Effects of an Anti-β Monoclonal Antibody on the Interaction of the *Escherichia coli* RNA Polymerase with the *lac* and TAC Promoters†

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ABSTRACT: The effects of an inhibitory monoclonal antibody (mAb) raised against the β subunit of the *Escherichia coli* RNA polymerase were determined on the kinetics and structural interactions during formation of the open promoter complex (RP_o). Analysis of the kinetics of abortive initiation on linear and supercoiled templates of the *lac* and TAC16 promoters showed that abortive synthesis by mAb 210E8–RNA polymerase varied as a function of DNA topology. A kinetic analysis of RP_o formation on the supercoiled *lac* UV5 promoter showed that mAb 210E8 effected a slight alteration in the isomerization rate and no effect on the initial rate of RNA polymerase binding to the promoter. The potent inhibition of initiation with linear promoters by mAb 210E8 was not apparent when the promoters were assayed in their supercoiled forms. Abortive synthesis with the TAC16 promoter was accompanied by an mAb 210E8 induced hindrance of ApUpU but not UpGpU synthesis. The data indicate that the inhibition by mAb 210E8 with the supercoiled TAC16 promoter is further alleviated when the spacer length is shifted from 16 base pairs (ApUpU formation) to 18 base pairs (UpGpU formation). When DNase I and dimethyl sulfate were used to probe DNA structure, mAb 210E8 was found to alter polymerase interactions with the *lac* promoter. DNase I footprinting indicated that the structural interactions for *lac* P^+ promoter–RNA polymerase complexes were slightly altered in the presence of mAb 210E8. Treatment of the RNA polymerase–*lac* UV5 complex with dimethyl sulfate revealed an alternate mode of RNA polymerase interaction with essential guanine contacts which was intermediate between a fully protected and free promoter. The single-stranded region revealed by the dimethyl sulfate reactivity of the RNA polymerase–*lac* UV5 complex was not detectable when the promoter complex was formed in the presence of mAb 210E8. The results of the kinetic and binding studies with linear *lac* UV5 suggest that mAb 210E8 elicits the formation of polymerase–DNA complexes that are partially active and sensitive to dissociation by $d(A\text{-}T)_n$. mAb 210E8 appears to trap RNA polymerase in an intermediate conformation that precedes the formation of a stable and active RP_o. The demonstration that mAb 210E8 elicits a slight effect on the lag time required for RP_o formation on the supercoiled *lac* UV5 promoter suggests that the antibody effects may not be completely diminished during initiation with the supercoiled template. A consideration of these results along with those showing a greater inhibition with the supercoiled TAC16 (ApU) relative the TAC16 (UpG) implies that mAb 210E8 may be probing an essential RNA polymerase–promoter interaction that is relieved by supercoiling and is sensitive to changes in spacer length. The presence of the anti-β mAb 210E8 during the steps leading to RP_o formation with a linear promoter may hinder the critical positioning of RNA polymerase with respect to sites on the promoter and impede the subsequent melting of the DNA.

Gene expression in prokaryotes is regulated primarily at the level of transcriptional initiation. The rate of RNA chain initiation occurs with widely varying frequencies, and the efficiency of transcription by RNA polymerase appears to be modulated by promoter-specific DNA sequences and various regulatory factors [for recent reviews, see von Hippel et al. (1984) and McClure (1985)]. Chemical, mutational, and kinetic analyses of the interaction of RNA polymerase with various promoters (Siebenlist et al., 1980; Hawley & McClure, 1982; Stefano & Gralla, 1982a) have provided evidence that the structural basis for promoter strength can be assigned to two conserved hexamers which lie 35 and 10 base pairs upstream from the initiation nucleotide site. Mutational studies with the *lac* promoter (Stefano & Gralla, 1982b) and the *ANT* promoter of the *Salmonella* phage P22 (Grana et al., 1985) as well as kinetic analyses of the TAC promoters (Mulligan et al., 1985) indicate that alterations in the spacer length between the conserved hexamers may serve as an additional determinant of promoter strength. In addition, a demonstration of two distinct RNA transcripts in the TAC16 promoter (Mulligan et al., 1985; Brosius et al., 1985) indicated that a shift in promoter activity may result from the recognition by RNA polymerase of an alternate start site.

It has been proposed from studies with T7 DNA (Chamberlin, 1974) that transcription proceeds via a transition from a closed promoter complex (RP_c)[1] between RNA polymerase and DNA to the formation of a stable open promoter complex (RP_o). A kinetic analysis of the rates of abortive initiation devised by McClure (1980) attempts to quantify promoter strength. In this analysis, measurement of the conversion from the closed to an open promoter complex yields independent estimates of the initial binding of RNA polymerase to DNA (K_B) and the final isomerization (k_2) to a stable RP_o. The

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[1] Abbreviations: RP_o, open promoter complex; RP_i, intermediate promoter complex; RP_c, closed promoter complex; mAb, monoclonal antibody; BSA, bovine serum albumin; ApA, adenylyl(3′–5′)adenosine; ApU, adenylyl(3′–5′)uridine; UpG, uridylyl(3′–5′)guanosine; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; CRP, cAMP receptor protein.

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nature of this transition now appears to involve more than a simple single-step mechanism for polymerase–promoter interactions. Kinetic studies (Buc & McClure, 1985) and DNA structural analyses (Spassky et al., 1985) have shown that the conversion of RP_c to RP_o on the linear *lac* UV5 promoter involves the formation of a transient intermediate complex, RP_i (Buc & McClure, 1985). Formation of intermediate complexes has also been postulated to explain kinetic estimates of RNA polymerase binding to the λ P_R promoter (Roe et al., 1984).

In the present study, a monoclonal antibody raised against the β subunit of the *Escherichia coli* RNA polymerase has been used to study interactions of RNA polymerase during promoter utilization with different templates. This anti-β antibody, mAb 210E8, was previously shown to inhibit the $d(A-T)_n$-directed synthesis of $r(A-U)_n$ (Rockwell et al., 1985). In the present study, the abortive initiation assay (McClure et al., 1978) is employed to show that mAb 210E8 can serve as an effective probe with which to detect differences in the response of RNA polymerase to alterations in DNA topology and spacer length. Antibody-induced structural changes in polymerase–promoter interactions were determined by comparing the DNase I and dimethyl sulfate reactivity patterns of mAb–RNA polymerase–DNA complexes with complexes formed in the absence of antibody (Schmitz & Galas, 1979; Spassky et al., 1984, 1985). This approach was employed to correlate mAb 210E8 related effects on the abortive initiation reaction with any structural changes effected by the antibody on the promoter during RP_o formation.

