Effects of an Anti-α Monoclonal Antibody on Interaction of *Escherichia coli* RNA Polymerase with *lac* Promoters†

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ABSTRACT: The anti-α monoclonal antibody, mAb 126C6, has been used to investigate the role of the α subunit in transcription initiation. mAb 126C6 strongly inhibits cAMP–CRP-dependent abortive initiation with *lac* P^+, partially inhibits abortive initiation with the *lac* L8UV5 promoter, and is without effect on the $d(A\text{-}T)_n$-directed synthesis of $r(A\text{-}U)_n$. DNase I footprinting shows that the preformed mAb 126C6–RNA polymerase complex does not bind to cAMP–CRP–*lac* P^+; RNA polymerase specific protection is largely lost after incubation of the preformed RP_o with mAb 126C6. Kinetic analysis of open complex formation by mAb 126C6–RNA polymerase with *lac* L8UV5 showed that changes in both the binding and the rate of isomerization account for the observed inhibition, with the isomerization step affected to a greater extent. Binding of cAMP–CRP to *lac* L8UV5 is RNA polymerase dependent. DNase I footprints show that as a consequence of mAb 126C6 binding of the preformed cAMP–CRP–*lac* L8UV5–RNA polymerase RP_o, CRP dissociates from its site on the promoter. RNA polymerase protection of the promoter upstream from −41 is also lost. DNase I footprinting of mAb 126C6–RNA polymerase complexed with cAMP–CRP–*lac* P^+ or −*lac* L8UV5 suggests that interactions between CRP and RNA polymerase are affected by binding of the anti-α mAb 126C6 to RNA polymerase. Protection methylation studies demonstrate that the formation of the mAb 126C6–RNA polymerase–*lac* L8UV5 open complex occurs at a slower rate and that nonoptimal contacts are established between mAb 126C6–RNA polymerase–*lac* L8UV5 promoter. After incubation of the preformed mAb 126C6–RNA polymerase with cAMP–CRP–*lac* P^+ the cytosines near the transcription start site remain inaccessible to methylation. After incubation of the preformed cAMP–CRP–*lac* P^+–RNA polymerase open complex with mAb 126C6 the cytosines near the start site remain unpaired.

The bacterial RNA polymerases are complex multisubunit enzymes. The RNA polymerase (EC 2.7.7.6) from *Escherichia coli* comprises four different subunits; the holoenzyme active during steps in initiation of transcription has the structure $\alpha_2\beta\beta'\sigma$. Although considerable information is available regarding the structure of the RNA polymerase subunits (Burgess et al., 1987) less is known regarding the role of each of the subunits. The β subunit is involved in the catalytic activity of the enzyme (Lisitsyn et al., 1988). Rifampicin and streptolydigin resistance has been mapped within the β subunit. The involvement of the σ subunit in promoter recognition has been clearly defined (Reznikoff et al., 1985). Less is known regarding the role played by the β' and α subunits in RNA polymerase. The involvement of the β' subunit in template binding and interaction with the σ subunit has been suggested (Kumar, 1981). The requirement for ADP ribosylation of the α subunit during T4 phage infection of *E. coli* suggests a possible role of this subunit in T4-specific transcription (Goldfarb & Palm, 1981). The α subunit has been assigned a role in the assembly of the RNA polymerase core enzyme (Ishihama, 1981). Monoclonal anti-α antibodies have been used to study the properties of the α subunit of the *E. coli* RNA polymerase (Riftina et al., 1989). Reassembly of the RNA polymerase core is blocked by two of the anti-α monoclonal antibodies studied. The α domain in which the epitope for mAb 126C6 residues is not impeded by subunit interactions in the RNA polymerase. The data obtained also suggest that in the holoenzyme the σ subunit may be positioned close to one of the α subunits. The affinity of the inhibitory mAb 126C6 for assembled α is greater than for free α, indicating that this subunit undergoes conformational changes during RNA polymerase assembly.

In the present study the inhibitory anti-α monoclonal antibody, mAb 126C6, has been used to investigate the possible role of the α subunit during transcription initiation.

Materials and Methods

Materials. Reagents were obtained as follows: cAMP, ApA,[1] Sigma Chemical Co.; $d(A\text{-}T)_n$, ribonucleoside triphosphates, Pharmacia; [^{3}H]UTP, [γ-^{32}P]ATP, and [α-^{32}P]dATP, ICN; T4 polynucleotide kinase, alkaline phosphatase, DNA polymerase I Klenow fragment, and restriction endonucleases, Boehringer Mannheim; DNase I, Cooper Biochemical; urea, Schwarz-Mann; Dimethyl sulfate, piperidine, and hydrazine, Aldrich; formamide, Amresco; acrylamide, Serva; Scintisol, Isolab.

