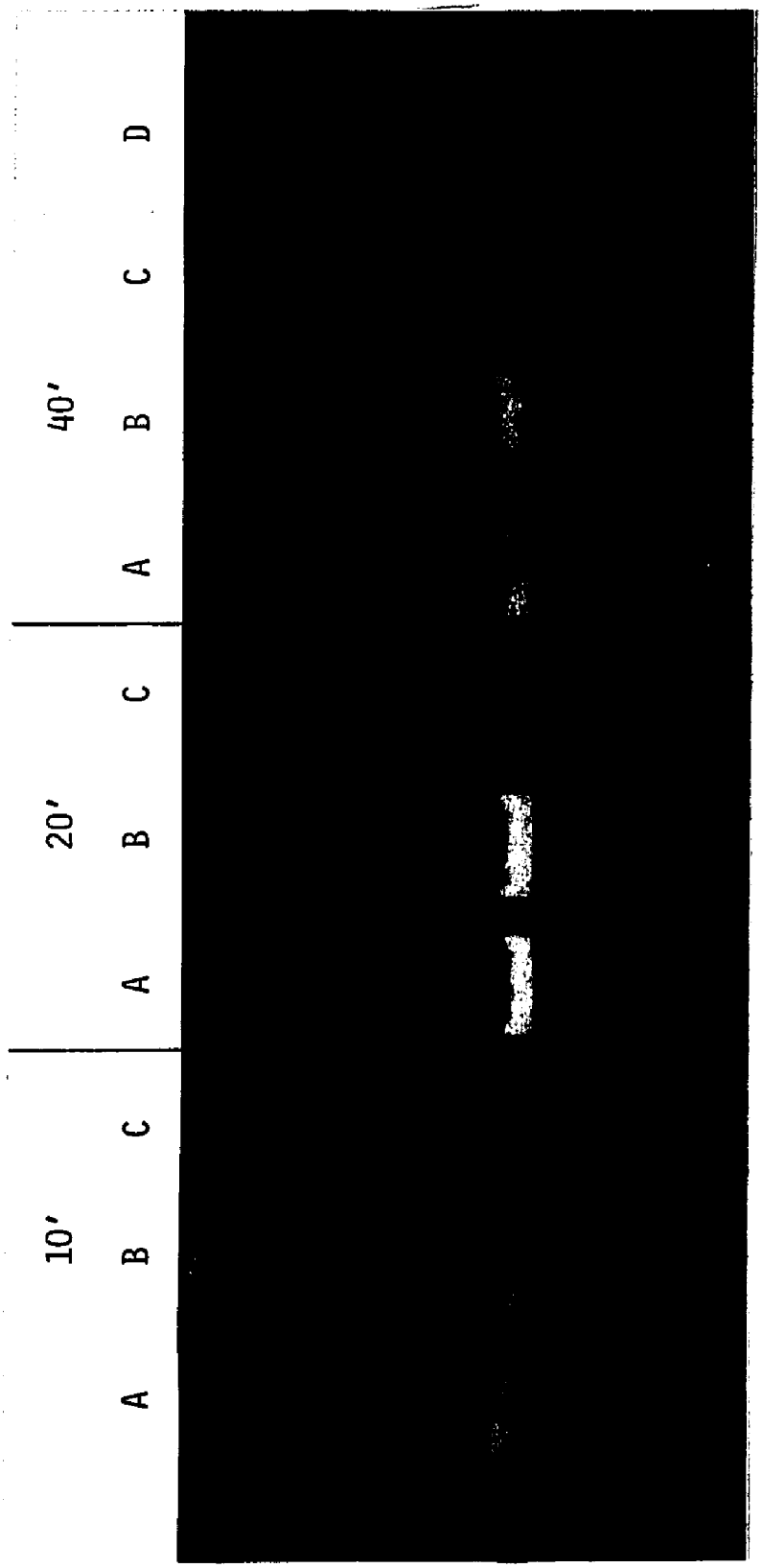

10'
A
B
C
20'
A
B
C
40'
A
B
C
D

Figure 12. Protection by RNA polymerase of the HpaII site of the lac UV5 and the HindII site of the TAC16 promoters in the presence and absence of the anti-beta mAb 210E8. The protocol employed for both promoter assays is as described in the Legend to Figure 10. Protection patterns were determined under conditions of abortive initiation (1): the lac UV5 directed synthesis of ApApUpU or the TAC16 directed synthesis of ApUpU. (A) control polymerase-lac UV5, (B) anti-beta mAb 210E8-polymerase-lac UV5, (C) unprotected lac UV5, (D) control polymerase-TAC16, (E) anti-beta mAb 210E8-polymerase-TAC16, (F) unprotected TAC16.

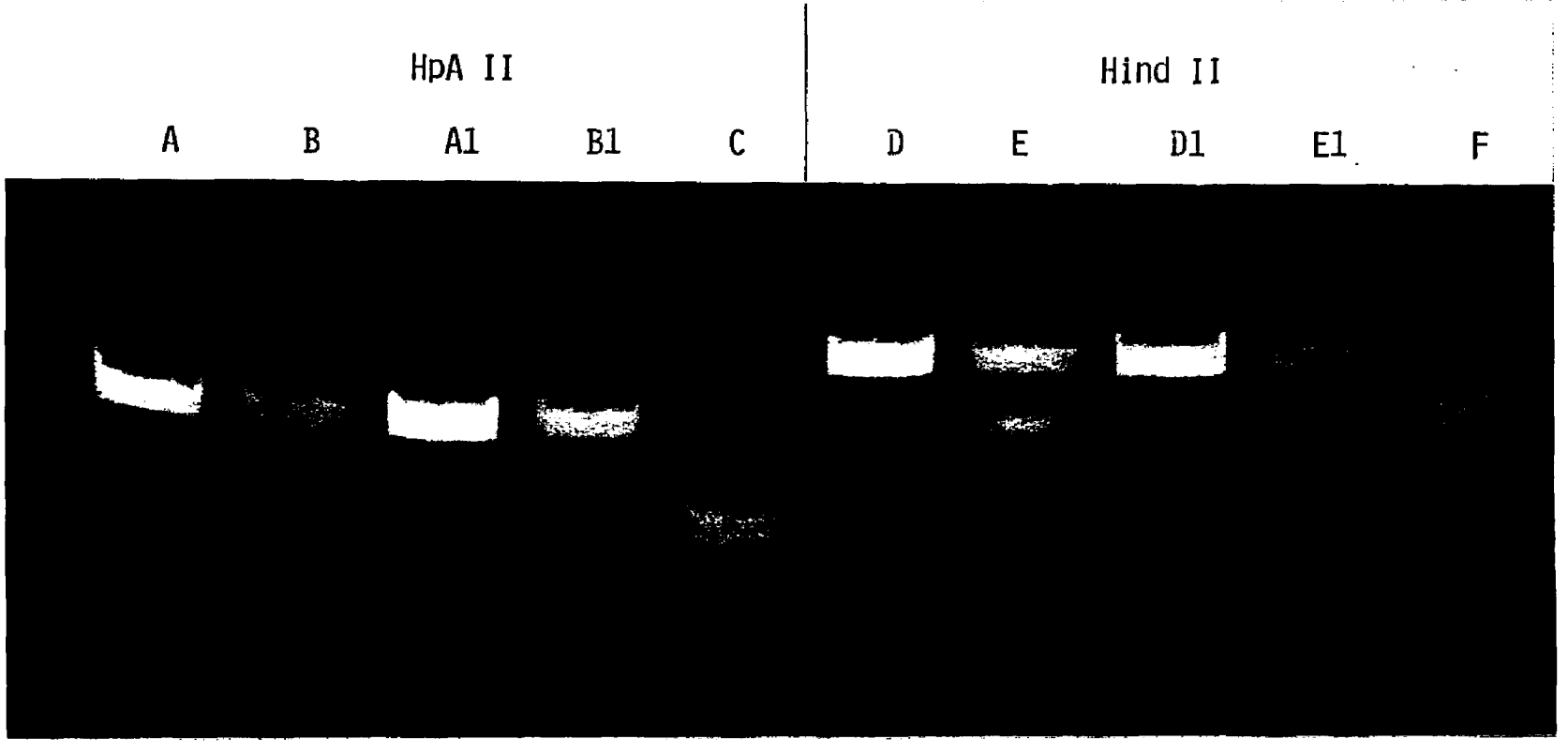

HpA II
Hind II
A
B
A1
B1
C
D
E
D1
E1
F

Figure 13. Protection by RNA polymerase over time of the HindII site of the TAC16 promoter in the presence and absence of the anti-beta' mAb 311G2. The protocol employed is as described in the Legend to Figure 10. (A) control polymerase, (B) anti-beta' mAb 311G2-polymerase, (C) unprotected TAC16, (D) unrestricted TAC16.