Materials and Methods

Materials. Reagents were obtained as follows: T4 polynucleotide kinase, alkaline phosphatase, DNA polymerase I Klenow fragment, and restriction endonucleases, Boehringer Mannheim; DNase I, Cooper Biomedical; adenylyl(3′–5′)-adenosine (ApA), adenylyl(3′–5′)uridine (ApU), and uridylyl(3′–5′)guanosine (UpG), radioimmunoassay-grade bovine serum albumin (BSA), and cyclic AMP, Sigma Chemical Co.; goat anti-mouse IgG peroxidase, Hyclone; $d(A-T)_n$, ribonucleoside triphosphates, and protein A–Sepharose, Pharmacia; [γ-^{32}P]ATP, [α-^{32}P]dATP, and [^{3}H]UTP, ICN; dimethyl sulfate, piperidine, and hydrazine, Aldrich; urea, Schwarz/Mann; formamide, Amresco; acrylamide, Serva; Fluorosil, Isolab.

Buffers. P60–BSA buffer consisted of 20 mM potassium phosphate (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 60% glycerol, and 1 mg/mL BSA. Storage buffer was composed of 50 mM potassium phosphate (pH 7.5), 150 mM KCl, and 0.05% NaN_3. WASP solvent was water, saturated ammonium sulfate, and 2-propanol (18:80:2) adjusted to pH 8 with ammonium hydroxide (Hansen & McClure, 1979).

Preparation of Enzyme and Antibody. E. coli RNA polymerase was prepared by a modification of the method of Burgess and Jendrisak (1975). Holoenzyme was isolated by chromatography on denatured calf thymus DNA–agarose as described by Lowe et al. (1979). Protein concentration was determined by using the extinction coefficient $E^{1\%}_{280nm} = 6.7$ (Levine et al., 1980). Monoclonal antibodies were prepared as described by Rockwell et al. (1985) and were isolated by chromatography on protein A–Sepharose (Ey et al., 1978). Immunoglobulin concentration was determined by using the extinction coefficient $E^{1\%}_{280nm} = 14.0$ (Ey et al., 1978). The cAMP receptor protein was prepared by the method of Eilen et al. (1978). CRP concentration was determined by using the extinction coefficient $E^{1\%}_{280nm} = 8.8$ (Aiba & Krakow, 1981).

DNA Preparations. pMB9 *lac* P$^+$ and pMB9 *lac* L8UV5 constructed by Dr. F. Fuller were obtained from Dr. A. Revzin. Plasmid pNI171 containing the TAC16 promoter (Amann et al., 1983) was provided by Dr. N. Irwin. Plasmids were isolated by alkaline extraction and then purified on glass powder by the method of Marko et al. (1982). The 203 bp *lac* P$^+$ and *lac* UV5 promoter fragments were isolated following digestion with *Eco*RI. The 250 bp TAC16 fragment was obtained by restriction of plasmid with *Eco*RI and *Hin*dIII. Fragments were purified from vector DNA by polyacrylamide gel electrophoresis according to Maxam and Gilbert (1980). The eluted DNA fragments were recovered by using a Schleicher & Schuell Elutip; purity was assessed by electrophoresis on a 0.8% agarose gel.

The supercoiled templates employed in these studies were the purified plasmid preparations carrying the promoter insert. Agarose gel analysis of each preparation showed that the plasmid isolation procedure employed yielded mostly form I along with some form II DNA.

The 5′ and 3′ ^{32}P *lac* P$^+$ or ^{32}P *lac* UV5 promoter fragments were labeled with [γ-^{32}P]ATP by polynucleotide kinase or with [α-^{32}P]dATP using the DNA polymerase Klenow fragment. The labeled fragments were then restricted with *Pvu*II which cuts the DNA at −123 and yields promoter fragments uniquely labeled on either the lower or the upper strand.

Abortive Initiation Assays. The abortive initiation assay of McClure et al. (1978) was employed. Standard reaction mixtures contained (final volume 50 μL) 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 μg of bovine serum albumin. The RNA polymerase concentration used was 20–40 nM (1–2 pmol), and where indicated, mAb 210E8 was included at a molar ratio (mAb 210E8:RNA polymerase ratio) of 20:1. The *lac* and TAC promoters were assayed at a final concentration of 2 nM linear fragment or 1 nM supercoiled plasmid. Unless otherwise indicated, the substrate concentrations were 0.5 mM dinucleoside monophosphate and 50 μM [^{3}H]UTP (200 cpm/pmol). For the *lac* P$^+$ abortive initiation assays, 20 nM cAMP receptor protein and 100 μM cAMP were included.

For each assay, 2-μL aliquots of RNA polymerase in P60–BSA buffer were preincubated in 10 or 15 μL of storage buffer with and without the inclusion of the indicated amount of mAb for 1 h at 0 °C. The appropriate template was then added, and the incubation was continued for 15 min at 37 °C. Following addition of the appropriate substrates, the incubation was continued for the times indicated. Aliquots (40 μL) were removed and applied to Whatman 3MM paper prespotted with 10 μL of 0.5 M EDTA. Reaction products were then resolved by paper chromatography as described by McClure et al. (1978).

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 (picomoles per minute) versus the reciprocal of the varied substrate concentration.

Lag Time Assays. These assays were performed according to a kinetic analysis of promoter strength devised by McClure (1980). 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 Scheme I.

Scheme I

$$R + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o$$

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. The time required for polymerase to form an active RP_o is measured as a promoter-specific lag time (τ_{obsd}) in an approach to the steady-state rate of abortive initiation. The lag time measurements obtained can then be used to partition polymerase–promoter interaction during RP_o formation into two functional parameters. A measurement of τ_{obsd} at various RNA polymerase concentrations is used to derive 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 employment of the pseudo-first-order experimental conditions where the concentration of polymerase is in excess of promoter, the relationship of τ_{obsd} to the concentrations of RNA polymerase can be expressed as the simplified equation:

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

A plot of τ_{obsd} vs 1/RNA polymerase concentration (τ 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 in the present study to compare promoter lag time measurements in the presence and absence of mAb 210E8. Steady-state control values were obtained by incubating an aliquot of preincubated RNA polymerase with template for 10 min at 37 °C and then initiating the reaction by the addition of substrates. Lag time measurements in the presence and absence of mAb 210E8 were obtained by preincubating two separate mixtures of the template and substrates for 10 min at 37 °C and then initiating both reactions by adding aliquots of preincubated RNA polymerase to one mixture and RNA polymerase–mAb 210E8 to the other. Aliquots (10 μL) were removed at the times indicated, and reaction products were resolved by paper chromatography as described above.