Preparation of RNA Polymerase and Antibody. RNA polymerase was purified from *E. coli* K12 cells by a modification of the procedure of Burgess and Jendrisak (1975). Holoenzyme was isolated by chromatography on denatured calf thymus DNA–agarose as described by Lowe et al. (1979). RNA polymerase holoenzyme concentration was determined by using the extinction coefficient $E^{1\%}_{280nm} = 6.7$ (Levine et al., 1980). The anti-α monoclonal antibody, mAb 126C6, was prepared as indicated in Rockwell et al. (1985). Immuno-

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[1] Abbreviations: CRP, cAMP receptor protein; mAb, monoclonal antibody; RP_o open promoter complex; RP_i, intermediate promoter complex; RP_c, closed promoter complex; ApA, adenylyl(3′–5′)adenosine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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globulin concentration was determined by using the extinction coefficient $E^{1\%}_{280nm}$ = 14.6 (Ey et al., 1978). CRP was purified by the method of Eilen et al. (1978) and 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 (Fuller, 1982) were obtained from Dr. A. Revzin (Michigan State University, East Lansing). Plasmids were isolated essentially as presented in Rockwell and Krakow (1988).

The 3′- and 5′-[^{32}P]*lac* P$^+$ or [^{32}P]*lac* L8UV5 promoter fragments were prepared by labeling with [α-^{32}P]dATP using the DNA polymerase Klenow fragment or with [γ-^{32}P]ATP by T4 polynucleotide kinase. The labeled fragments were then restricted with *Pvu*II, which cuts the DNA at −123 yielding promoter fragments uniquely labeled on either the upper or lower strand.

Assay of d(A-T)$_n$-Directed r(A-U)$_n$ Synthesis. The mAb 126C6–RNA polymerase complex was formed by incubation of 2 pmol of RNA polymerase holoenzyme with 20 pmol of mAb 126C6 for 30 min at 37 °C. After addition of 30 nmol of d(A-T)$_n$, the mixture was incubated for 10 min at 37 °C. Synthesis of r(A-U)$_n$ was carried out in a reaction mixture (90 μL) that contained 40 mM Tris-HCl (pH 8.0), 13 mM potassium phosphate (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 400 nmol of ATP, 100 nmol of [^{3}H]UTP (44 000 cpm/nmol), and 2.5% glycerol. After incubation at 37 °C for 20 min, the r(A-U)$_n$ was precipitated with 5% trichloroacetic acid, collected on glass fiber filters (Whatman GFC), and counted in Scintisol.

Abortive Initiation Assays. A modification (Rockwell & Krakow, 1988) of the abortive initiation assay of Malan et al. (1984) was used to determine the effect of the monoclonal antibody on transcription from the *lac* L8UV5 and *lac* P$^+$ promoters. The reaction mixture (final volume, 50 μL) contained the following: 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5% glycerol, 3 nM *lac* P$^+$ (plus 40 nM CRP and 0.1 mM cAMP) or *lac* L8UV5 DNA fragment, 50 nM RNA polymerase, and 250 nM mAb 126C6 unless otherwise indicated in the legends. Monoclonal antibody–polymerase complexes were formed by incubation of the antibody with RNA polymerase for 30 min at 37 °C or for 1 h on ice. After an additional preincubation with the template for 10 min at 37 °C, 1 mM ApA and 50 μM [^{3}H]UTP (190 cpm/pmol) were added. The reaction was allowed to proceed at 37 °C for the time indicated and was then terminated by addition of 10 μL of 0.5 M EDTA. The radioactive products were resolved by ascending paper chromatography in WASP solvent (McClure, 1980). The amount of ApApUpU synthesized was estimated by determining the radioactivity of appropriate 1-cm segments in Scintisol.

Lag Time Assays. The procedure of McClure (1980) was used to determine the binding (K_B) and isomerization (k_2) constants for mAb 126C6–RNA polymerase directed by *lac* L8UV5. Two protocols differing in the initiation step were employed. Steady-state control values were obtained by preincubating RNA polymerase with the promoter fragment for 15 min at 37 °C to allow for the formation of the RP_o. The reaction was initiated by addition of a mixture of ApA and [^{3}H]UTP and 50-μL aliquots were taken for paper chromatography at the times indicated. Lag time measurements in the presence and absence of mAb 126C6 were obtained by preincubating the mixture of template and substrates at 37 °C and then initiating transcription by addition of RNA polymerase or mAb 126C6–RNA polymerase complex preincubated at 37 °C. Aliquots (50 μL) were removed at the times indicated and the reaction products were resolved as described above.

Table I: Effect of Anti-α mAb 126C6 on Reactions Directed by d(A-T)$_n$ and *lac* Promoters[a]

	% residual activity[b]		
	lac P$^+$	*lac* L8UV5	d(A-T)$_n$
mAb 126C6	7[c]	42	95

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

DNase I Footprinting, Guanine and Cytosine Methylation. The reactions were carried out essentially as presented in Rockwell and Krakow (1988). The standard binding mixtures contained (final volume, 50 μL) the following: 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5% glycerol, 3 nM 3′- or 5′-[^{32}P]*lac* fragment, and where indicated 50 nM RNA polymerase, 40 nM CRP, 0.1 mM cAMP, and 250 nM mAb 126C6. Monoclonal antibody–polymerase complexes were formed by incubation of mAb 126C6 with RNA polymerase for 30 min at 37 °C or for 1 h on ice. The times of incubation at 37 °C are given in the figure legends.

