Binding of *Escherichia coli* Ribonucleic Acid Polymerase Holoenzyme to a Bacteriophage T7 Promoter-Containing Fragment: Selectivity Exists over a Wide Range of Solution Conditions†

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ABSTRACT: The selectivity of binding of *Escherichia coli* RNA polymerase holoenzyme to a promoter-containing fragment of T7 DNA has been investigated over a range of solution conditions by using a double-label nitrocellulose filter binding assay. A ^{32}P-labeled HaeIII restriction fragment of T7 D111 DNA containing the A1 and D promoters for the *E. coli* enzyme and a ^{3}H-labeled nonpromoter HaeIII fragment of comparable size were incubated with σ-saturated holoenzyme and filtered through a nitrocellulose membrane filter. We find that the extent of binding of polymerase to the promoter-containing fragment decreases dramatically with increasing salt concentrations and with increasing pH and increases moderately with increasing temperature in the range 0–37 °C. By contrast, the nonspecific interaction of polymerase with the nonpromoter fragment is known to be relatively insensitive to pH and temperature, though a strong function of salt concentration [deHaseth, P. L., Lohman, T. M., Burgess, R. R., & Record, M. T., Jr. (1978) *Biochemistry 17*, 1612–1622]. Selectivity of binding of RNA polymerase in our assay is demonstrated by a greater fractional retention of the promoter-containing fragment than of the nonpromoter fragment on the filter. We observe selective binding over the temperature range from 0 to 37 °C near neutral pH and over a wide range of Na^+ concentrations, in the presence or absence of Mg^{2+}. Because of the different dependences of promoter and nonpromoter binding on pH and temperature, the extent of selectivity increases with increasing temperature and decreases with increasing pH. Quantitative treatment of these binding data [Strauss, H. S., Burgess, R. R., & Record, M. T., Jr. (1980) *Biochemistry* (second paper of four in this issue)] confirms these conclusions and shows that selectivity is a function of ion concentration as well.

The selective binding of *Escherichia coli* RNA polymerase holoenzyme to bacterial or viral promoters is central to the control of transcription and to the overall regulation of gene expression. It is not known whether the thermodynamics or the kinetics of the binding reaction are more important in determining the observed in vivo selectivity. von Hippel et al. (1974) accounted for the observed levels of repression in the *E. coli* lactose operon by using an equilibrium model which included repressor and RNA polymerase binding to both specific and nonspecific sites. On the other hand, Maquat & Reznikoff (1978), Seeburg & Schaller (1975), and Seeburg et al. (1977) find good correlation between in vitro association rates and in vivo promoter strengths of wild-type and mutant *lac* promoters.

Determination of the relative affinities of RNA polymerase for promoter-containing and nonpromoter fragments provides a measure of the equilibrium selectivity of RNA polymerase for promoter over nonpromoter sites. In addition, investigation of the equilibria of promoter and nonpromoter binding reactions as functions of solution variables such as monovalent and divalent cation concentration, pH, and temperature provides information about the molecular details of the interactions (Record et al., 1978). Recently, deHaseth et al. (1977a,b) and Revzin & von Hippel (1977) used such a physicochemical approach to study the details of the interaction of *lac* repressor with nonoperator DNA. Comparison of their data with that of Riggs et al. (1970a,b) on *lac* repressor–operator interactions revealed molecular differences between the two modes of binding (Record et al., 1977).

The binding of *E. coli* RNA polymerase to promoter DNA has been investigated for several bacteriophage and bacterial promoters, although generally not over a wide range of solution variables. The promoters studied include those of T7 (Hinkle & Chamberlin, 1972a,b), fd, f1, M13 (Seeburg & Schaller, 1975; Seeburg et al., 1977; Giacomoni et al., 1977a,b), T5 (von Gabain & Bujard, 1977), and the lactose operon (Maquat & Reznikoff, 1978). The interactions of RNA polymerase holoenzyme and core enzyme with nonpromoter DNA have been investigated by deHaseth et al. (1978) using a chromatographic technique. The literature of *E. coli* RNA polymerase–DNA interactions has been reviewed by Chamberlin (1976).