The lag time (τ_{obsd}) for *lac* UV5, TAC16 (ApU), and TAC16 (UpG) was determined by a least-squares analysis of those data points that were at least 5 times the estimated lag time (McClure, 1980). The lag time analysis is based on the a priori decision to sequentially exclude data points of increasing time increments until the slopes of the three curves of a τ analysis, each estimated through linear regression, are not significantly different. The lag time is estimated as the distance between the intercept generated by this method and the origin. The binding constant, K_B, and the isomerization constant, k_2, for the *lac* UV5 insert in the presence and absence of mAb 210E8 were calculated from a τ plot of $1/\tau_{obsd}$ 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.

To determine whether reactions run in the presence of monoclonal antibodies achieve steady-state conditions similar to the controls, slope values were obtained from regression analyses of only those data points of a reaction assumed to be in the steady state (>4 min). Slope values were generated for each of the three reactions run at the RNA polymerase concentration indicated and then compared by using Student's t test to determine if any significant differences ($p \leq 0.05$) were apparent. If the slopes of the three curves did not differ significantly, it was assumed that the reactions had reached a similar steady state.

Promoter Binding Assays: Protection and DNA Enzymatic and Chemical Modification. For the DNA protection studies (Garner & Revzin, 1982), binding reactions (20 μL) contained 2.5 pmol of *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-min incubation at 37 °C, reaction mixtures containing *lac* UV5 promoter complexes were diluted with the same buffer without KCl to adjust the assay conditions to 32 mM KCl for restriction by *Hpa*II. After the addition of $d(A\text{-}T)_n$ to 11 μM and 9 units of *Hpa*II, reactions were reincubated at 37 °C for the times indicated. To terminate restriction, mixtures were adjusted to contain 333 μg/mL heparin, 17 mM EDTA, and 0.1% sodium dodecyl sulfate. A one-fifth volume of a solution containing 2 parts 50% glycerol and one part 0.1% bromphenol blue was then added. Reactions were immediately applied to a nondenaturing 7.5% polyacrylamide gel. After electrophoresis, gels were stained with ethidium bromide (1 μg/mL) to determine DNA protection patterns. In these experiments, a 4-fold ratio of polymerase to DNA was sufficient to give full protection. Titration of *Hpa*II with the appropriate free promoter fragment was initially performed to determine the amount of *Hpa*II sufficient to ensure complete restriction of the DNA within the time limits of the assay.

The DNase I footprinting and guanine methylation experiments were carried out according to the procedure of Spassky et al. (1985) using the conditions for the abortive initiation assays. The N-3 cytosine methylation reaction was performed by using the procedure of Kirkegaard et al. (1983). The DNase I and chemical modification reactions were performed with binding assays containing 4 nM ^{32}P *lac* promoter fragment and 180 nM RNA polymerase in the presence and absence of the indicated monoclonal antibody at a molar ratio of antibody to polymerase of 10 to 1. After a 15-min incubation at 37 °C, DNase I or dimethyl sulfate was added. Footprints were determined with both *lac* promoters by adding DNase I to 80 ng/mL while methylation of guanine and cytosine residues of the *lac* UV5 promoter was studied by incubation with dimethyl sulfate at a concentration of 50 mM for 1 min or 200 mM for 2 min, respectively. The DNase I reaction was stopped by the addition of 2 M ammonium acetate, 100 μg/mL tRNA, and 20 mM EDTA followed by phenol extraction, ethanol precipitation, and reprecipitation and drying. The DNA pellet was resuspended in formamide loading buffer and analyzed by electrophoresis on 8% denaturing sequencing gels according to Maxam and Gilbert (1980). The methylation reactions were terminated with 2 M ammonium acetate, 100 μg/mL tRNA, 20 mM EDTA, and 1 M 2-mercaptoethanol followed by ethanol precipitation. For the guanine methylation experiments, the DNA was then reprecipitated, washed, dried, and subjected to piperidine treatment before electrophoresis. To obtain preferential cleavage at N-3 methylcytosines, the dried DNA pellet was treated with hydrazine prior to reaction with piperidine (Kirkegaard et al., 1983). After electrophoresis, the gels were autoradiographed at −70 °C using Kodak XAR-5 film and a Dupont Cronex Hi-Plus intensifying screen.

Results

To ascertain the effects of the inhibitory mAb 210E8 on RNA polymerase–promoter interactions, the rates of abortive initiation on the *lac* and TAC16 promoters were examined in the presence and absence of antibody. The TAC16 promoter is a hybrid construct of the −35 consensus region of the *trp* promoter joined to the −10 consensus region of the *lac* UV5 promoter (Amann et al., 1983) that has been shown to initiate transcription with ApA, ApU, or UpG in the presence of UTP (Mulligan et al., 1985). The data presented in Table I show

Table I: Effect of mAb 210E8 on Abortive Initiation Directed by Supercoiled and Linear *lac* UV5 and TAC16 Promoters[a]

promoter	mAb 210E8	[^{3}H]UMP incorpd (pmol/pmol of promoter) s/c	linear
lac P$^+$	–	59	28
	+	48	2
lac UV5	–	55	48
	+	47	8
TAC16 (AUU)	–	96	36
	+	46	2
TAC16 (UGU)	–	37	14
	+	30	2

[a] Preincubation and reaction conditions are described under Materials and Methods. RNA polymerase (2 pmol) preincubated in the presence and absence of mAb 210E8 was incubated with promoter DNA (0.1 pmol of linear; 0.05 pmol of supercoiled) for 15 min at 37 °C. The substrates were added to give a final concentration of 0.5 mM ApA and 50 μM [^{3}H]UTP. The results are expressed as picomoles of [^{3}H]UMP incorporated per picomole of promoter per minute. s/c, supercoiled.