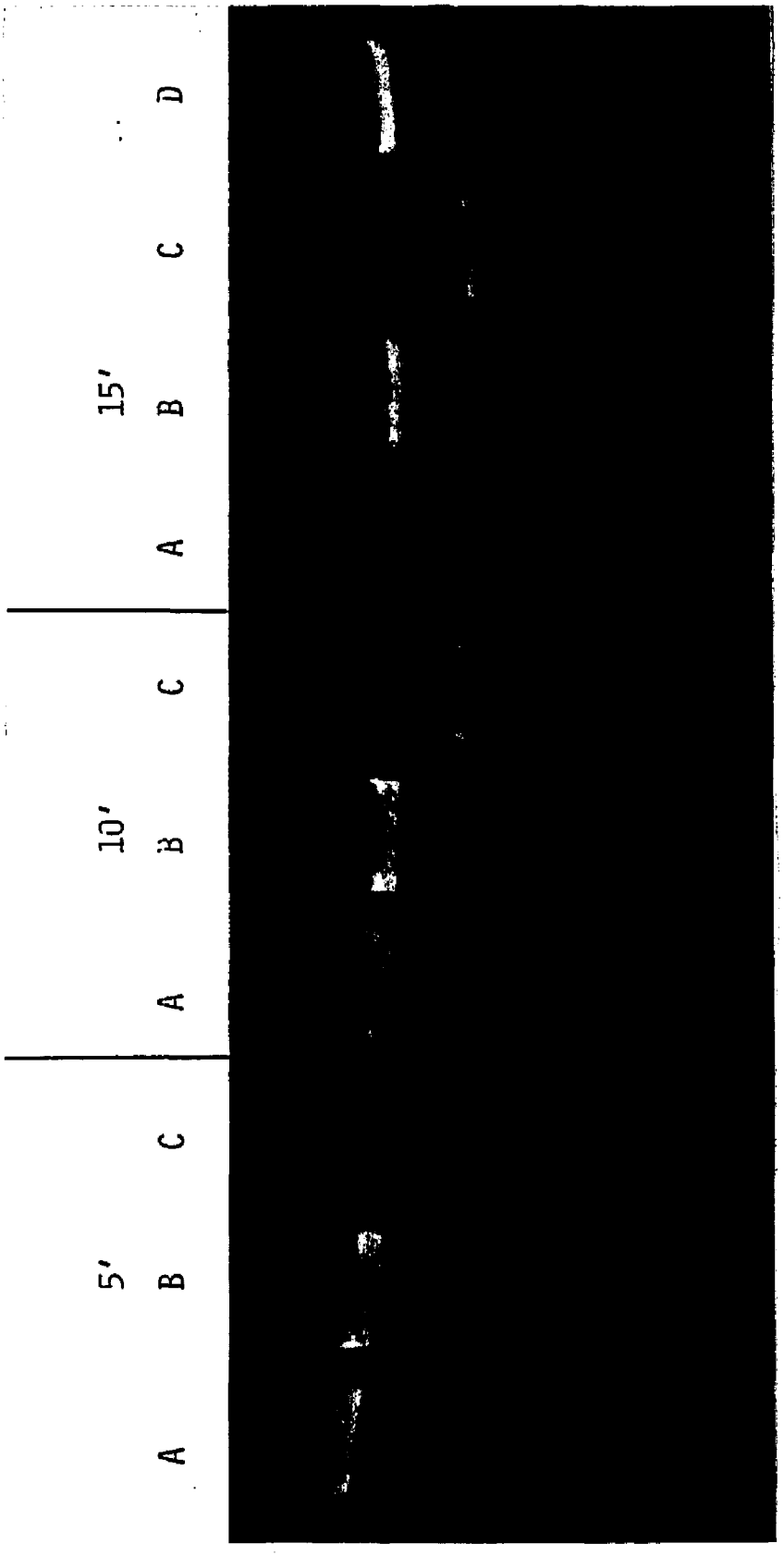
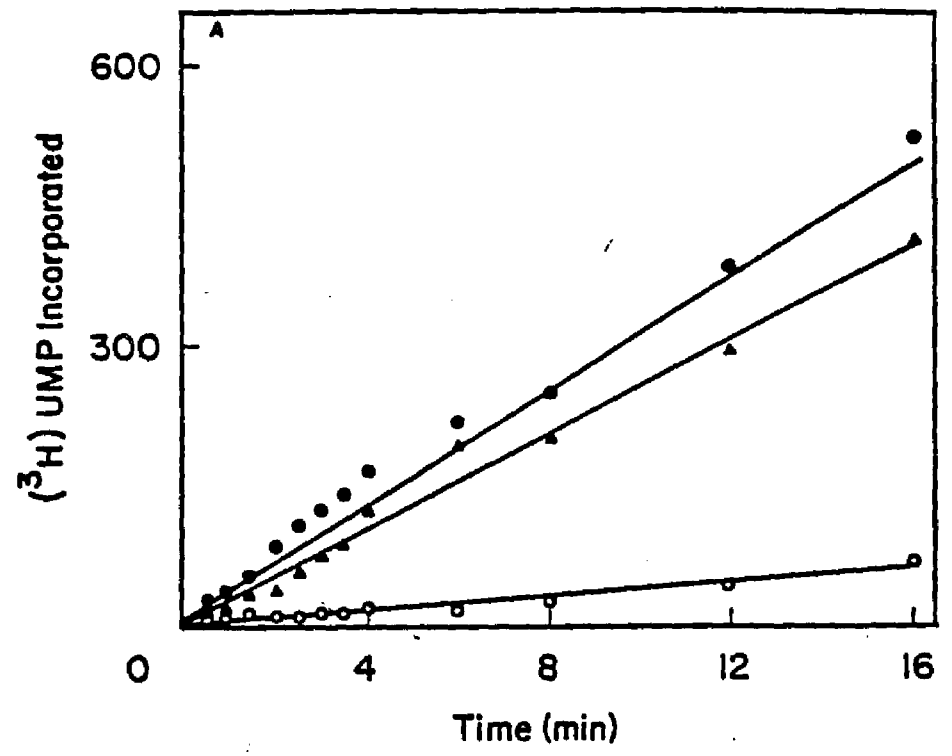

5'
10'
15'
A
B
C
A
B
C
A
B
C
D

Figure 14. Protection by RNA polymerase of the HpaII and Alu1 sites of the lac UV5 promoter in the presence and absence of the inhibitory anti-beta mAb 210E8 and anti-beta' 311G2. The protocol employed is as described in the Legend to Figures 10 and 12. Protection patterns were obtained in the presence of the abortive substrates ApA + UTP (1) to form ApApUpU and the substrates ATP + UTP + GTP (2) to form a 9 base pair transcript. (A) control polymerase, (B) anti-beta mAb 210E8-polymerase, (C) anti-beta' mAb 311G2-polymerase, (D) unprotected lac UV5.


HpA II
A1
B1
C1
A2
B2
C2
Alu 1
A2
B2
C2
D

Figure 15. Effect of anti-beta mAb 210E8 on the tau_{obs} of the lac UV5 promoter. Each lag assay was performed as described under "Materials and Methods". Table 12 shows the RNA polymerase concentrations employed, the tau_{obs} measurements and the percent residual activities for each promoter assay. Plots are presented for tau_{obs} measurements on linear (A) and supercoiled (B) templates. The effects of mAb 210E8 on lag times were determined by performing three parallel abortive reactions: a steady state control (●) in which a reaction mixture containing preformed polymerase-DNA complexes was initiated by the addition of substrates and two separate lag assay in which reaction mixtures containing DNA and substrates were initiated by the addition of polymerase alone (▲) and mAb 210E8-polymerase complexes (○).


A
600
300
0
4
8
12
16
(^{3}H) UMP Incorporated
Time (min)

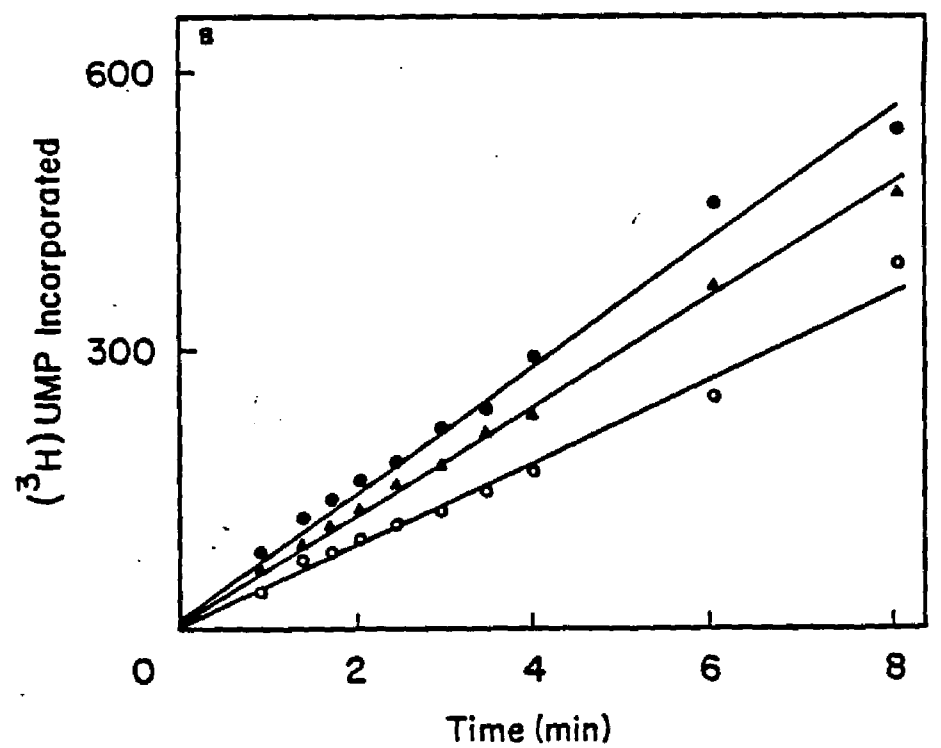
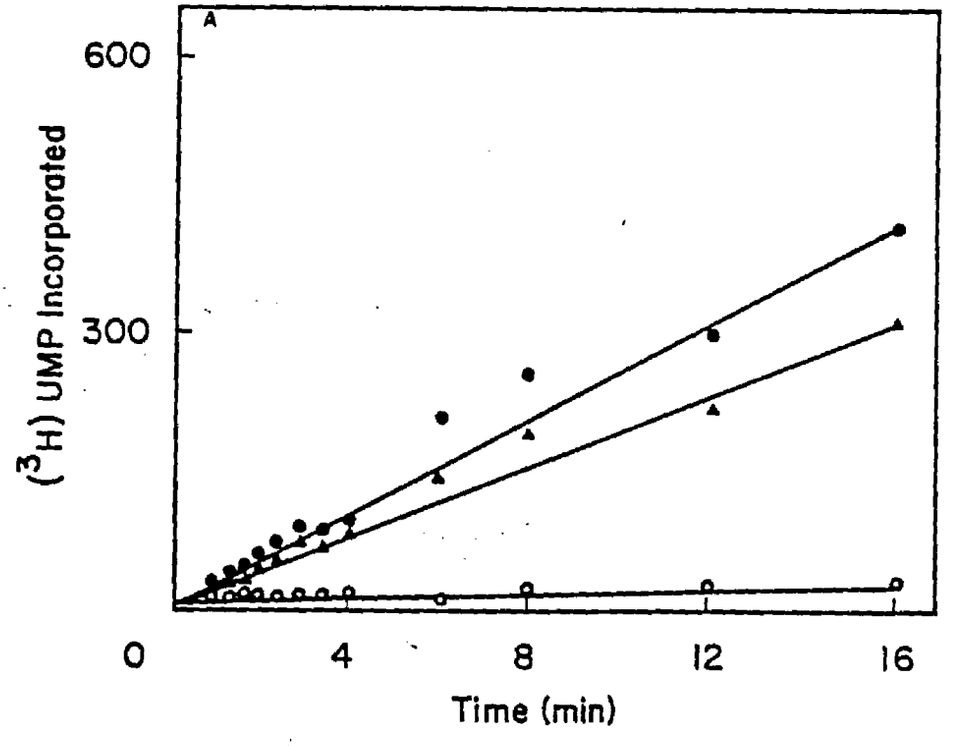