Results

Shown in Table I are the effects of the anti-α monoclonal antibody used in this study on reactions directed by d(A-T)$_n$ and the *lac* P$^+$ and *lac* L8UV5 promoters. The mAb 126C6 raised against the purified α subunit contains G2b heavy chains and κ light chains (Riftina et al., 1989). Specificity of mAb 126C6 for α was verified by immunoblotting using RNA polymerase holoenzyme resolved by SDS–polyacrylamide gel electrophoresis and by solid-phase ELISA using purified α, β, and β′ subunits (data not shown). The data presented in Table I show that mAb 126C6 does not inhibit the d(A-T)$_n$-directed synthesis of r(A-U)$_n$, strongly inhibits cAMP–CRP-dependent initiation with *lac* P$^+$, and to a lesser extent inhibits initiation with the *lac* L8UV5 promoter. The data indicate that reactions involving a functional promoter are more sensitive to inhibition by mAb 126C6 than is the reaction directed by d(A-T)$_n$.

Formation of the RP_o with the *lac* P$^+$ promoter is CRP-dependent. The DNase I footprint of the RP_o formed with RNA polymerase, CRP, and the 3′-^{32}P end-labeled (nontemplate) strand of the *lac* P$^+$ promoter shows a pattern of interactions spanning −70 to +20 on the DNA (Figure 1, lane d). When mAb 126C6–RNA polymerase is incubated with the preformed CRP–*lac* P$^+$ complex, binding by RNA polymerase is not observed (Figure 1, lane h). RNA polymerase specific protection of the *lac* P$^+$ promoter is lost after a 10-min incubation of the preformed RP_o with mAb 126C6 (Figure 1, lane e). The enhancements seen in the region between −20 and −25 persist even after a 40-min incubation of the RP_o with mAb 126C6, suggesting that RNA polymerase has not completely dissociated from the promoter (Figure 1, lane g). The footprint after 40 min is similar to that seen for RNA polymerase binding to *lac* P$^+$ in the absence of CRP. It seems unlikely that this is due to the binding of mAb 126C6–RNA polymerase to the *lac* P2 site, since the CRP present at its site

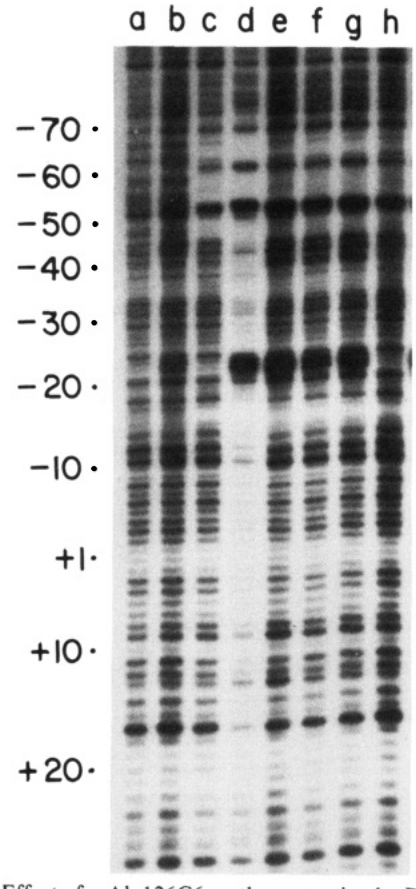

FIGURE 1: Effect of mAb 126C6 on the protection by RNA polymerase of the 3′-[^{32}P]*lac* P$^+$ promoter (nontemplate strand) against attack by DNase I. Conditions are described under Materials and Methods with 3 nM 3′-^{32}P end-labeled (nontemplate) strand of *lac* P$^+$ promoter, 50 nM RNA polymerase, 40 nM CRP, and 100 μM cAMP. Lane a, [^{32}P]*lac* P$^+$ only; lane b, RNA polymerase; lane c, cAMP–CRP; lane d, RP_o; lane e, RP_o incubated with mAb 126C6 for 10 min at 37 °C; lane f, RP_o incubated with mAb 126C6 for 20 min at 37 °C; lane g, RP_o incubated with mAb 126C6 for 40 min at 37 °C; lane h, mAb 126C6–RNA polymerase incubated with cAMP–CRP–[^{32}P]*lac* P$^+$ for 30 min at 37 °C.