In this paper we report the results of double-label filter binding experiments on the interactions of *E. coli* RNA polymerase holoenzyme with a mixture of promoter-containing and nonpromoter restriction fragments of T7 DNA. The promoter fragment (end labeled with ^{32}P) was a 1550 base pair HaeIII restriction fragment of T7 D111 DNA containing the A1 and D promoters for the bacterial polymerase. The nonpromoter fragment (^{3}H labeled) was a T7 fragment of comparable size. Under conditions where selectivity of binding exists (i.e., where polymerase has a higher affinity for promoter sites than for nonspecific sites), a larger fraction of promoter fragments than nonpromoter fragments is retained on the filter. We find that selectivity of holoenzyme for this promoter fragment is exhibited over a range of temperatures (0–37 °C), pH, and ionic conditions. However, the extent of selectivity (or ratio of binding constants for promoter and nonpromoter binding) varies greatly with the conditions examined. For rigorous discussion of the extent of selectivity, quantitative statistical analysis of the double-label filter binding experiments

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is required. This analysis will be presented in the following paper (Strauss et al., 1980).

Materials and Methods

(*a*) *E. coli RNA Polymerase.* *E. coli* RNA polymerase was purified by the method of Burgess & Jendrisak (1975), with additional purification on a single-stranded DNA agarose column (Lowe et al., 1979). The σ subunit and core polymerase were prepared from purified holoenzyme on a Bio-Rex 70 column (Burgess, 1976; Lowe et al., 1979). σ-Saturated RNA polymerase was prepared by adding purified σ to purified holoenzyme to a final molar ratio of σ to core of approximately 2:1 and stored in a 50% glycerol storage buffer at −70 °C. Concentrations were determined by absorbance (corrected for light scattering) at 280 nm (Burgess, 1976).

σ-Saturated RNA polymerase was characterized with respect to its ability to initiate transcription in a rifampicin challenge assay (Mangel & Chamberlin, 1974). Polymerase (0.32 μg) and T7 D111 DNA (4.4 μg) were preincubated for 20 min at 37 °C in a 100-μL volume of 0.01 M Hepes[1] buffer, pH 7.5, 0.01 M $MgCl_2$, 0.10 M NaCl, 0.001 M DTT, 10^{-4} M EDTA, and 50 μg/mL BSA. (The input polymerase/DNA mole ratio was approximately 4:1.) The preformed complexes were presented simultaneously with 5 μg/mL rifampicin (Sigma Chemical Co.), 420 μM ATP, GTP, CTP (P-L Biochemicals), 100 μM UTP (P-L Biochemicals), and 4 μCi of [^{3}H]UTP (New England Nuclear). After a 90-s incubation, the reaction was quenched with 5% trichloroacetic acid (0 °C). Precipitated RNA was collected on filters and subjected to liquid scintillation counting. In all experiments, we observed 90–100% of the RNA synthesis obtained in control experiments in which rifampicin was omitted. Similar results were obtained with 0.01 M Tris buffer, pH 7.4.

Rifampicin challenge experiments were carried out after preincubation (20 min) at pH 6.2, 6.8, 8.1, and 8.6, corresponding to the pH range of the binding experiments reported here. If the 90-s transcription assay is carried out at the pH of the preincubation, more than 90% of the transcription is again insensitive to rifampicin, but the amount of precipitable RNA decreases with decreasing pH. Presumably this result is a reflection of the decrease in average RNA chain length with decreasing pH [cf. Wienand et al. (1978)]. In any case, it is not the result of irreversible denaturation of the enzyme, since if the transcription assay is performed at pH 7.5 after preincubation for 20 min at pH 6.2–8.6, both the level of rifampicin resistance and the level of transcription are normal.