B
600
300
0
2
4
6
8
(^{3}H) UMP Incorporated
Time (min)

Figure 16. Effect of anti-beta mAb 210E8 on the tau_{obs} of the TAC16(ApU) promoter. The protocol employed is as described in the Legend to Figure 15.


A
600
300
0
4
8
12
16
(^{3}H) UMP Incorporated
Time (min)

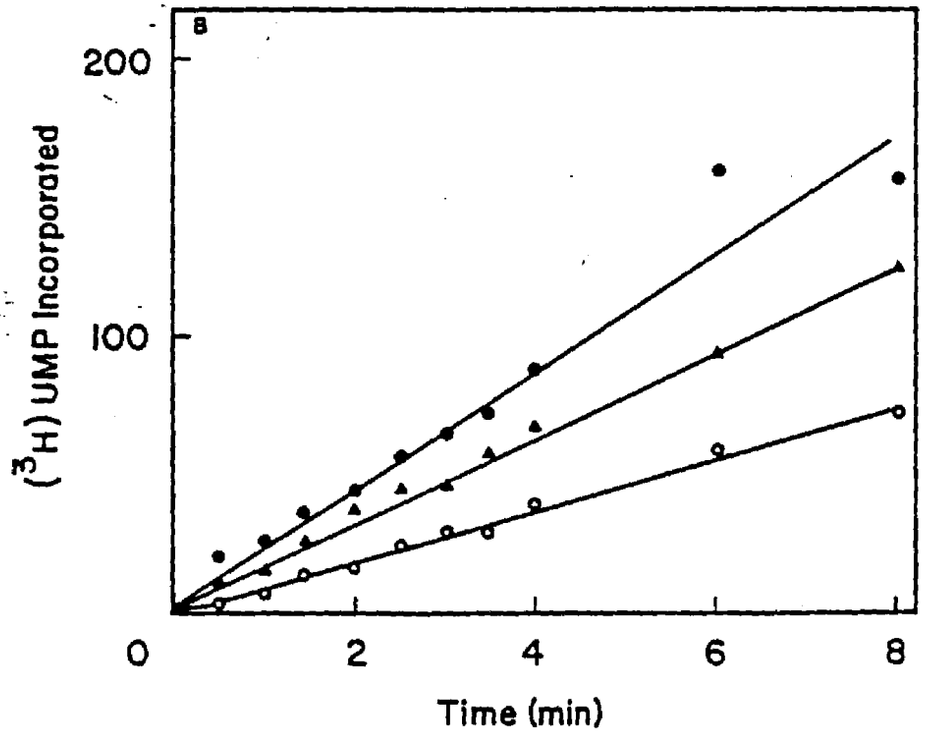
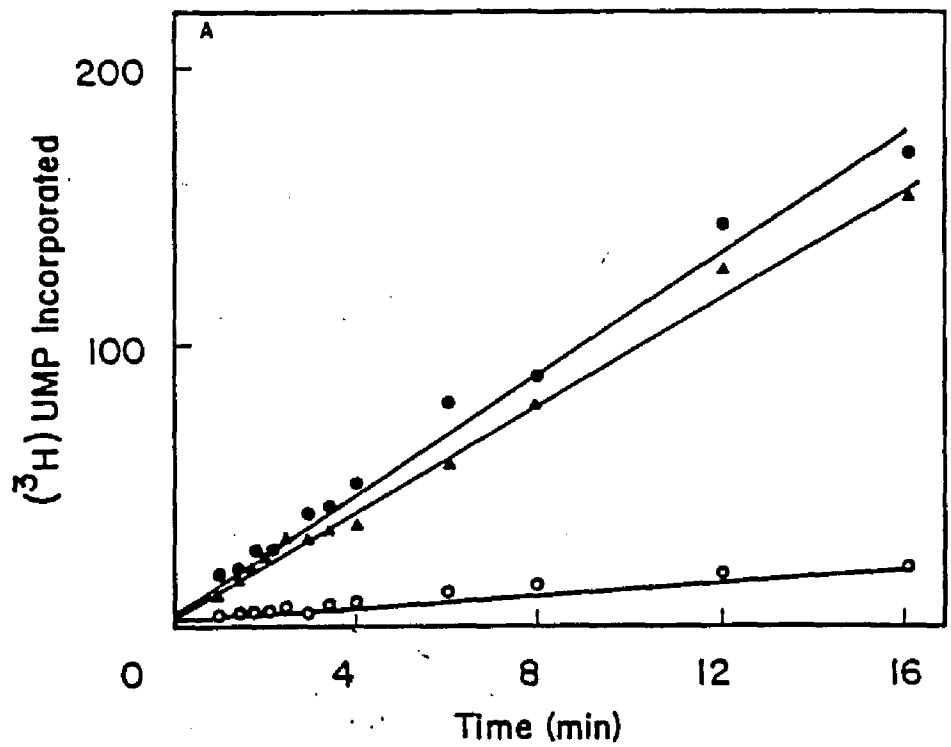

B
200
100
0
2
4
6
8
(^{3}H) UMP Incorporated
Time (min)

Figure 17. Effect of anti-beta mAb 210E8 on the tau_{obs} of the TAC16(UpG) promoter. The protocol employed is as described in the Legend to Figure 15.


A
200
100
0
4
8
12
16
Time (min)
(^{3}H) UMP Incorporated

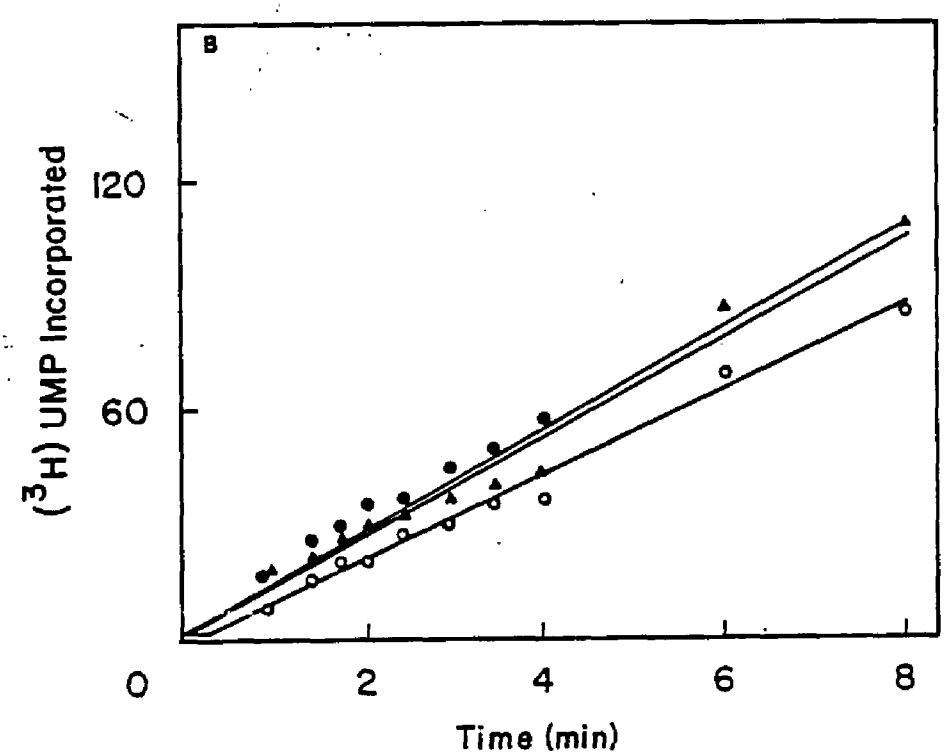

B
120
60
0
2
4
6
8
Time (min)
(^{3}H) UMP Incorporated

Figure 18. Effect of anti-beta mAb 210E8 on the rate of RP_O formation on the T7 A1, A3 Promoters (Product: CpApU) Standard preincubation and reaction conditions were employed. Lag time assays were performed as described in the Legend to Figures 15-17. Table 13 lists the polymerase and DNA concentrations employed and the percent residual activities obtained. Assays contained 2 mM primer (CpA) and 50 uM [^{3}H]UTP; (▲) steady state control; (x) lag control, (●) lag + mAb 210E8.