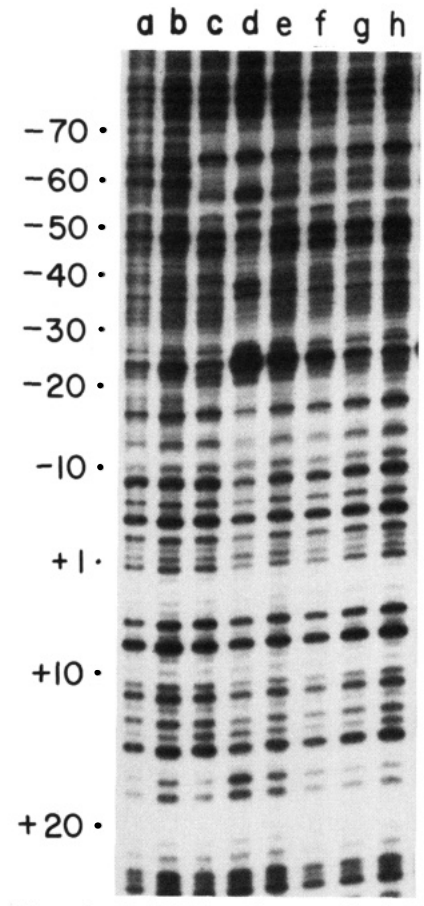

FIGURE 2: Effect of mAb 126C6 on the protection by RNA polymerase of the 5′-[^{32}P]*lac* P$^+$ promoter (template strand) against attack by DNase I. Conditions are described under Materials and Methods with 3 nM 5′-^{32}P end-labeled (template) strand of *lac* P$^+$ promoter, 50 nM RNA polymerase, 40 nM CRP, and 100 μM cAMP. Lane a, [^{32}P]*lac* P$^+$ only; lane b, RNA polymerase; lane c, cAMP–CRP; lane d, RP_o; lane e, RP_o incubated with mAb 126C6 for 10 min at 37 °C; lane f, RP_o incubated with mAb 126C6 for 20 min at 37 °C; lane g, RP_o incubated with mAb 126C6 for 40 min at 37 °C; lane h, mAb 126C6–polymerase incubated with cAMP–CRP–[^{32}P]*lac* P$^+$ for 30 min at 37 °C.

would interfere with binding of RNA polymerase to the P2 site.

As observed with the 3′-[^{32}P]*lac* P$^+$ lower (nontemplate) strand, the preformed mAb 126C6–RNA polymerase complex also does not yield a defined footprint with the 5′-[^{32}P]*lac* P$^+$ upper (template) strand complexed with CRP (Figure 2, lane h). However, the RP_o formed with the labeled template strand appears to be more sensitive to incubation with mAb 126C6. The enhancement at −38 is lost by 10 min of incubation of the RP_o with mAb 126C6 while that at −25 decreases until its disappearance by 40 min. RNA polymerase appears to associate more strongly with the 3′-^{32}P-labeled nontemplate strand. Effects of the anti-α mAb 126C6 are also seen in the CRP-binding domain of the template strand of the *lac* promoter, implying that interactions between RNA polymerase and CRP are affected. The enhancement at −52 and −54 is reduced to that seen with cAMP–CRP in the absence of RNA polymerase and the protection at −57 and −58 is decreased (Figure 2, lanes e–g).

The rate-determining step for initiation is the formation of the open promoter complex (RP_o). Kinetic analysis of abortive initiation allows for an evaluation of the binding and isomerization constants during formation of the open promoter complex (McClure, 1980). A steady-state rate of ApApUpU synthesis is reached by the mAb 126C6–RNA polymerase–*lac* L8UV5 complex after a lag of 15 min with 50 nM RNA polymerase; the rate of incorporation approaches (90%) that obtained in the absence of the antibody (data not shown). The data presented in Table II indicate that the isomerization rate (k_2) is more sensitive to inhibition by mAb 126C6 than is promoter binding (K_B).

Table II: Effects of mAb 126C6 on the Kinetics of RP_o Formation on the *lac* L8UV5 Promoter[a]

	K_B, μM^{-1}	k_2, s^{-1}
RNA polymerase	431 ± 65	0.033 ± 0.005
mAb 126C6–RNA polymerase	195 ± 19	0.0076 ± 0.0008

[a] The protocol employed is described under Materials and Methods. RNA polymerase was incubated with mAb 126C6 for 30 min at 37 °C. The molar ratio of antibody to α was 5 to 1. The τ_{obs} measurements were obtained from lag time assays performed at different RNA polymerase concentrations in the presence and absence of mAb 126C6.

The data derived from the lag time experiments indicate that mAb 126C6 increased the time required for formation of the RP_o. The *lac* L8UV5 RP_o formed with mAb 126C6–RNA polymerase after incubation for 15 min shows a pattern of enhancement and protection similar to that of the control RP_o, but the overall interaction appears slightly weaker (Figure 3, panel A). Incubation of mAb 126C6 with the preformed RP_o for 30 min does not markedly affect its stabililty. The *lac* L8UV5 promoter contains a double mutation; the UV5 mutation in the −10 consensus region enables RNA polymerase