the effects of mAb 210E8 on abortive initiation from the *lac* P$^+$, *lac* UV5, and TAC16 promoters in their supercoiled and purified fragment forms. The extent of inhibition elicited by mAb 210E8 varied with the promoter assayed. The demonstration that the control rates of abortive initiation are independent of supercoiling for the *lac* UV5 promoter and are slightly lowered for linear *lac* P$^+$ is consistent with the earlier studies of Malan et al. (1984) on the effects of supercoiling on the *lac* promoter. The strong inhibition elicited by mAb 210E8 on the linear *lac* UV5 and TAC16 (UpG) promoters was not apparent when these promoters were assayed in their supercoiled forms. In contrast, the inhibition by mAb 210E8 of abortive synthesis on the linear TAC16 (ApU) promoter was lessened but still evident on a supercoiled template. In these experiments, an excess of mAb 210E8 was used; the molar ratio of mAb 210E8 to RNA polymerase was 20 to 1. Titration of mAb 210E8 with RNA polymerase indicated that maximal inhibition with the *lac* UV5 and TAC16 (ApU) promoters can be attained at a molar ratio of 1.4 mAb 210E8 per RNA polymerase (data not shown). This is consonant with the binding of mAb 210E8 to a single antigenic determinant on RNA polymerase.

The response of mAb 210E8–RNA polymerase to promoter strength was examined by using a quantitative analysis of the abortive initiation assay (McClure, 1980). The presence of mAb 210E8 elicited different effects on RNA polymerase activity when the lag time required for RP_o formation was measured with linear and supercoiled forms of the *lac* and TAC16 promoters. Typical lag time assays to measure the τ_{obsd} for linear and supercoiled *lac* UV5 templates are shown in Figure 1A,B. Similar plots were obtained for the linear versus supercoiled templates for the TAC16 (ApU) and TAC16 (UpG) promoters (data not shown). The lag time measurements obtained from these plots are presented in Table II. These results indicate 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 all three linear promoters 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 min) for each promoter assayed. For each analysis the slope values of the RNA polymerase initiated reaction were compared with those

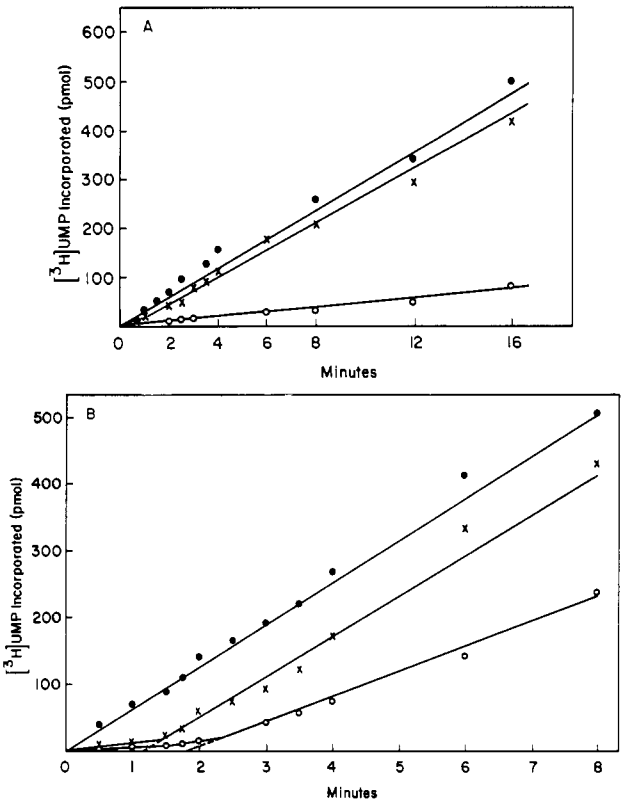

FIGURE 1: Effect of mAb 210E8 on the τ_{obsd} of the *lac* UV5 promoter. Each lag assay was performed as described under Materials and Methods. Table II gives the RNA polymerase concentration employed, the τ_{obsd} measurements, and the percent residual activities for each promoter assay. Plots are presented for τ_{obsd} measurements with (A) linear and (B) supercoiled promoters. 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 RNA polymerase–DNA complexes was initiated by the addition of substrates and two separate lag assays in which reaction mixtures containing DNA and substrates were initiated by the addition of RNA polymerase alone (×) and the mAb 210E8–RNA polymerase complex (○).

Table II: Effect of mAb 210E8 on τ_{obsd} Obtained with Supercoiled and Linear *lac* UV5 and TAC16 Promoters[a]

promoter	mAb 210E8	RNA polymerase (nM)	τ_{obsd} (s) (±SE)[b]	residual act.[c] (%)
lac UV5 (s/c)	–	25	66 ± 17*	
	+	25	107 ± 21*	96
lac UV5 (lin)	–	40	20 ± 17	
	+	40	nd[d]	17
TAC16 (AUU) (s/c)	–	13	8 ± 14	
	+	13	44 ± 8*	75
TAC16 (AUU) (lin)	–	40	34 ± 8*	
	+	40	nd	7
TAC16 (UGU) (s/c)	–	20	23 ± 19	
	+	20	36 ± 15*	90
TAC16 (UGU) (lin)	–	40	19 ± 27	
	+	40	nd	14

[a] The protocol employed is as described in the legend to Figure 1; the linear and supercoiled templates were included at 0.1 and 0.05 pmol, respectively. [b] Standard errors of τ_{obsd} were estimated by using the δ method modified from Rao (1952) by Rockwell et al. (1987). Note that those values marked with an asterisk differ significantly from 0 in that their lower 95% confidence limit is greater than 0. [c] Calculated from 1-h data points. The τ_{obsd} measurements were calculated by using a least-squares analysis as described under Materials and Methods. [d] nd, not determined; s/c, supercoiled; lin, linear.