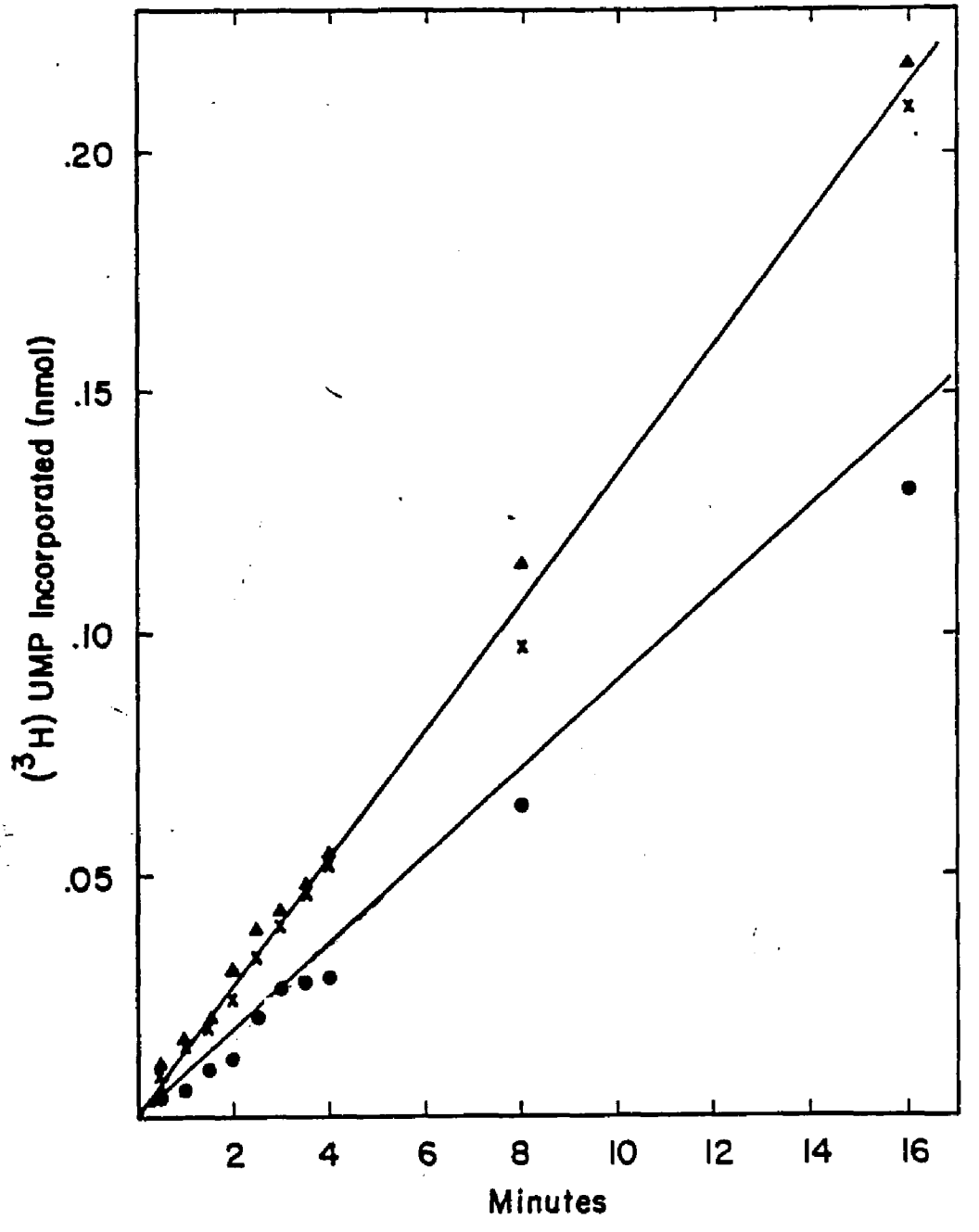

.20
.15
.10
.05
(^{3}H) UMP Incorporated (nmol)
2
4
6
8
10
12
14
16
Minutes

Figure 19. Effect of anti-beta mAb 210E8 on the rate of RP_O formation on T7 DNA (Product:pApU). Assay conditions are as described in the Legend to Figure 18. Assays contained 2 mM 5' AMP and 50 uM [^{3}H]UTP.

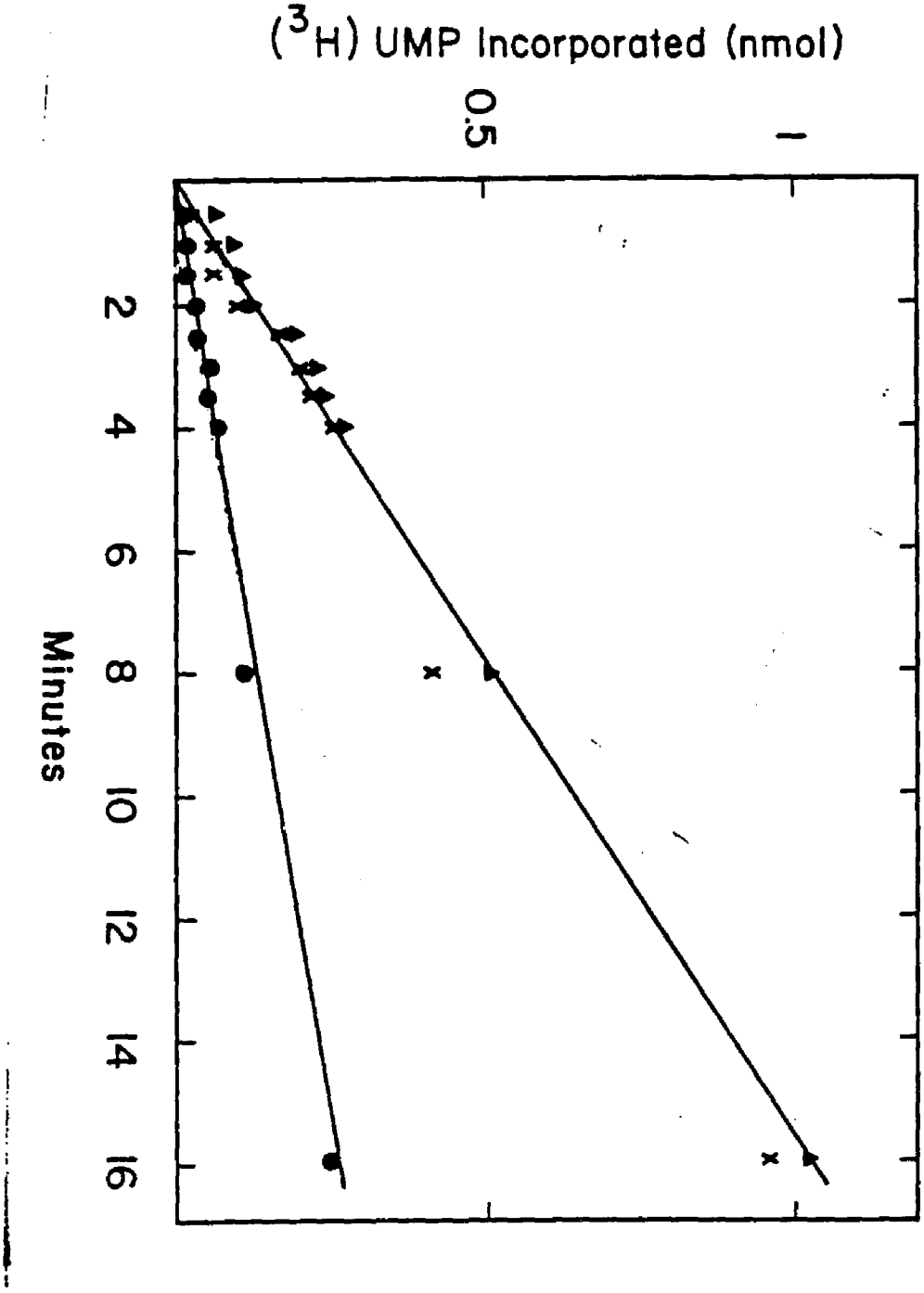

(^{3}H) UMP Incorporated (nmol)
0.5
1
Minutes
2
4
6
8
10
12
14
16

Figure 20. Effect of anti-beta mAb 210E8 on the rate of RP_o formation on $d(A-T)_n$ (Product: pApU). Assay conditions are as described in the Legend to Figure 19.