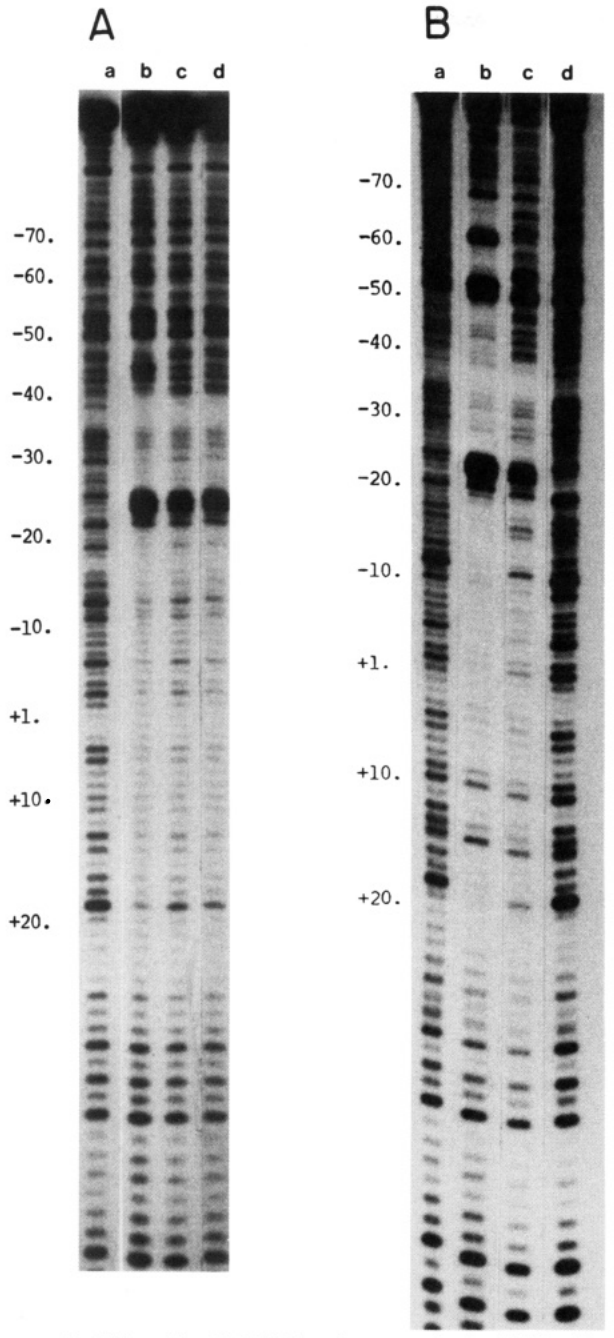

FIGURE 3: Effect of mAb 126C6 on the protection by RNA polymerase of the 3′-[^{32}P]*lac* L8UV5 promoter against attack by DNase I in the presence and absence of cAMP–CRP. Conditions are described under Materials and Methods with 3 nM 3′-^{32}P end-labeled (nontemplate) strand of *lac* L8UV5 promoter and 50 nM RNA polymerase and, where indicated, 40 nM CRP and 100 μM cAMP. Panel A: lane a, [^{32}P]*lac* L8UV5 only; lane b, RP_o; lane c, mAb 126C6–RNA polymerase incubated with [^{32}P]*lac* L8UV5 for 15 min at 37 °C; lane d, RP_o incubated with mAb 126C6 for 30 min at 37 °C. Panel B: lane a, [^{32}P]*lac* L8UV5 only; lane b, cAMP–CRP, RNA polymerase and [^{32}P]*lac* L8UV5 incubated 45 min at 37 °C; lane c, cAMP–CRP–[^{32}P]*lac* L8UV5–polymerase incubated for 15 min at 37 °C followed by incubation with mAb 126C6 for 30 min at 37 °C; lane d, cAMP–CRP and [^{32}P]*lac* L8UV5 incubated for 30 min at 37 °C.

to establish the RP_o in the absence of CRP, and the L8 mutation in the CRP binding site lowers its affinity for cAMP–CRP. Binding of CRP to the *lac* L8UV5 promoter is stabilized by RNA polymerase (Li & Krakow, 1988). When the RP_o is formed in the presence of cAMP–CRP, the pattern observed for cAMP–CRP binding to its site on the *lac* L8UV5 promoter is similar to that seen with the *lac* P$^+$ promoter as judged by the protection against DNase I attack (Figure 3, panel B, lane b). Incubation of the anti-α mAb 126C6 with the cAMP–CRP–*lac* L8UV5 RP_o results in dissociation of CRP from its site (Figure 3, panel B, lane c). This is accompanied by both the loss of the CRP-dependent pattern of protection and enhancement between −50 and −70 and also the extended region of protection within the RNA polymerase binding domain of the promoter upstream of −41 on the nontemplate strand of the *lac* L8UV5 promoter. The binding of mAb 126C6 to RNA polymerase interferes with the RNA polymerase–CRP contacts required to stabilize the interaction of CRP with the L8 site.

The observation that N-3 methylcytosine is more reactive to hydrazine than cytosine (Peattie & Gilbert, 1980) was employed by Kirkegaard et al. (1983) to map cytosines in the single-stranded regions of RNA polymerase–promoter complexes. Cytosine residues of the *lac* L8UV5 template strand at positions −1, −2, −4, and −6 were found to be in an unpaired region. The method of Kirkegaard et al. (1983) was applied to determine the accessibility of the N-3 position of cytosine during incubation of the mAb 126C6–RNA polymerase complex with *lac* L8UV5. The data presented in Figure 4 show the helix unwinding near the transcription start site that occurs during formation of the RP_o. The relatively rapid formation of the RP_o by RNA polymerase is evidenced by the appearance of the bands at positions −1, −2, −4, and −6 within a 1-min incubation. Formation of the RP_o by mAb 126C6–RNA polymerase occurs at a slower rate (Figure 4, lanes b–f). The pattern of helix unwinding appears to be the same for both RNA polymerase and mAb 126C6-RNA polymerase, indicating that the positioning of free and antibody-bound RNA polymerases on the promoter is comparable.

The data presented in Figure 5 compare the accessibility of cytosines in the cAMP–CRP–*lac* P$^+$–RNA polymerase RP_o incubated with mAb 126C6 and in cAMP–CRP–*lac* P$^+$ incubated with the mAb 126C6–RNA polymerase complex. The data show that even after a 40-min incubation of the RP_o with mAb 126C6 the cytosines near the start site of transcription remain unpaired (Figure 5, lanes b–d). The preformed mAb 126C6–RNA polymerase complex incubated with cAMP–CRP–*lac* P$^+$ does not form the open promoter complex (Figure 5, lane e). The latter finding correlates with the inhibition of initiation from *lac* P$^+$ by mAb 126C6–RNA polymerase. The results are puzzling in that the results of DNase I footprinting (Figures 1 and 2) indicate that incubation of the preformed RP_o with mAb 126C6 results in extensive loss of RNA polymerase contacts with the promoter.