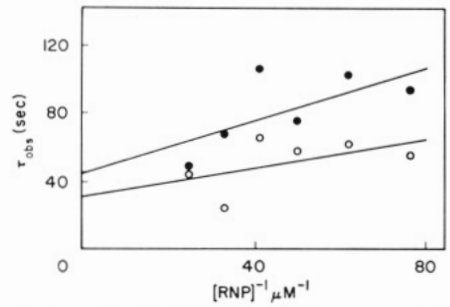

FIGURE 2: Effects of mAb 210E8 on the kinetics of RP_o formation on the supercoiled *lac* UV5 promoter. The protocol employed is described under Materials and Methods. The τ_{obsd} measurements were obtained from lag time assays performed at different RNA polymerase concentrations in the presence (●) and absence (○) of mAb 210E8. Linear regressions of these points are indicated as straight lines in the figure. Slopes, intercepts, and their respective standard errors are as follows: for the upper regression, slope = 0.79 ± 0.25 and intercept = 44.71 ± 12.98; for the lower regression, slope = 0.42 ± 0.20 and intercept = 31.20 ± 10.16. These estimates are used to calculate K_B and k_2. The standard errors of the plotted means are from left to right: 0.80, 0.95, 3.52, 0.86, and 3.21 for the control (○) and 1.05, 1.93, 5.73, 3.35, 5.61, and 5.68 for the measurements obtained in the presence of mAb 210E8 (●). In the absence of mAb 210E8, k_2 was 0.032 s^{-1} and K_B was 1.4×10^{-8} M^{-1}. In the presence of mAb 210E8, k_2 was 0.022 s^{-1} and K_B was 1.7×10^{-8} M^{-1}. Each data point is the mean of three independent experiments.

values obtained for a steady-state control and the RNA polymerase initiated reaction in the presence of mAb 210E8. According to these criteria, only the slope values for the mAb 210E8–RNA polymerase initiated reactions on linear templates were found to differ significantly ($p \leq 0.05$) from the control values. Even after 3-h 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 with the supercoiled promoters by mAb 210E8–RNA polymerase eventually reached steady-state rates which were not significantly different from the controls. The lack of inhibition observed in the reactions directed by supercoiled DNA is not a consequence of the dissociation of the mAb 210E8–RNA polymerase complex. Electrophoresis on 0.5% agarose gels showed that on addition of goat anti-mouse IgG peroxidase, the mAb 210E8–RNA polymerase–pMB9 (*lac* UV5) supercoiled DNA complex formed an immune aggregate which did not migrate out of the loading well; staining with ethidium bromide indicated that migration of the supercoiled DNA was prevented only in the presence of mAb 210E8–RNA polymerase; similar results were obtained with the noninhibitory mAb 221C7 (data not shown).

A τ plot showing the effects of mAb 210E8 on the rate of RP_o formation with the supercoiled *lac* UV5 promoter is presented in Figure 2. In the presence of mAb 210E8, the isomerization constant, k_2, was reduced by approximately 30% with little effect on the binding (K_B) of RNA polymerase to promoter. The control τ values obtained in this study are intermediate between those obtained by Malan et al. (1984) using a fully supercoiled *lac* UV5 insert. This probably reflects the fact that the method we used for plasmid isolation yields a small percentage of open circular forms of plasmid (Marko et al., 1982).

The kinetics of ApApUpU synthesis with linear and supercoiled *lac* UV5 promoters are shown in Table III. Inhibition by mAb 210E8 is noncompetitive with regard to primer concentration with the linear *lac* UV5 promoter. The V_{max} is greatly decreased while the apparent increase in K_m results in a value which falls within the limits of the K_m determined for the control. Under the same conditions, the kinetics of ApApUpU synthesis with the supercoiled *lac* UV5 promoter indicate that mAb 210E8 had little effect on both the affinity of RNA polymerase for substrate (K_m value) and the rate of the reaction (V_{max}) when compared to control values.

Table III: Effect of mAb 210E8 on the Kinetic Constants for Abortive Initiation on Linear and Supercoiled *lac* UV5 Promoters[a]

promoter	mAb 210E8	K_m (μM)	V_{max} (pmol/min)	residual act.[b] (%)
lac UV5 (s/c)	–	82 ± 35	100 ± 9.5	100
	+	136 ± 9	89 ± 11.7	85
lac UV5 (linear)	–	242 ± 35	20 ± 2.7	100
	+	424 ± 152	2 ± 0.8	10

[a] Preincubation and reaction conditions are described under Materials and Methods. The ApA concentration was varied from 0.2 to 2 mM in the presence of 50 μM [^{3}H]UTP. The RNA polymerase concentration was 2 pmol, and linear or supercoiled *lac* UV5 was included at 0.1 or 0.05 pmol, respectively. RNA polymerase preincubated in the presence or absence of mAb 210E8 was combined with DNA and incubated for 15 min at 37 °C. Substrates were then added, and the incubation was continued for an additional 5 min. The K_m and V_{max} values were obtained from slopes and intercepts estimated with least-squares analyses of double-reciprocal plots of 1/velocity (picomoles per minute) versus 1/ApA concentration. Standard errors were estimated by using the method indicated in Table II. [b] Data taken from highest titration point; s/c, supercoiled.

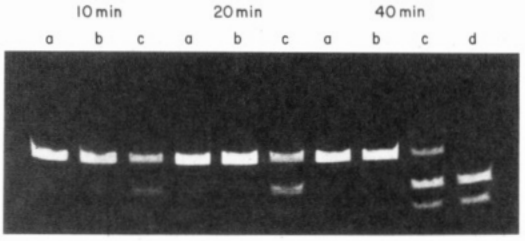

FIGURE 3: Protection by RNA polymerase over time of the *Hpa*II site of the *lac* UV5 promoter in the presence and absence of the inhibitory mAb 210E8 and the noninhibitory mAb 221C7. The binding conditions are as described under Materials and Methods. The reactions and their times of incubation are as indicated above each lane: a, control RNA polymerase; b, mAb 221C7–RNA polymerase; c, mAb 210E8–RNA polymerase; d, unprotected *lac* UV5.

The effect on RNA polymerase–promoter stability was examined by performing DNA protection studies with the RNA polymerase–*lac* UV5 complex in the presence and absence of the inhibitory mAb 210E8 and noninhibitory anti-β mAb 221C7. In this experiment, the accessibility of the *Hpa*II site (position −19) of the linear *lac* UV5 promoter to cleavage was examined after challenge with $d(A\text{-}T)_n$ (Figure 3). This restriction site occurs within a promoter region which interacts with RNA polymerase during RP_o formation (Kirkegaard et al., 1983; Siebenlist et al., 1980). RNA polymerase–promoter complexes formed in the presence and absence of the noninhibitory mAb served as controls. The *Hpa*II site was completely protected in the control RNA polymerase or mAb 221C7–RNA polymerase–*lac* UV5 complex while a slow cleavage at this site was noted with the mAb 210E8–RNA polymerase–*lac* UV5 complex. The reduced stability of the complex formed in the presence of mAb 210E8 was less pronounced when $d(A\text{-}T)_n$ was omitted.