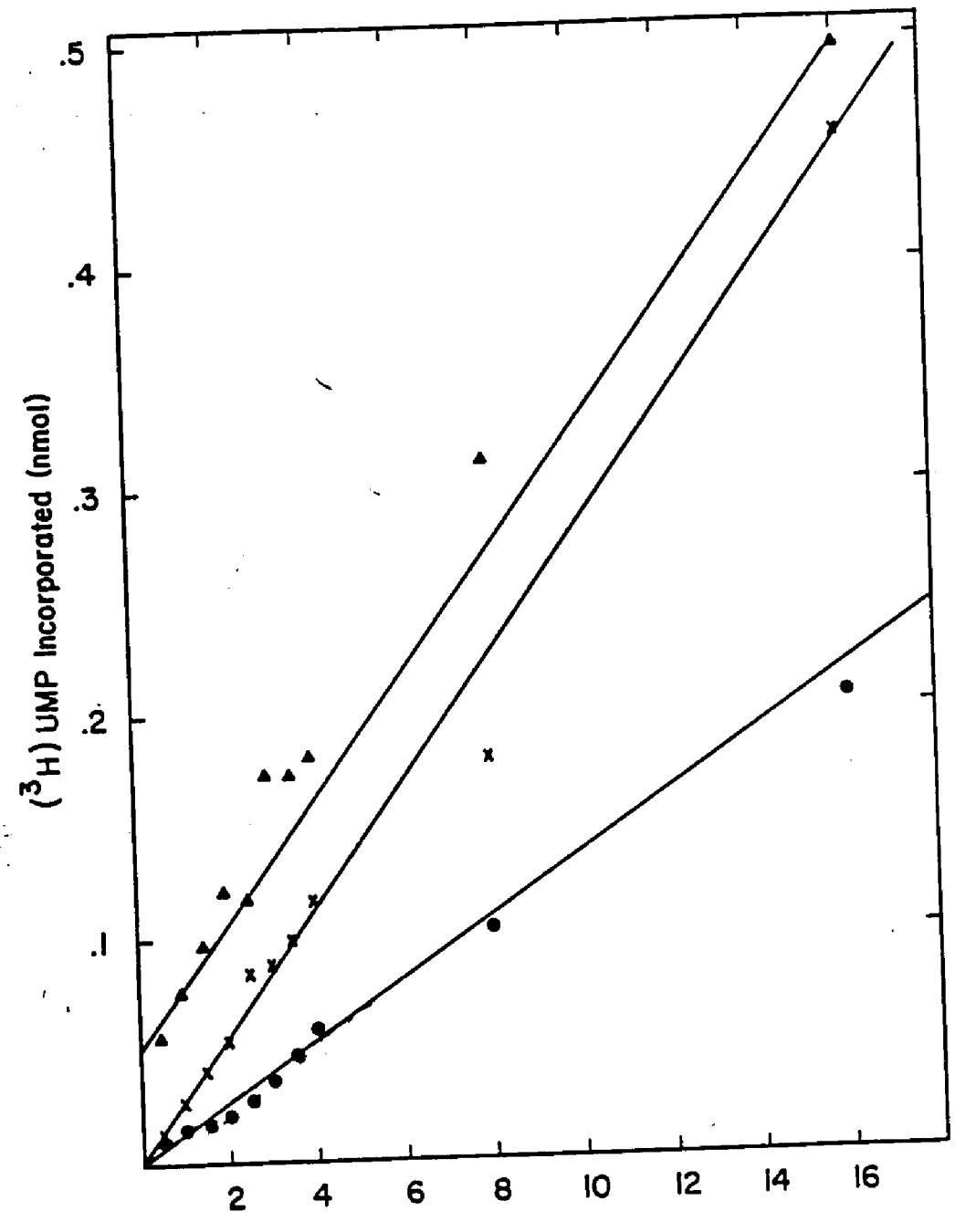

.5
.4
.3
.2
.1
(^{3}H) UMP Incorporated (nmol)
2
4
6
8
10
12
14
16

Figure 21. The effects of anti-beta mAb 210E8 on the kinetics of RP_o formation on the supercoiled lac UV5 promoter. The protocol employed is as described in "Materials and Methods". In each plot tau_{obs} is plotted versus the reciprocal of the RNA polymerase (RNP) concentration. The tau_{obs} measurements were obtained from lag time assays performed at different RNA polymerase concentrations in the presence (●) and absence (○) of mAb 210E8. In the absence of mAb the isomerization (k_2) was 0.022 s^{-1} the binding constant (K_B) was 1.4 x 10^{-8} M^{-1}.In the presence of mAb the k_2 was 0.032 s^{-1} and the K_B was 1.7 x 10^{-8} M^{-1}.

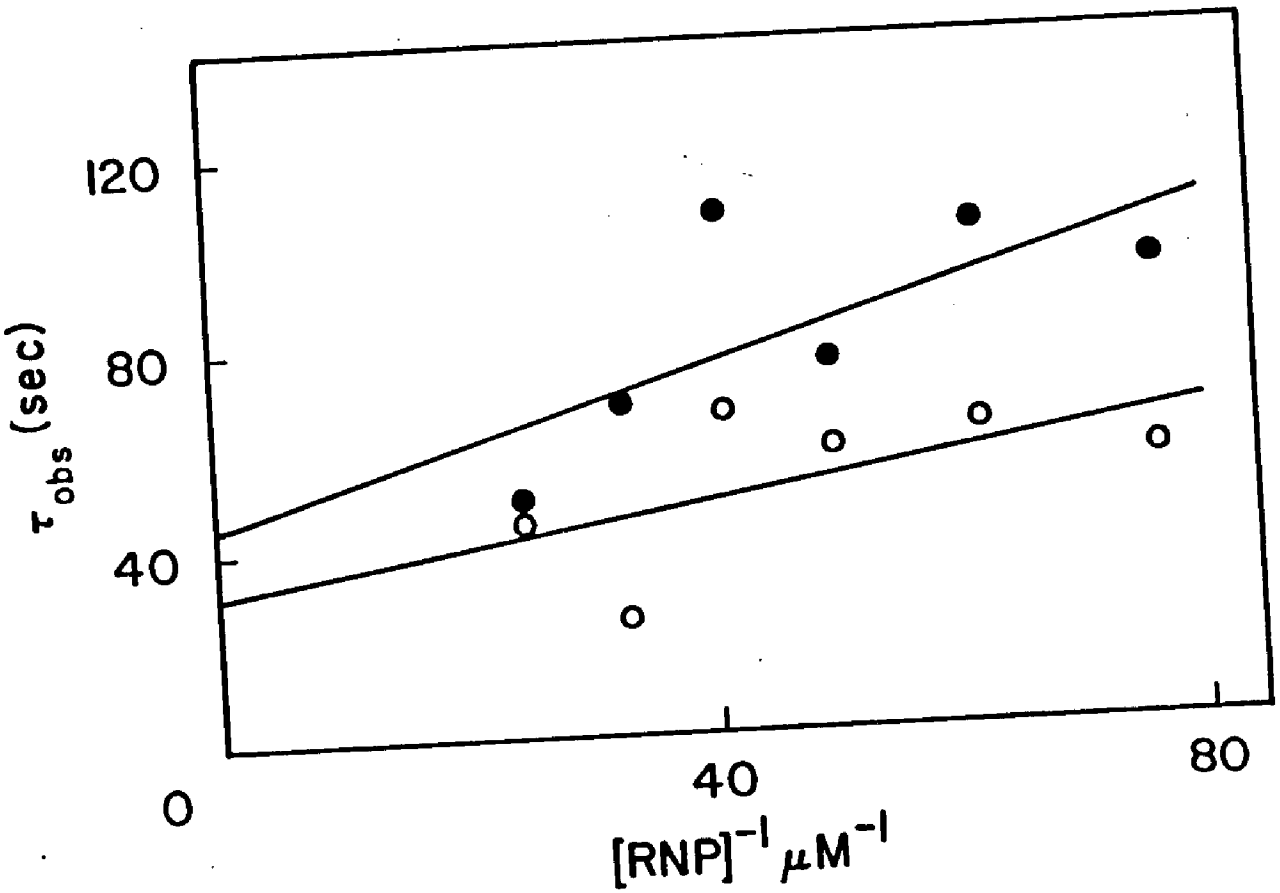

120
80
40
0
τ_{obs} (sec)
40
80
$[RNP]^{-1}\ \mu M^{-1}$

Figure 22. Effect of mAbs on the protection by RNA polymerase of the lac P^+ promoter against attack by DNase 1. Conditions are as described under "Materials and Methods" using 4 nM 5'-end labeled template (lower) strand of lac P^+ promoter and 180 nM RNA polymerase. Reaction mixtures also contained 20 nM CRP and 100 uM cAMP. The nucleotide positions are numbered relative to the start site of initiation. The mAb 210E8-induced difference in the normal reactivity pattern is indicated by an arrow. Lanes a-g show the DNase 1 footprints of cAMP-CRP-lac P^+ complexes formed with (a) no mAb (b) the inhibitory anti-beta mAb 210E8-polymerase, (c) the noninhibitory anti-beta mAb 211C7-polymerase, (d) the inhibitory anti-beta' mAb 311G2-polymerase, (e) the noninhibitory anti-beta' mAb 371D6-polymerase, (f) and without polymerase. Lanes g-k show that the inclusion of the abortive subatrates, ApA + UTP, did not change the DNase 1 cleavage pattern for the cAMP-CRP-lac P^+ complexes formed with (g) polymerase alone, (h) mAb 210E8-polymerase, (i) mAb 221C7-polymerase, (j) mAb 311G2-polymerase and (k) mAb-371D6-polymerase. The lane "G" is a guanine methylation pattern of the unprotected lac P^+ promoter (not shown).