Discussion

Apart from its involvement in the formation of the RNA polymerase core the role of the α subunit has not been defined. Data obtained from cross-linking studies have not provided evidence for contacts between α and DNA (Chenchick et al., 1981; Park et al., 1982) or between α and the nascent transcript (Bernhard & Meares, 1986; Stackhouse & Meares, 1988). The anti-α monoclonal antibody, mAb 126C6, used in this study has been shown to bind to an epitope that is available on both of the α subunits in the RNA polymerase core and holoenzyme (Riftina et al., 1989). Data were also obtained that suggested that part of the σ subunit was positioned close to one of the α subunits in the RNA polymerase holoenzyme. The mAb 126C6 almost completely inhibits abortive initiation from CRP-dependent *lac* P$^+$, partially inhibits abortive initiation from *lac* L8UV5, and is without apparent effect on the d(A-T)$_n$-directed synthesis of r(U-A)$_n$. In the present study the effects of mAb 126C6 on the for-

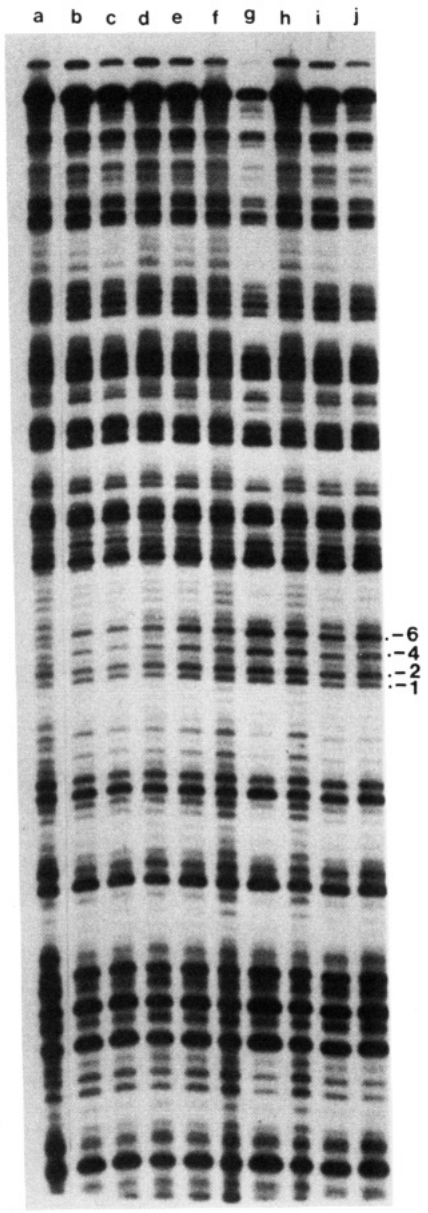

FIGURE 4: Effect of mAb 126C6 on the methylation of single-strand-specific cytosines of RNA polymerase–*lac* L8UV5 promoter complexes. Reaction conditions are described under Materials and Methods with 3 nM 5′-^{32}P end-labeled (template) strand of *lac* L8UV5 fragment and 50 nM RNA polymerase. Cytosine reactivity patterns are shown for *lac* L8UV5 complexes formed with the following: lane a, unprotected fragment; lanes b–f, mAb 126C6–RNA polymerase complex and [^{32}P]*lac* L8UV5 incubated at 37 °C for 1, 2.5, 5, 10, and 20 min, respectively; lanes g–j, RNA polymerase and [^{32}P]*lac* L8UV5 incubated at 37 °C for 0.5, 1, 2.5, and 20 min, respectively.