The effect of mAb 210E8 on RNA polymerase interactions at promoter-specific contacts during the binding process was assessed by probing the phosphodiester backbone of the DNA with DNase I and the base residues with dimethyl sulfate. The results of the DNase I footprint analysis showed that the binding of RNA polymerase to the *lac* P^+ promoter in the presence of mAb 210E8 gave a pattern which differed only

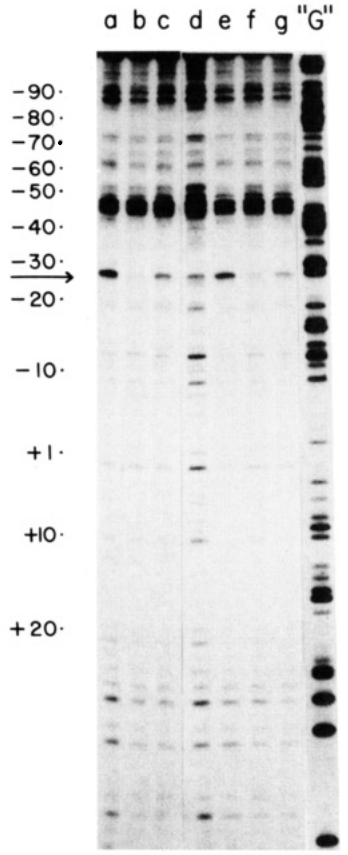

FIGURE 4: Effect of mAb 210E8 on the protection by RNA polymerase of the *lac* P^+ promoter against attack by DNase I. Conditions are described uner Materials and Methods using 4 nM 5′-^{32}P end-labeled (lower) strand of *lac* P^+ promoter, 180 nM RNA polymerase, 20 nM CRP, and 100 μM cAMP. The mAb 210E8 induced difference in the normal reactivity pattern is indicated by an arrow. Lanes a and e, no mAb; lanes b and f, mAb 210E8–RNA polymerase; lanes c and g, mAb 221C7–RNA polymerase; lane d, without RNA polymerase. Lanes e–g show that the DNase I patterns of RNA polymerase–cAMP–CRP–*lac* P^+ complexes formed are not altered when the abortive substrates, ApA + UTP, are included in the binding assay. Lane "G" is a guanine methylation pattern of the unprotected *lac* P^+ promoter.

slightly from the control patterns obtained in the absence of antibody or in the presence of the noninhibitory mAb 221C7. However, the enhancement of the band located between positions −20 and −30 when RNA polymerase binds to the *lac* P^+ promoter was consistently found to be suppressed in the presence of mAb 210E8 (Figure 4). The results presented in Figure 4 also show that the DNase I footprint was not appreciably changed when initiation complexes were formed on addition of ApA plus UTP.

Chemical modification studies with dimethyl sulfate revealed altered methylation patterns for the mAb 210E8–RNA polymerase–*lac* UV5 complex (Figure 5A). Significant enhancement and protection patterns for guanine contacts on the control RNA polymerase–promoter complex were consistent with the previous results of Siebenlist et al. (1980). On the template strand, the guanine residues at the −32- and −14-positions were found to be protected and enhanced, respectively. On the nontemplate strand, the guanine residues at the −6-, −13-, and −24-positions were protected against methylation while the guanine residues at the −1-, −17-, and −38-positions were more accessible to methylation. The mAb 210E8–RNA polymerase–promoter complexes displayed a dimethyl sulfate reactivity pattern which was intermediate with those obtained with the control RNA polymerase–promoter complex and the unprotected promoter fragment. With the mAb 210E8–RNA polymerase, the extent of protection of the guanine at position −32 and the enhancement of the guanine at position −14 of the template strand were always less than the reactivities obtained for the controls. Overexposure of the gel from this experiment revealed the protection by mAb 210E8–RNA polymerase of an adenine at position −36 on the template strand and the cytosines located downstream of the −10 region (Figure 5B). When compared to the control lanes, these patterns are only observed in the absence of RNA polymerase.

The effect of mAb 210E8 was further examined with regard to the reactivity pattern of the cytosines at the −6-, −4-, −2-, and −1-positions of the template strand of the RNA polymerase–*lac* UV5 complex (Figure 6). This approach was employed to detect unpaired cytosine residues within a region of the *lac* UV5 promoter that is known to be unwound by RNA polymerase binding (Kirkegaard et al., 1983). With this procedure, the N-3 positions of cytosines normally engaged in hydrogen bond formation in duplex DNA are methylated in the unpaired state. Subsequent hydrazine treatment of the modified DNA permits detection by preferential chain cleavage at the N-3 methylcytosines. The results in Figure 6 show that the methylation of unpaired cytosines during RP_o formation on *lac* UV5 could not be detected in the presence of mAb 210E8. Conversely, the chemical modification of the single-stranded region induced by RNA polymerase binding is clearly indicated for the complexes formed with RNA polymerase alone or with the noninhibitory mAb 221C7. The methylation patterns obtained in this study do not appear to be a reagent-induced effect since a DNase I footprint of complexes treated with dimethyl sulfate indicated that the conditions used for methylation did not appreciably alter RNA polymerase–DNA contacts in the presence or absence of antibody.

DISCUSSION

The monoclonal antibody used in this study was previously 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. Kinetic analysis showed that the inhibition by mAb 210E8 was competitive for UpA incorporation but noncompetitive with regard to UTP (Rockwell et al., 1985). In the present study, the effects of this anti-β monoclonal antibody on the steps leading to the formation of the open promoter complex have been characterized.

The data presented for the linear and superhelical forms of the *lac* and TAC promoters suggest that the inhibition of abortive synthesis in the presence of mAb 210E8 appears to correlate with changes in DNA topology. The greater inhibition of abortive synthesis with the linear promoters indicates that transcription by the mAb 210E8–RNA polymerase complex is favored on supercoiled templates. The mAb 210E8 induced hindrance of RNA polymerase activity on a linear promoter is demonstrated by the lag time studies. In the presence of mAb 210E8, abortive initiation on linear *lac* UV5 and TAC16 promoters proceeds at exceedingly slow rates. The τ_{obsd} measurements on linear promoters 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 in control RNA polymerase reactions.