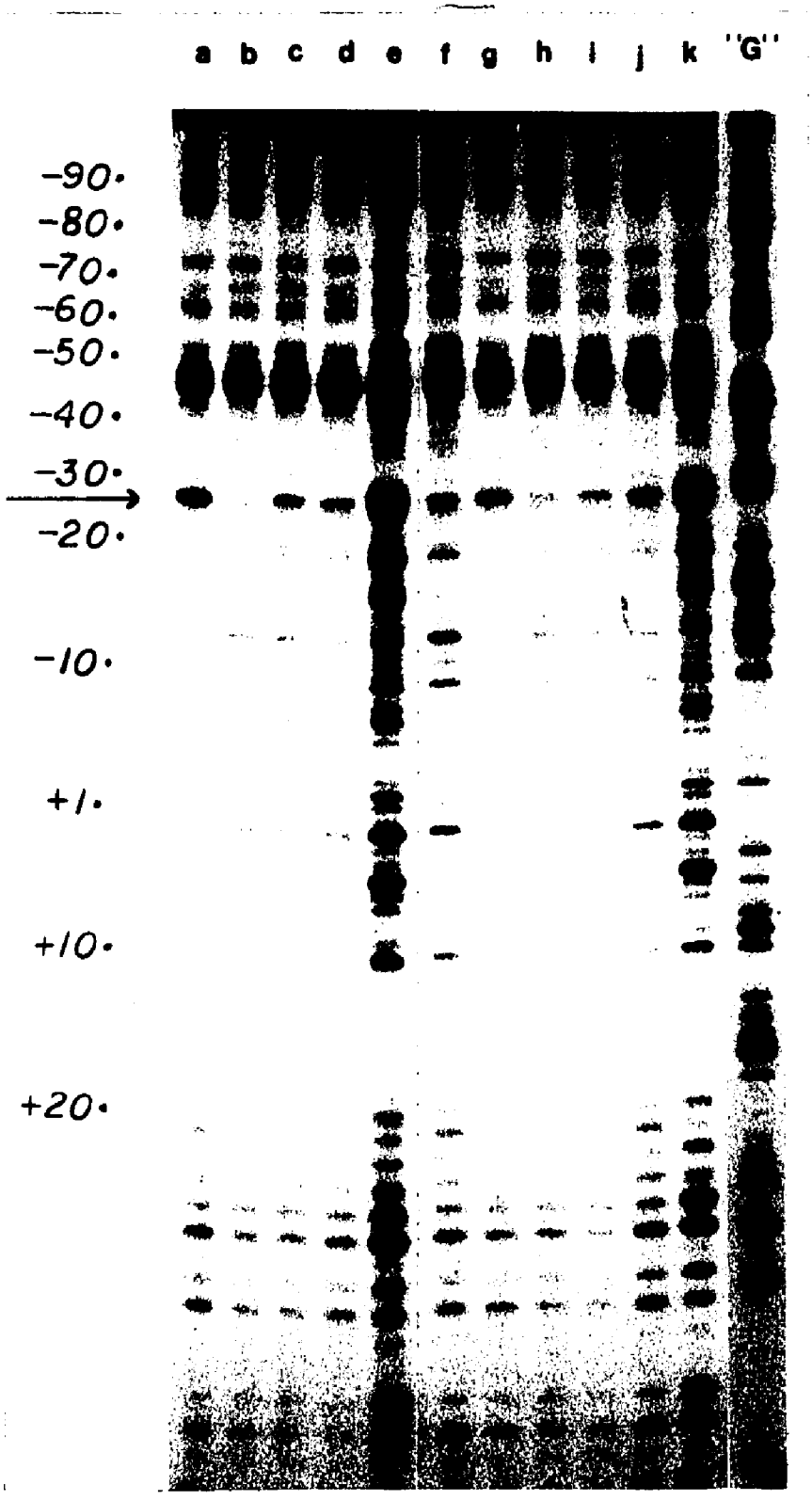
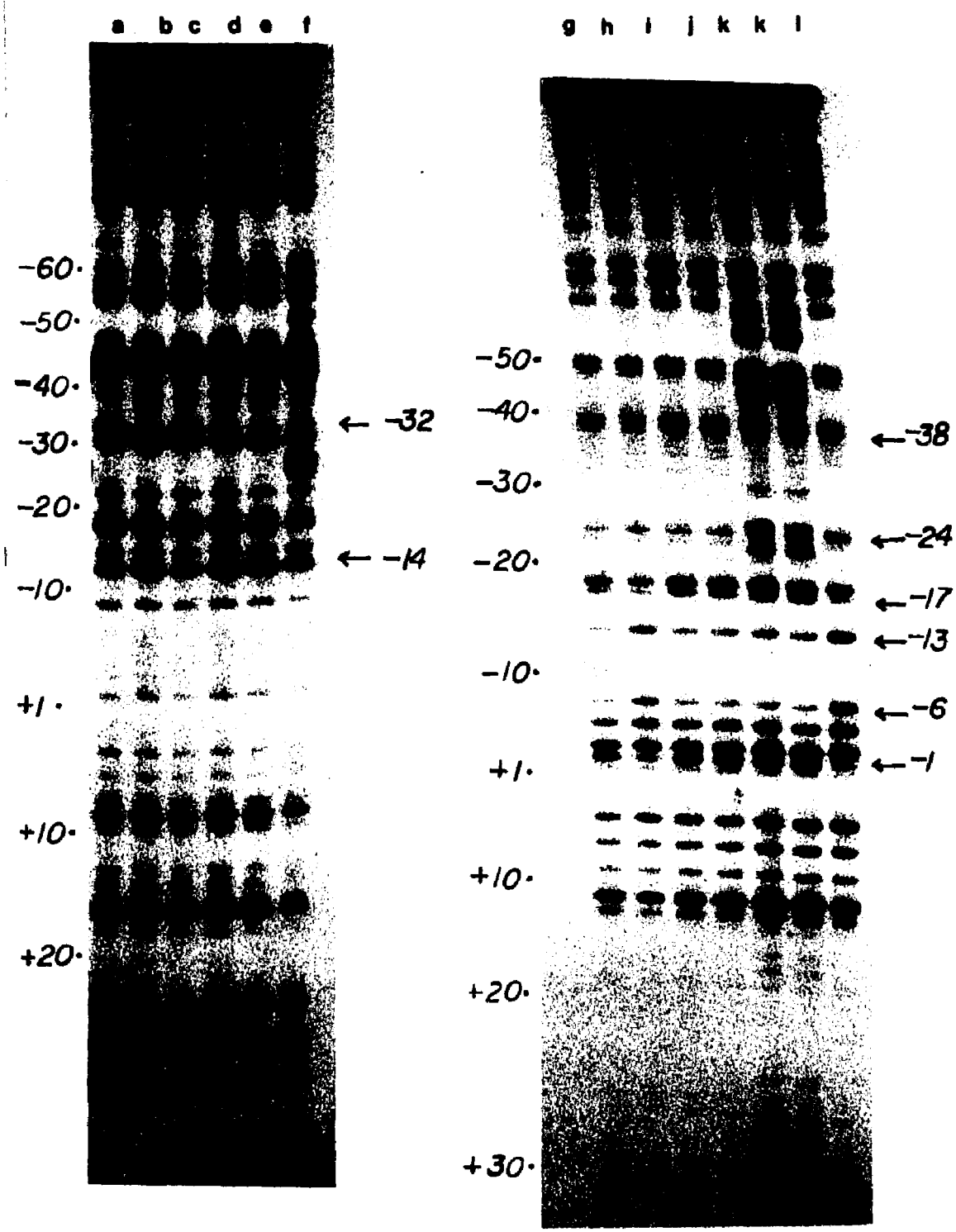

a b c d e f g h i j k "G"
-90·
-80·
-70·
-60·
-50·
-40·
-30·
-20·
-10·
+1·
+10·
+20·

Figure 23. Effect of mAbs on the reactivity of base residues of the RNA polyermase-lac UV5 promoter complex to methylation by dimethyl sulfate. Reaction conditions are as described under "Materials and Methods" using 4 nM 5'(template) and 3'nontemplate) end-labeled lac UV5 promoter fragments and 180 nM RNA polymerase. (Panel A) template (lower) strand. (Panel B) Nontemplate (upper) strand. Reactivity patterns are shown for lac UV5 complexes formed with (a) the unprotected fragment (b) polymerase alone (c) mAb 210E8-polymerase (d) mAb 221C7-polymerase (e) mAb 311G2-polymerase (f) 371D6- polymerase (g) polymerase alone (h) mAb 210E8-polymerase (i) mAb 221C7-polymerase (j) mAb 311G2-polymerase, (k) mAb 371D6-polymerase and (l) without polymerase. The inclusion of ApA + UTP did not alter the methylation patterns.



A
template
strand

B
nontemplate
strand

Figure 24. Effect of anti-beta' mAb 371D6 on the protection by RNA polymerase and CRP of the lac P^+ and lac UV5 promoters against attack by DNase 1. The protocol employed is as described in the Legend to Figure 22. Binding assays were performed with the lac UV5 (a) and lac P^+ (b) promoters. For each promoter complexes were formed with polymerase alone (1) and mAb 371D6 polymerase (2) in the presence (+) and absence (-) of cAMP-CRP. The arrow indicates the location of the CRP dependent bands for both promoters in lanes 2a and 2b. The presence of the additional band was also not observed in the DNase 1 footprint of unprotected lac P^+ and lac UV5 promoter fragments (not shown).