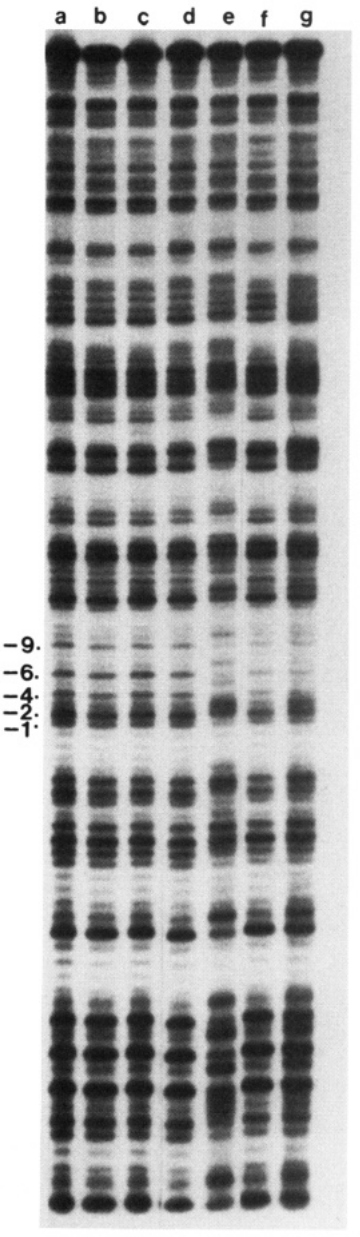

FIGURE 5: Effect of mAb 126C6 on the reactivity of base residues in the RNA polymerase–*lac* P$^+$ complex to methylation by dimethyl sulfate. Reaction conditions are described under Materials and Methods with 3 nM of 5′-^{32}P end-labeled (template) strand of *lac* P$^+$ promoter, 50 nM RNA polymerase, 40 nM CRP, and 100 μM cAMP. Lane a, RP_o; lane b, RP_o incubated with mAb 126C6 for 10 min at 37 °C; lane c, RP_o incubated with mAb 126C6 for 20 min at 37 °C; lane d, RP_o incubated with mAb 126C6 for 40 min at 37 °C; lane e, mAb 126C6–RNA polymerase complex incubated with cAMP–CRP and [^{32}P]*lac* P$^+$ for 30 min at 37 °C; lane f, RNA polymerase and [^{32}P]*lac* P$^+$ incubated for 30 min at 37 °C; lane g, cAMP–CRP and [^{32}P]*lac* P$^+$ incubated for 30 min at 37 °C.

mation of open complexes with the *lac* P$^+$ and *lac* L8UV5 promoters have been characterized.

The two-step model for the formation of open promoter complexes (Chamberlin, 1974) has been extended to include an additional intermediate, RP_i (Buc & McClure, 1985; Spassky et al., 1985):

$$R + P \rightleftarrows RP_c \rightleftarrows RP_i \rightleftarrows RP_o$$

Spassky et al. (1985) correlated the conversion of a closed promoter complex (RP_c) to an intermediate (RP_i) with the strict positioning on the polymerase surface of the −10 and −35 regions of the promoter with respect to each other. The postulated intermediate, RP_i, detected at low temperature, was characterized in temperature shift experiments (Buc & McClure, 1985; Spassky et al., 1985) and separated from RP_o by gel electrophoresis (Straney & Crothers, 1987). The RP_i is transcriptionally inactive and the cytosines near the start site remain hydrogen bonded (Buc & McClure, 1985; Spassky et al., 1985). The rate-limiting step in the formation of the *lac* RP_o in this sequence is the formation of the RP_i. The conversion of the RP_i to the RP_o occurs rapidly at 37 °C and is associated with the unstacking of DNA bases and an implicit cooperative transconformation of RNA polymerase (Spassky et al., 1985). An alternate mode of polymerase–*lac* promoter contacts was demonstrated in a study of the effect of the anti-β mAb 210E8 on the formation of the RP_o with linear and supercoiled *lac* L8UV5 promoters (Rockwell & Krakow, 1988). Binding of mAb 126C6 to RNA polymerase produces

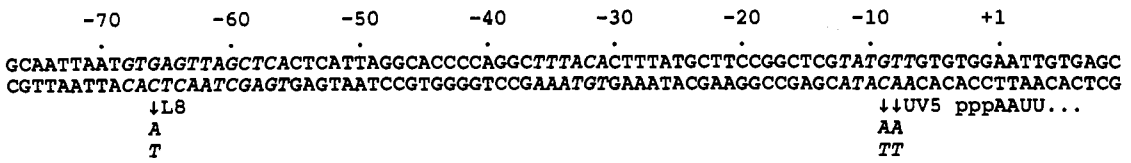

FIGURE 6: Diagram of the *lac* promoter region. The binding region for CRP and the −10 and −35 regions of the RNA polymerase binding domain of the *lac* promoter are shown in italics.

a greater effect on the rate of isomerization (k_2) than on the initial binding to the *lac* promoter (K_B). The ability to ultimately form a functional *lac* L8UV5 RP_o in the presence of mAb 126C6 indicates that the RNA polymerase α subunits probably do not directly interact with residues in the promoter. Heumann et al. (1988) have demonstrated by neutron small-angle scattering that RNA polymerase undergoes conformational changes during promoter binding. Their results indicate that there is a decrease in the cross section along with an elongation of RNA polymerase bound to the T7 A1 promoter. The retardation of the rate of isomerization by mAb 126C6 suggests that one or both of the α subunits may be involved in the conformational changes in RNA polymerase required to form the RP_o. The mAb 126C6 bound to RNA polymerase may be acting directly by slowing a conformational change in one or both of the α subunit(s) or indirectly by sterically hindering the conformational transition of a domain in a proximal subunit.