In contrast, the kinetics of abortive initiation by mAb 210E8–RNA polymerase on the supercoiled *lac* UV5 promoter approached steady-state rates that paralleled those of the control. The τ plot obtained with the supercoiled *lac* UV5

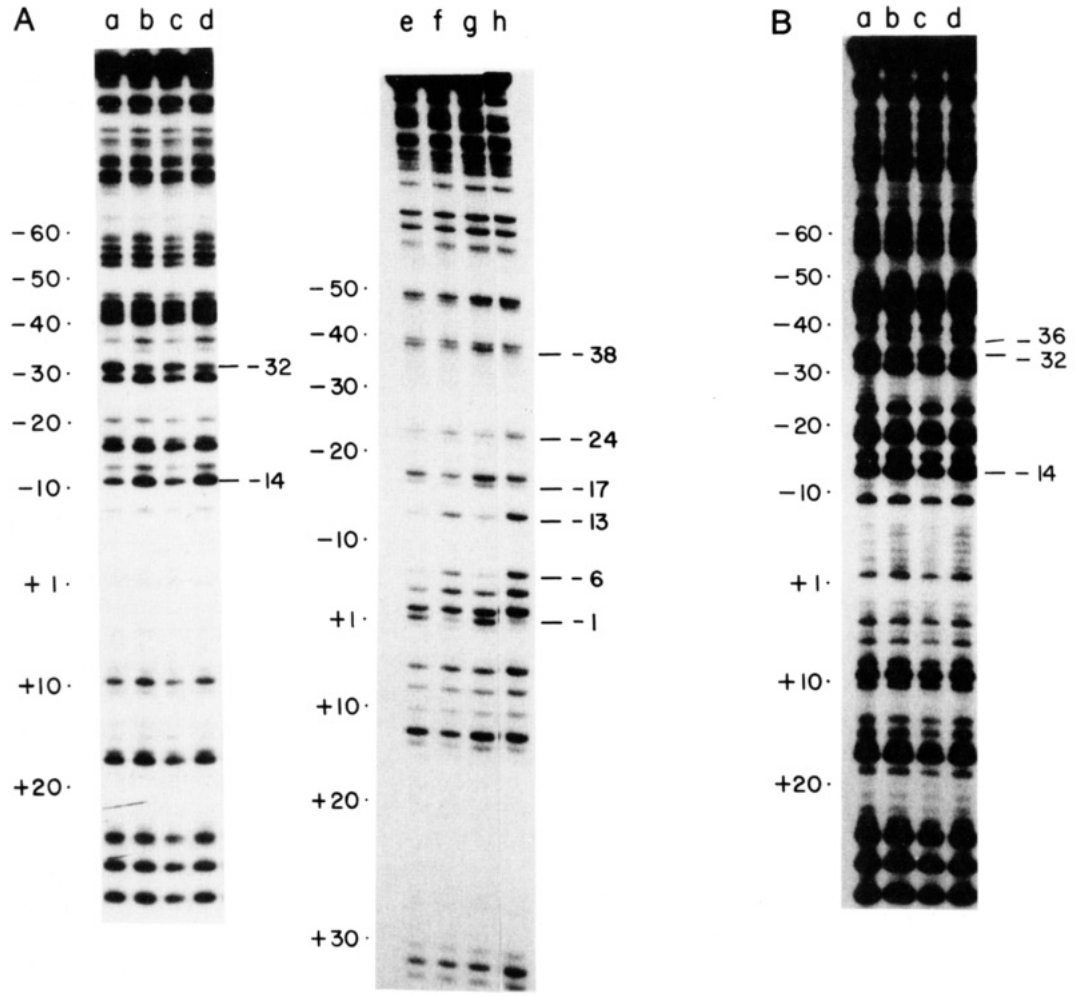

FIGURE 5: (A) Effect of mAb 210E8 on the reactivity of base residues of the RNA polymerase–*lac* UV5 promoter complex to methylation by dimethyl sulfate. Reaction conditions are described under Materials and Methods using 4 nM 5′ (template) and 3′ (nontemplate) ^{32}P-labeled *lac* UV5 promoter fragments and 180 nM RNA polymerase. Panel A: Template (lower) strand, a–d; nontemplate (upper) strand, e–h. Reactivity patterns are shown for *lac* UV5 complexes formed with (lanes a and h) the unprotected fragment, (lanes b and e) RNA polymerase alone, (lanes c and f) mAb 210E8–RNA polymerase, and (lanes d and g) mAb 221C7–RNA polymerase. Panel B: Overexposure of the methylation pattern for the template strand presented in panel A, a–d.

insert indicated that mAb 210E8 exerts only a slight decrease in the rate of isomerization from the closed to the open promoter complex without affecting the initial rate of promoter binding. The difference in the rates of abortive initiation on linear and supercoiled *lac* UV5 indicates that supercoiling alleviated a constraint on promoter utilization by the mAb 210E8–RNA polymerase complex. Kinetic measurements indicate that stable binding of RNA polymerase to a negatively coiled DNA is due to an enhanced rate of association and a reduced rate of dissociation (Wang, 1982). 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, for *lac* UV5 is greatly increased on a supercoiled promoter while the rate of isomerization, k_2, is decreased (Malan et al., 1984). The similar K_B value obtained 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 minor change in the k_2 value seen for the mAb 210E8–RNA polymerase implies that the presence of antibody may exert some 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). On the basis of analyses of the properties of complexes formed between RNA polymerase and *lac* UV5 fragment, an additional intermediate has been identified. This intermediate species, RP_i, has been characterized by kinetic (Buc & McClure, 1985) and footprint (Spassky et al., 1985) analysis. These authors have proposed Scheme II in which the formation of RP_i is the rate-limiting step.