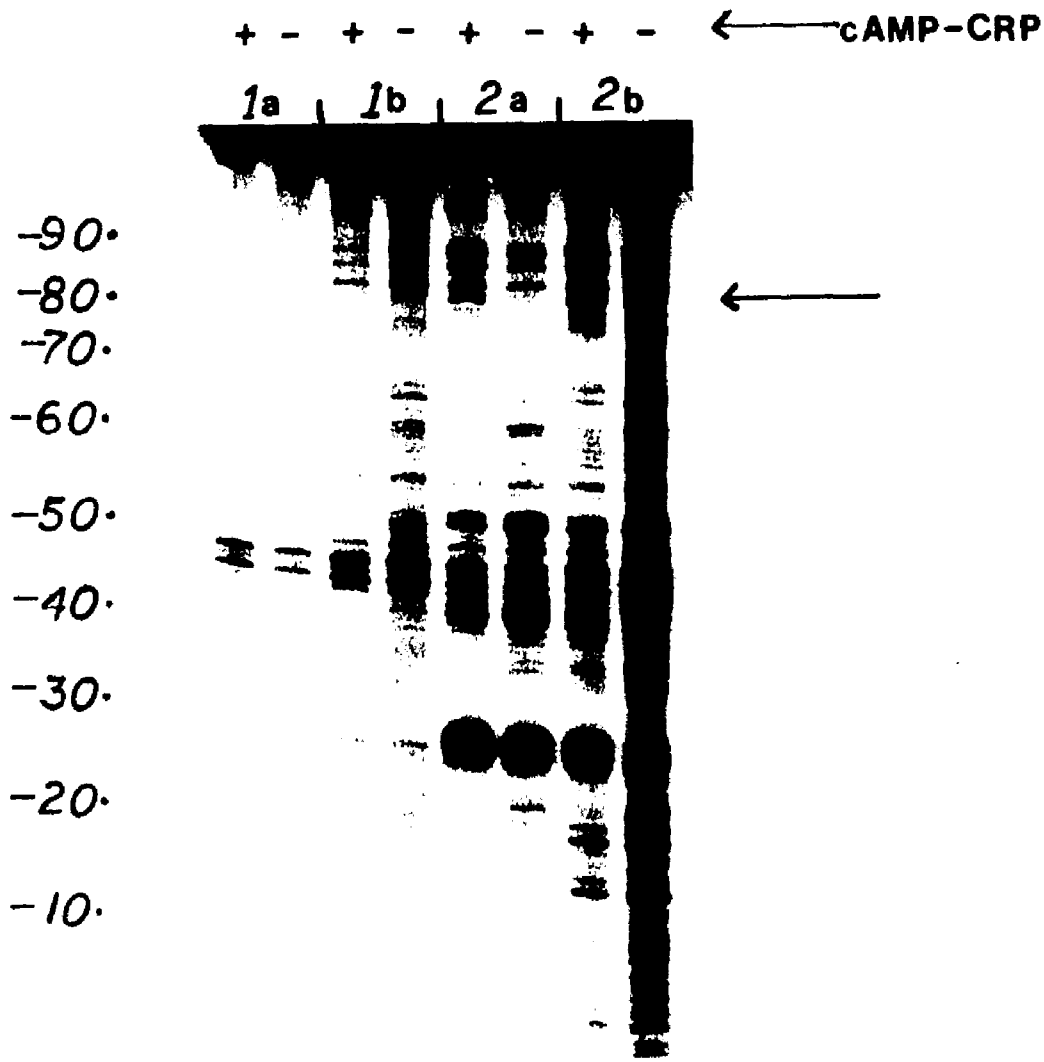

+ - + - + - + -
cAMP-CRP
1a
1b
2a
2b
-90·
-80·
-70·
-60·
-50·
-40·
-30·
-20·
-10·

Figure 25. Effect of the inhibitory mAbs on the methylation of single strand specific cytosines of RNA polymerase-lac UV5 promoter complexes. Reaction conditions are as described under "Materials and Methods" using 4 nM 5' end- labeled template (lower) strand of lac UV5 and 180 nM RNA polymerase. Cytosine reactivity patterns are shown for lac UV5 complexes formed with (a) polymerase alone, (b) mAb 210E8-polymerase, (c) mAb 221C7-polymerase, (d) the unprotected fragment and (e) mAb 311G2-polymerase.

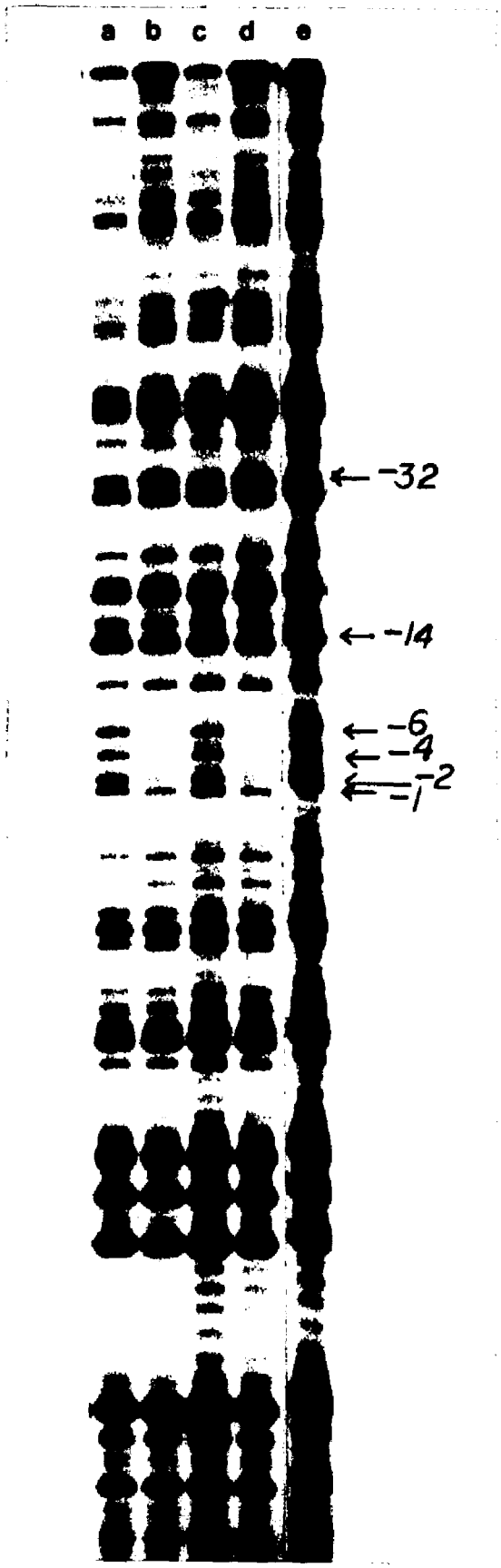

a
b
c
d
e
-32
-14
-6
-4
-2
-1

Figure 26. DNase 1 footprint of polymerase-lac UV5 promoter complexes formed in the presence and absence of 25% glycerol and the inhibitory mAbs anti-beta 210E8 and anti-beta' 311G2. The protocol employed is as described in the legend to Figure 22. DNA was labeled on the nontemplate (upper) strand. In lanes a-d promoter complexes were formed with (a) polymerase alone, (b) mAb 210E8-polymerase, (c) mAb 311G2-polymerase in the presence of 25% glycerol. In lanes d-f, (d) polymerase alone, (e) mAb 210E8-polymerase and (f) mAb 311G2-polymerase were complexed with lac UV5 at 2% glycerol. The unprotected fragment is shown in lane g.

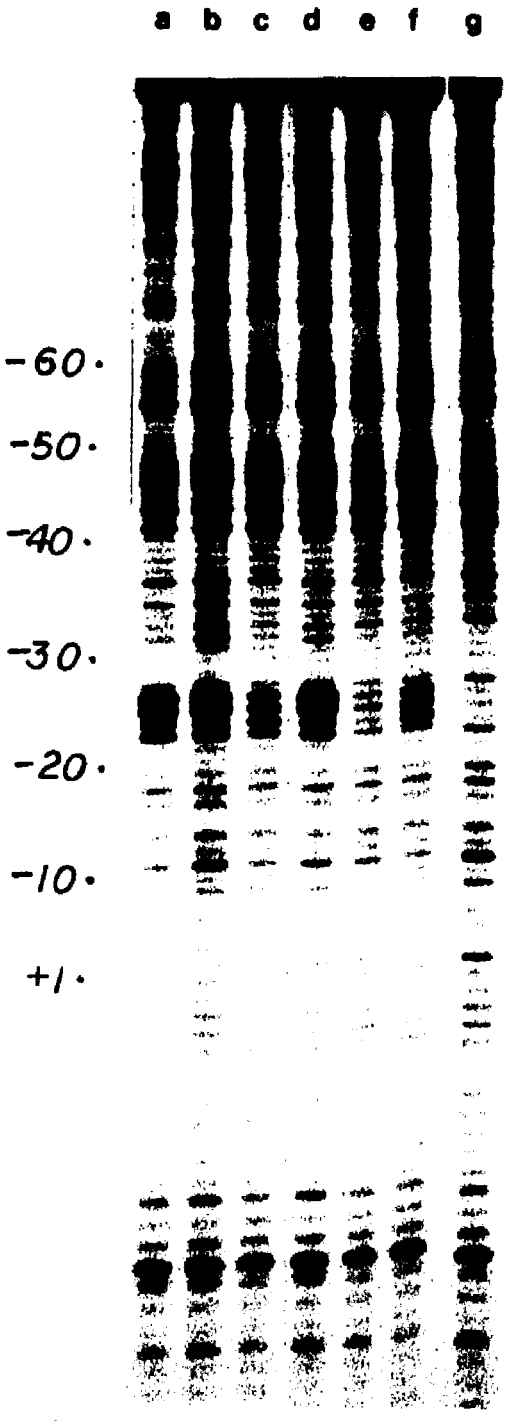

a b c d e f g
-60·
-50·
-40·
-30·
-20·
-10·
+1·

Figure 27. Guanine methylation of the lac UV5 promoter complexes with mAb 210E8-polymerase (or polymerase alone) in the presence of 25% glycerol. The protocol employed is as described in the legend to Figure 23. DNA was labeled on the nontemplate (upper) strand.The methylation patterns of protein-promoter complexes formed at 25% glycerol are shown on the left. Control reactions performed in the presence of 2% glycerol are shown on the right for comparison. The control reactivity patterns (2% glycerol) were obtained under the same conditions as those shown in Figure 23. Reactivity patterns are shown for lac UV5 complexes formed with (a)polymerase alone, (b) mAb-210E8 polymerase, (c) mab 221C7-polymerase, (d) mAb 311G2-polymerase, (e) mAb 371D6-polymerase and (f) without polymerase.