(*b*) *T7 (D111) DNA.* The T7 deletion mutant, D111, which lacks the A2, A3, and B promoters (Studier, 1975), was obtained as a phage stock from F. W. Studier. Unlabeled D111 phages were grown on *E. coli* C. ^{3}H-Labeled phages were grown on a *thy*$^-$ mutant of *E. coli* C, using [*methyl*-^{3}H]thymidine (New England Nuclear) in the growth medium at 1.1 μCi/mL. Standard procedures were used to purify the phage and extract the intact phage DNA.

(*c*) *Buffers.* Tris (Trizma base), Hepes, and Bicine were from Sigma Biochemicals; salts were reagent grade. BSA was from Miles (Pentex). Stock solutions were filter sterilized by using 0.22-μm filters (Millipore). All solutions were periodically streaked out on agar plates to check for possible mold and bacterial contamination. DNA was stored in either DNA/T buffer [0.01 M Tris (pH 7.9 at 25 °C), 0.05 M NaCl, and 0.001 M Na_3EDTA] or DNA/H buffer [0.01 M Hepes (pH 7.43 at 25 °C), 0.05 M NaCl, and 0.001 M Na_3EDTA.

(*d*) *Restriction Fragments.* Homogeneous samples of individual restriction fragments (with blunt ends and a minimum of single-strand scissions) were required for the binding studies. In the preparation of these fragments, sterile plastic or siliconized labware was used. All enzymes and other proteins used were tested for exonuclease activity by incubation with ^{3}H-labeled DNA at 37 °C for 12 h, followed by Cl_3AcOH precipitation and scintillation counting of both supernatant and precipitate (Dynan et al., 1977). No exonuclease activity was detected in any protein preparation used in this work.

HaeIII fragments were obtained by mixing 1.0–1.5 mg of D111 DNA with sufficient HaeIII restriction endonuclease (provided by D. Noble and J. Slightom) to give complete digestion in 12 h at 37 °C in HaeIII buffer [0.006 M Tris (pH 7.9), 0.006 M $MgCl_2$, 0.001 M DTT, and 50 μg/mL BSA]. The final volume was 2.0–2.5 mL. Digestion was stopped with the addition of 1 mL of sample buffer (50% v/v glycerol, 0.02% bromphenol blue, and 0.1 M EDTA). The reaction mixture was layered onto a 4% polyacrylamide–25% glycerol slab gel (Blakesley & Wells, 1975) and electrophoresed for 12 h at 160 V. The gel was stained for 5 min with 0.5 μg/mL ethidium bromide, destained for 5 min in distilled water, and illuminated from below at 366 nm with a long-wavelength UV lamp (UV Products C60) to visualize the bands. This wavelength was chosen to minimize the damage to the DNA from photoinduced nicking reactions (Brunk & Simpson, 1977).

DNA was eluted from the gel by either diffusion or electrophoresis. In both methods, the DNA bands were cut out of the gel and the gel strips were squeezed through a syringe (without needle). In the diffusion method, the gel slurry was placed in a second syringe which contained a sterile, siliconized glass wool plug. Three to five milliliters of buffer [0.1 M Tris (pH 7.9) and 0.7 M NaCl) was added, and the mixture was allowed to sit for 24–36 h in the dark at room temperature. The DNA was separated from the polyacrylamide by applying pressure to the syringe plunger. In the electrophoresis method, the gel slurry was placed in a dialysis bag and electrophoresed against Tris–borate–EDTA buffer (Peacock & Dingman, 1968) at 15 mA for 2 h. The DNA was separated from the polyacrylamide with a stoppered syringe as described above. Yields were variable but were in the range of 60–95% by either technique for fragments in the molecular weight range of interest ($\sim 10^6$ daltons). Ethidium bromide was removed from the fragments [in a buffer adjusted to 0.7 M NaCl and 0.1 M Tris (pH 7.9)] by four extractions with 1-butanol at 0 °C.