The precise mechanism by which cAMP–CRP stimulates transcription remains to be defined. Direct CRP–RNA polymerase interaction was initially proposed by Majors (1975) and experimental results support this proposal (Li & Krakow, 1987; Li & Krakow, 1988; Ren et al., 1988; Pinkney & Hogget, 1988; Straney et al., 1989). Bending of the DNA by CRP bound to its site has been proposed to facilitate RNA polymerase–CRP interactions (Wu & Crothers, 1984; Liu-Johnson et al., 1986). Studies of the effects of monoclonal antibodies directed against CRP suggested that contact between CRP and RNA polymerase occurs when both are bound to the *lac* P^+ or L8UV5 promoter (Li & Krakow, 1988). DNase I footprinting indicates that when the mAb 126C6–RNA polymerase complex is incubated with cAMP–CRP–*lac* P^+ the essential interactions between RNA polymerase and the promoter are not established; this may be a consequence of a lack of interaction between RNA polymerase and CRP. This would explain the marked inhibition of transcription from the *lac* P^+ promoter by mAb 126C6. Since the primary binding site for cAMP–CRP on *lac* P^+ (−50 to −70 bp) is adjacent to the RNA polymerase binding site on the promoter (Figure 6), steric interference of contact between cAMP–CRP and mAb 126C6–RNA polymerase appears possible. DNase I footprinting shows that binding of mAb 126C6 to the preformed RP_o occurs, and as a consequence, the interactions between the *lac* P^+ promoter and RNA polymerase are severely affected. Nevertheless, the mAb 126C6–RNA polymerase complex remains bound to the *lac* P^+ promoter. This is indicated by the residual enhancement of DNase I cutting at −23 and −24 on the nontemplate strand and by the retention of the unwound region between −1 and −6. The DNase I footprints resulting from incubation of the RP_o with mAb 126C6 resemble those of the *lac* P^+–RNA polymerase complex formed in the absence of cAMP–CRP. However, the effect of RNA polymerase on the CRP bound at its site is not seen; the enhancements within the CRP binding site at −52 and −54 on the template strand are reduced to that observed with cAMP–CRP alone. DNase I footprints of RNA polymerase bound to the *lac* promoters in the absence of cAMP–CRP do not show the enhancements at −52 and −54. The effect of mAb 126C6 on the interactions in the CRP binding site on *lac* P^+ and *lac* L8UV5 supports the involvement of contact between CRP and RNA polymerase.

Binding of cAMP–CRP to the *lac* L8UV5 mutant is RNA polymerase dependent. Incubation of mAb 126C6 with the RP_o formed with cAMP–CRP–*lac* L8UV5 promoter results in dissociation of cAMP–CRP from its site. Although mAb 126C6 has a moderate effect on the interaction of RNA polymerase with the *lac* L8UV5, the relative decrease of protection against the DNase I attack in the promoter region between −41 and −50 proximal to the CRP binding domain is more pronounced. These contacts may contribute to the stability of the cAMP–CRP–*lac* L8UV5–RNA polymerase complex. A steric effect of mAb 126C6 on dissociation of CRP from the promoter seems possible. This may occur if one of the α subunits in RNA polymerase is proximal to the CRP binding domain. Binding of mAb 126C6 to this α subunit may prevent contact of CRP with RNA polymerase. The effect of the anti-α mAb 126C6 on interactions of cAMP–CRP with RNA polymerase in the *lac* L8UV5 complex suggests that at least one of the α subunits may be proximal to the CRP in an open promoter complex.

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Effect of Base-Pair Sequence on the Conformations and Thermally Induced Transitions in Oligodeoxyribonucleotides Containing Only AT Base Pairs†

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ABSTRACT: T_m curves, CD spectra, and kinetics results of the self-complementary DNA dodecamers d(A_6T_6), d($A_3T_3A_3T_3$), d($A_2T_2A_2T_2A_2T_2$), d(ATATATATATAT), and d(T_6A_6) demonstrate that the thermal transitions of these oligomers at low salt concentration involve a hairpin intermediate. At high salt concentrations (>0.1 M Na^+) only a duplex to denatured-strand transition appears to occur. The temperature and salt-concentration regions of the transitions are very sequence dependent. Alternating-type AT sequences have a lower duplex stability and a greater tendency to form hairpins than sequences containing more nonalternating AT base pairs. Of the two nonalternating sequences, d(T_6A_6) is significantly less stable than d(A_6T_6). Both oligomers have CD curves that are very similar to the unusual CD spectrum of poly(dA)·poly(dT). The Raman spectra of these two oligomers are also quite similar, but at low temperature, small intensity differences in two backbone modes and three nucleoside vibrations are obtained. The hairpin to duplex transition for the AT dodecamers was examined by salt-jump kinetics measurements. The transition is faster than transitions for palindromic-sequence oligomers containing terminal GC base pairs. Stopped-flow kinetics studies indicate that the transition is second order and has a relatively low activation energy. The reaction rate increases with increasing ionic strength. These results are consistent with a three-step mechanism for the hairpin to duplex reaction: (i) fraying of the hairpin oligomers' terminal base pairs, (ii) a rate-determining bimolecular step involving formation of a cruciform-type intermediate from two hairpin oligomers with open terminal base pairs, and (iii) base-pair migration and formation in the intermediate to give the duplex.

In addition to the well-characterized A, B, and Z double-helical states of DNA (Cantor & Schimmel, 1980; Dickerson et al., 1982; Saenger, 1984; Wells & Harvey, 1987; Blackburn & Gait, 1990), there are nonstandard structures, such as bent, triple-helical, and cruciform-hairpin conformations, which are thought to play important roles in crucial biological processes such as control of gene expression and recombination (Lee et al., 1984; Lyamichev et al., 1986; Moser & Dervan, 1987; Hanvey et al., 1988; Htun & Dahlberg, 1988; Lilley et al., 1988; Petrillo et al., 1988; Chen et al., 1988). Pure AT sequences in double-helical DNA, for example, are known to exhibit significant variations in structure and in interactions with ligands as the sequence on each chain is varied from nonalternating to alternating purine–pyrimidine bases [cf. Saenger (1984) and Yoon et al. (1988)]. Nonalternating AT base pair sequences can lead to curvature of the DNA helix

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