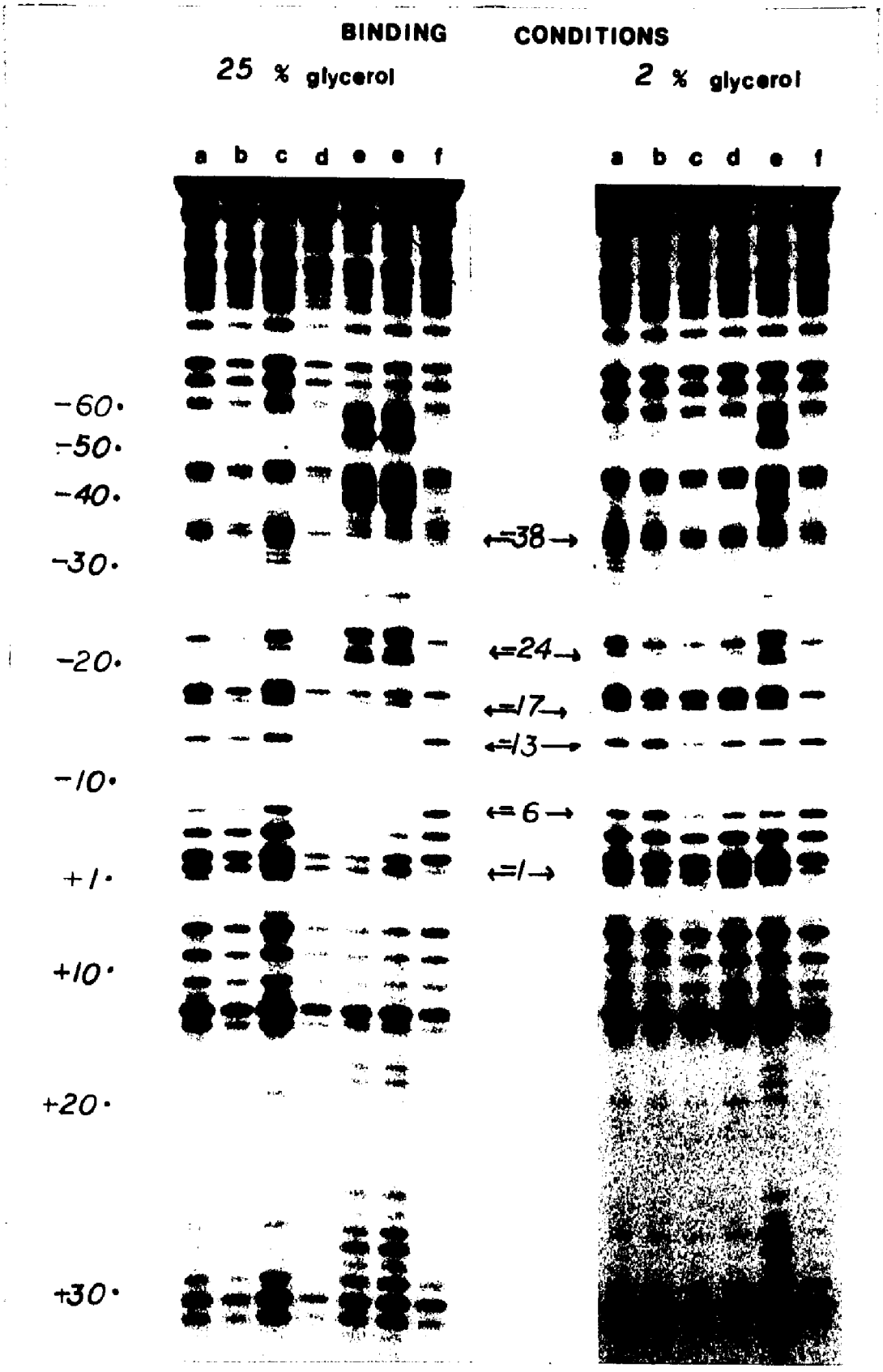
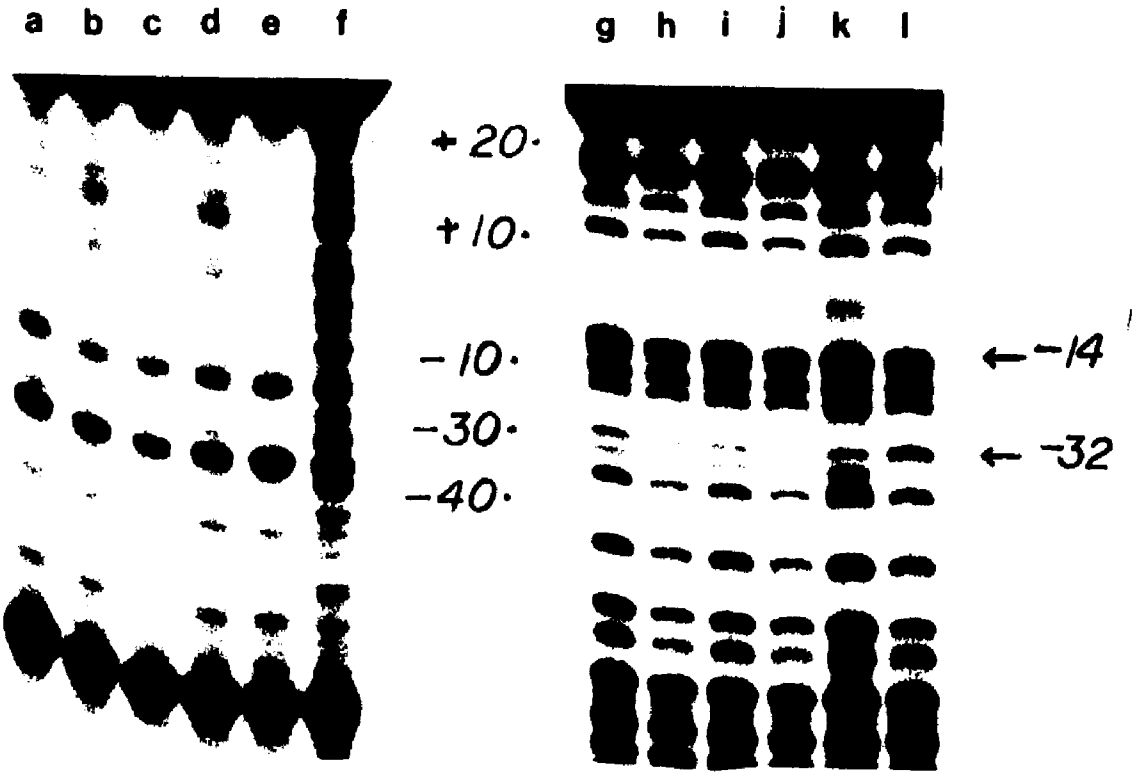

BINDING CONDITIONS
25 % glycerol
2 % glycerol
a b c d e e f
a b c d e f
-60·
-50·
-40·
-30·
-20·
-10·
+1·
+10·
+20·
+30·
←38→
←24→
←17→
←13→
←6→
←1→

Figure 28. DNase 1 footprint and guanine methylation pattern of polymerase-TAC16 promoter complexes formed in the presence and absence of mAbs. The protocol employed is described in the legends to Figures 22 and 23. DNA was labeled in the 3'-end labeled template (lower) strand. Lanes a-f show the DNase 1 footprints for lac UV5 complexes formed with (a) polymerase alone, (b) mAb 210E8-polymerase, (c) mAb 221C7-polymerase, (d) mAb 311G2-polymerase, (e) mAb 371D6-polymerase and (f) without polymerase. Lanes g-l show the "G" reactions for lac UV5 complexes formed with (g) polymerase alone, (h) mAb 210E8-polymerase, (i) mAb 221C7-polymerase, (j) mAb 311G2-polymerase, (k) mAb 371D6-polymerase and (l) without polymerase. The inclusion of the abortive substrates, ApU + UTP or UpG + UTP, resulted in DNase 1 footprints and guanine methylation patterns that resembled patterns obtained in the absence of substrates.


a b c d e f
g h i j k l
+20·
+10·
-10·
-30·
-40·
←-14
←-32

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