After butanol extraction, ^{3}H-labeled fragments were diluted to 0.1–0.2 M NaCl and loaded onto 1-mL DEAE-cellulose (Whatman DE-52) columns (Hirsh & Schleif, 1976). Columns were washed with DNA/T buffer adjusted to 0.35 M NaCl and the fragments were eluted at 0.6 M NaCl. Fragments were precipitated with 3–4 volumes of 100% ethanol and resuspended in DNA/H buffer.

To prepare ^{32}P-end-labeled fragments, we used the T4 polynucleotide kinase one-step exchange reaction (Van de Sande et al., 1973). Fragments were isolated, purified through the butanol extractions as described above, ethanol precipitated, and resuspended in 0.5 mL of 0.05 M Tris (pH 7.9), 0.01 M $MgCl_2$, 0.12 M NaCl, and 0.005 M DTT. After addition of 1 nmol of ADP, 0.15 nmol of ATP, and 0.3 mCi of γ-^{32}P-labeled ATP [provided by E. Calva and W. Dynan; prepared

[1] Abbreviations used: DTT, dithiothreitol; BSA, bovine serum albumin; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; bp, base pair.

as described by Miller & Burgess (1978)], 6 units of T4 polynucleotide kinase (New England Biolabs) was added, and the reaction mixture (final volume 0.7 mL) was incubated for 30 min at 37 °C. Subsequently, the DNA fragments were separated from unreacted ATP and ADP by passage over a 10-mL Sephadex G-50 (superfine) column and loading the void peak directly onto a 1-mL DEAE-cellulose column. Fragments were eluted as above, ethanol precipitated, and resuspended in DNA/H buffer. Chromatography on a poly(ethylenimine) plate (Brinkman) and autoradiography (Miller & Burgess, 1978) were performed to test for [γ-^{32}P]ATP contamination of the fragments. No contamination was observed. The final recovery of fragments ranged between 15 and 40%.

Homogeneity of the various fragment preparations was determined by analytical gel electrophoresis. DNA concentrations were determined by ethidium bromide fluorescence (LePecq & Paoletti, 1966) using an Aminco Bowman spectrofluorometer. Accurate determinations could be performed on as little as 10 ng of DNA.

(*e*) *Nitrocellulose Filter Binding Assay.* Since the extent of binding of RNA polymerase to DNA is a sensitive function of temperature, pH, and ionic conditions, these variables were carefully controlled in all filter binding assays. Final buffer solutions were made by mixing concentrated stock solutions to the desired concentration. Salt concentrations in all experiments were verified by conductivity measurements on a Radiometer COM 2e conductivity meter. Solution pH values were measured at the temperature and ionic conditions of the experiment with a Radiometer 26 pH meter. The temperature of the incubation was always within 0.5 °C of the stated value.

Nitrocellulose filters (Schleicher & Schuell; BA 85; 24 mm) were treated with 0.4 N KOH for 20 min (Lin & Riggs, 1972), neutralized, and stored in the appropriate filter buffer at room temperature (see figure legends). Filtrations were performed at constant suction (2 cmHg lower than atmospheric pressure; this corresponds to a flow rate of 4–5 mL/min).

The experiments presented here were performed according to one of two protocols, A and B. In protocol A, σ-saturated RNA polymerase (1 μL of a stock solution at 55 μg/mL) was added to 50 μL of the appropriate binding buffer (for exact composition see figure legends) usually containing 95 ng of ^{32}P-labeled A1/D promoter fragment and 64 ng of ^{3}H-labeled nonpromoter fragments of comparable size. (In some experiments, the radiolabels were reversed.) The reaction mixture was incubated for 20 min at the appropriate temperature and diluted to 0.5 mL with binding buffer. A 0.45-mL portion was filtered and immediately washed with 1.0 mL of filter buffer. Less than 6 s elapsed between dilution and filtration. (Filter buffer was identical with binding buffer except the BSA and DTT were omitted.) The filter was dried, dissolved in 0.5 mL of 2-ethoxyethanol (Kobayashi & Harris, 1978), and counted in a toluene-based scintillation fluid.