Scheme II

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

Spassky et al. (1985) suggest that the formation of RP_i involves the initial positioning of RNA polymerase with the −35 and −10 regions of the promoter. At 37 °C, this is immediately followed by a rapid isomerization to form RP_o. They also suggest that the transition from RP_i to RP_o involves a cooperative conformational transition of enzyme and DNA in the binary complex. The appropriate contacts between amino acid chains and DNA sites are then established, and the consequent unwinding of the DNA around position +1 occurs to yield the active RP_o complex. Buc and McClure (1985) also indicate that in the case of a superhelical promoter the system is driven toward formation of the open complex under conditions which do not favor a transient RP_i complex. The effect of mAb 210E8 on abortive initiation on linear and supercoiled promoters suggests that RNA polymerase–promoter interactions vary with DNA topology. The data indicate that supercoiling appears to compensate for the inhibitory

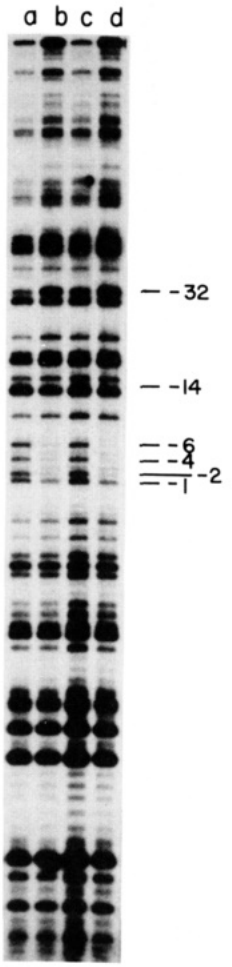

FIGURE 6: Effect of the inhibitory mAb 210E8 on the methylation of single-strand-specific cytosines of RNA polymerase–*lac* UV5 promoter complexes. Reaction conditions are described under Materials and Methods using 4 nM 5′ end-labeled template (lower) strand of *lac* UV5 and 180 nM RNA polymerase. Cytosine reactivity patterns are shown for *lac* UV5 complexes formed with (lane a) RNA polymerase alone, (lane b) mAb 210E8–RNA polymerase, (lane c) mAb 221C7–RNA polymerase, and (lane d) the unprotected fragment.

effect of mAb 210E8 by allowing the conversion of the mAb 210E8–RNA polymerase–*lac* UV5 promoter complex to an active open promoter complex.

The DNase I footprints of mAb 210E8–RNA polymerase–*lac* promoter complexes suggest that the positioning of RNA polymerase on the DNA is not greatly altered by the inhibitory antibody. The suppression of the enhancement between positions −20 and −30 does indicate a partial loss of a significant binding contact within the *lac* P^+ promoter. However, the obvious difference in the reactivity of mAb 210E8–RNA polymerase–*lac* UV5 complexes to methylation by dimethyl sulfate indicated an alternate mode of RNA polymerase binding to the *lac* promoter. The mAb 210E8–RNA polymerase appears to interact with essential guanine residues on both the template and nontemplate strands of the promoter in a form which is intermediate between fully protected and unprotected. The inability to methylate cytosine residues at positions −6, −4, −2, and −1 on the template strand indicates that the mAb 210E8–RNA polymerase–promoter complex more closely resembles a closed promoter complex. This failure to detect single-strand-specific cytosines in the mAb 210E8–RNA polymerase–*lac* UV5 complex suggests that the antibody hinders the RNA polymerase mediated unpairing of specific base pairs and thus the transition from a "closed" to a catalytically active "open" promoter complex. That the mAb 210E8–RNA polymerase does not form a stable RP_o was also indicated by the slow cleavage of the *Hpa*II site of the *lac* UV5 promoter. The antibody appears to trap the RNA polymerase in a conformation that hinders the final conversion to a stable RP_o. The consistent low level of abortive synthesis with linear promoters suggests that the transformation of the mAb 210E8–RNA polymerase–promoter complex to the open complex is not favored.

According to the model for RP_o formation cited above, the conversion of RP_c to RP_o on the linear *lac* UV5 promoter involves 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 the 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 and temperature-dependent complex forms prior to RP_o formation (Buc & McClure, 1985). This RP_i was unable to initiate but was stable to dissociation by $d(A\text{-}T)_n$. The mAb 210E8 mediated RP_i proposed in the present study represents a different form of intermediate promoter complex which exhibits a low rate of product formation at 37 °C and is slowly dissociated by $d(A\text{-}T)_n$. Assuming that the inhibition effected by mAb 210E8 is mediated through a conformational effect on RNA polymerase in the RP_i complex, we would propose that nonoptimal contacts are established with the linear promoters. This 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.

The demonstration that supercoiling compensates for the effect of mAb 210E8 was complicated by the mAb 210E8 induced hindrance of ApUpU but not UpGpU synthesis on the supercoiled TAC16 promoter 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. It has been postulated from previous studies (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 & Gralla, 1982c) and the P22 phage promoter from *Salmonella typhimurium* (Grana et al., 1985) have shown that promoter activity is sensitive to changes in spacer length. On the basis of 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.

Complexes of mAb 210E8–RNA 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 supercoiled TAC16 (ApU) promoter insert. The lack of inhibition with 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–RNA polymerase complex. On the TAC16 promoter, the presence of two 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 different effects of mAb 210E8 for the *lac* UV5

and the TAC16 (ApU) inserts may also reflect differences in spacer length or an alternate interaction in the −35 region. These data may also reflect the demonstration by Kirkegaard et al. (1983) that certain base contacts downstream from the −10 region of the *lac* UV5 and TAC16 promoters display different patterns of interaction with RNA polymerase during RP_o formation.

The results of the structural and kinetic analyses imply that the mAb 210E8 is probing a polymerase interaction that can be modulated by both supercoiling and changes in the spacer length. 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 mAb-induced hindrance of either the simultaneous alignment of RNA polymerase with the consensus regions of a promoter, the topological unwinding of the DNA, or the subsequent melting of the −10 region. The data also suggest a close involvement of the β subunit in the positioning of polymerase preceding RP_o formation. In a previous study, it was shown that mAb 210E8 partially inhibited both initiation and elongation in reactions directed by $d(A\text{-}T)_n$ (Rockwell et al., 1985). The effect of mAb 210E8 on elongation may be related to that demonstrated for initiation in that both activities involve a local unwinding of the DNA helix.

Considering the large size of an immunoglobulin, the mAb 210E8 induced hindrance of the correct alignment of RNA polymerase with promoter contacts could render certain amino acid residues inaccessible to essential base contacts during the transition to the tight interaction required for RP_o. Binding of the large antibody to RNA polymerase may impede RP_o formation directly by steric hindrance or indirectly by altering an essential promoter interaction at RNA 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 a function of DNA topology. The added constraint by mAb 210E8 on the spacer length of the supercoiled TAC16 promoter is a further demonstration that mAb 210E8 may be probing polymerase–promoter interactions that are modulated by factors that are known to affect promoter strength.

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Registry No. ApA, 2391-46-0; RNA polymerase, 9014-24-8.

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