Protocol B differed from protocol A in that σ-saturated RNA polymerase (1 μL of a stock solution at 180 μg/mL) was added to 0.5 mL of the appropriate binding buffer containing 55 ng of ^{32}P-labeled A1/D promoter fragment and 55 ng of ^{3}H-labeled nonpromoter fragments. Again the reaction mixture was incubated for 20 min at the appropriate temperature and a 0.45-mL portion was filtered, followed by a 1-mL wash of filter buffer. Protocols A and B yield similar results.

(*f*) *Data Presentation.* The data are presented in terms of θ_P and θ_D, the fractions of promoter (θ_P) and nonpromoter (θ_D) fragments retained by the filter. Generally the promoter fragment was ^{32}P labeled and the nonpromoter fragment was ^{3}H-labeled. In this case

$$\theta_P = \frac{^{32}\text{P dpm} - \text{background } ^{32}\text{P dpm}}{\text{total } ^{32}\text{P dpm}}$$

$$\theta_D = \frac{^{3}\text{H dpm} - \text{background } ^{3}\text{H dpm}}{\text{total } ^{3}\text{H dpm}}$$

where ^{3}H dpm and ^{32}P dpm are crossover-corrected disintegrations per minute of ^{3}H and ^{32}P, respectively. The background dpm were determined in parallel experiments in which labeled DNA was filtered in the absence of RNA polymerase (at the same DNA concentration and in a representative buffer). The maximum dpm were determined by spotting the total amount of DNA originally in the solution onto a nitrocellulose filter (in 1–10-μL volume). The filter was dried and treated as the others. Ninety percent of this crossover-corrected value (only 0.45 out of 0.50 mL was actually filtered) was taken to be the total dpm. Experimental points plotted in the figures are generally averages of triplicate assays. Error bars represent one standard deviation from the mean.

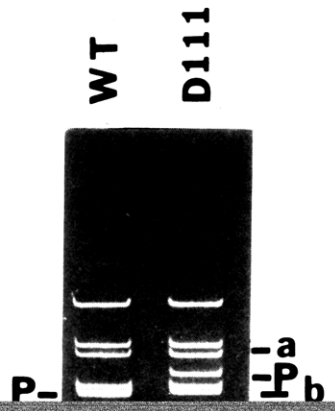
Results

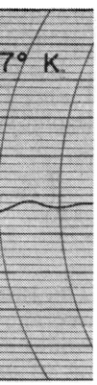
(*1*) *Filter Binding Assay: Definition of Selectivity.* The filter binding assay is based on the observation that RNA polymerase, like many other proteins, can be quantitatively retained on nitrocellulose filters (Hinkle & Chamberlin, 1972a). We have verified that 100% of the RNA polymerase is retained by the filter under all solution conditions used here (H. S. Strauss, R. Boston, R. R. Burgess, and M. T. Record, Jr., unpublished experiments). When a solution containing RNA polymerase and double-stranded DNA is filtered, those DNA molecules to which polymerase is bound are retained on the filter. The majority of uncomplexed DNA molecules pass through the filter. However, in the absence of polymerase or under conditions where polymerase does not bind to DNA, there is a low background level of retention of DNA on the filters which we have attempted to minimize by KOH pretreatment of the filters (Lin & Riggs, 1972) and by use of a 1-mL wash. The DNA background never exceeded 6% and was usually less than 3% of the input counts.

The filter assay procedure used here differs significantly from many previously reported [cf. Lin & Riggs (1972) and Hinkle & Chamberlin (1972a)]. Preliminary experiments indicated that for either intact T7 DNA (with 7 promoter sites and 7.7×10^4 nonpromoter sites) or the HaeIII restriction fragment investigated here (with 2 promoter sites and 3.1×10^3 nonpromoter sites; see below), the amount of polymerase bound to nonpromoter sites was *always* a significant fraction of the total amount of bound polymerase under all solution conditions examined. Polymerase molecules bound to nonpromoter sites can retain DNA molecules on nitrocellulose filters (see below). In previous studies, polyanionic competitors were used to remove polymerase from these nonspecific complexes before filtering. Here, however, we have used the level of nonpromoter binding of polymerase as a point of reference in the investigation of selective binding.

The equilibrium selectivity ratio is defined in terms of the binding constant ratio $K_{obsd}^{RP}/K_{obsd}^{RD}$, where K_{obsd}^{RP} is the promoter binding constant and K_{obsd}^{RD} is the binding constant for the interaction of RNA polymerase with a nonpromoter site (von Hippel et al., 1974; Record et al., 1977). Evidence supporting the assumption that the specific and nonspecific binding reactions of RNA polymerase have reached equilibrium under the incubation conditions employed here is pres-

ented in the following paper (Strauss et al., 1980). Each nucleotide residue is a potential nonpromoter site (McGhee & von Hippel, 1974); both the promoter and nonpromoter fragments used in this study contain ~3100 nonpromoter sites. Selectivity exists under conditions where $K_{obsd}^{RP}/K_{obsd}^{RD} > 1$. Moreover, the value of the ratio $K_{obsd}^{RP}/K_{obsd}^{RD}$ is a convenient measure of the extent of selectivity; selectivity increases as this ratio increases. From the filter assay, one obtained θ_P and θ_D, the fractional extents of retention of promoter and nonpromoter fragments. The quantity θ_D may be interpreted as the fraction of promoter-containing molecules which whould be filter bound in the absence of selective promoter binding. If $\theta_P > \theta_D$, then one may conclude without ambiguity that selectivity exists under those assay conditions. For θ_P to be





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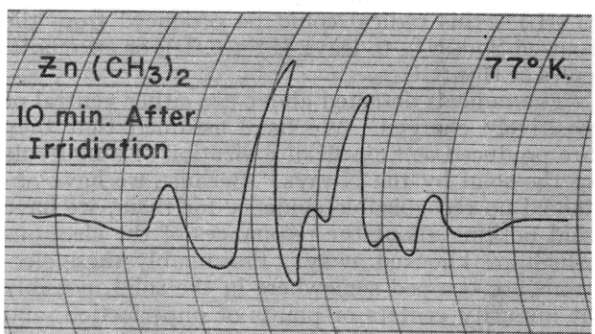
cal postulated in irradiated mercury diethyl could be produced as a primary effect of the ionization. The C_2H_5 would simply break off the ionized molecule to leave $(HgC_2H_5)^+$. Possibly the greater inertia of the two parts over those of mercury dimethyl prevents further reactions at 77°K.

Zinc Dimethyl.—Immediately after irradiation at 77°K. $Zn(CH_3)_2$ gave a quartet superimposed upon a weaker triplet, as shown in the upper curve of Fig. 2. Ten days later the quartet was



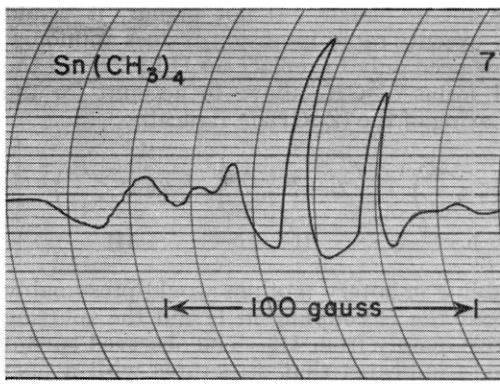


Fig. 3.—Electron spin resonance at 9 kMc. of methyl in the solid state. The tracing represents t derivative of the actual absorption curve.

perature and for propionic acid at the tem of liquid air. Since the same quartet has b observed for irradiated $Zn(CH_3)_2$, it seems that the quartet with a total spread of gauss is indeed the "fingerprint" of the me radical. There seems to be no other radic to be produced in irradiated zinc dimethy could give the observed pattern.

Now that we believe the 75 gauss quart arises from the methyl free radical, we a

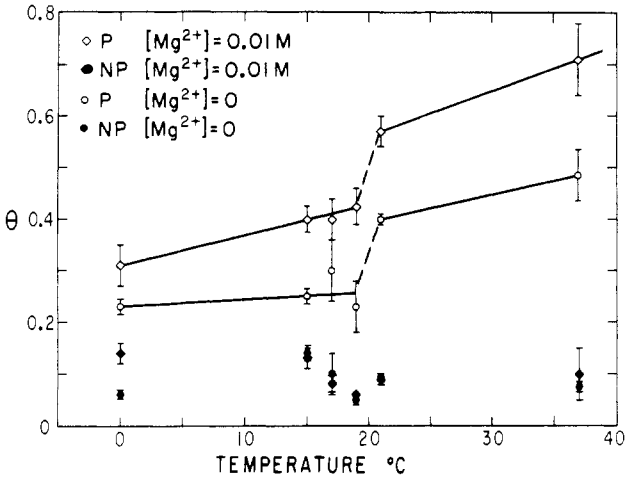


FIGURE 2: Fraction of DNA retained on the filter as a function of incubation temperature. Protocol A. Concentrations based on 50-μL volume: RNA polymerase, 2.2×10^{-9} M; ^{32}P-labeled promoter fragment, 1.80×10^{-9} M; ^{3}H-labeled nonpromoter fragments, 1.03×10^{-9} M. (For DNA, the concentrations are moles of fragment/liter.) $[Mg^{2+}]$ = 0.01 M; buffer, 0.01 M Hepes, 7.35 (at 37 °C), 10^{-3} M DTT, 10^{-4} M Na_3EDTA, 50 μg/mL BSA, 0.1 M NaCl, and 0.01 M $MgCl_2$. $[Mg^{2+}]$ = 0; buffer, 0.01 M Hepes, 7.35 (at 37 °C), 10^{-3} M DTT, 10^{-3} M Na_3EDTA, 50 μg/mL BSA, and 0.20 M NaCl. Background was 4% for ^{32}P for ^{32}P and 3% for ^{3}H. Maximum dpm were 403 for ^{32}P and 1573 for ^{3}H. Symbols: (◇) ^{32}P-labeled promoter, $[Mg^{2+}]$ = 0.01 M; (○) ^{32}P-labeled promoter, $[Mg^{2+}]$ = 0; (◆) ^{3}H-labeled promoter, $[Mg^{2+}]$ = 0.01 M; (●) ^{3}H-labeled nonpromoter, $[Mg^{2+}]$ = 0.

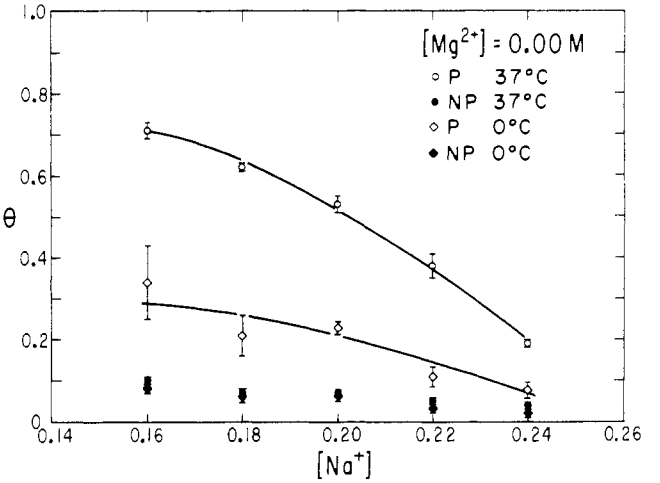


FIGURE 3: Fraction of DNA retained on the filter as a function of added sodium ion concentration at 0 and 37 °C in the absence of Mg^{2+}. Protocol A. Concentrations based on 50-μL volume: RNA polymerase, 2.2×10^{-9} M; ^{32}P-labeled promoter fragment 1.80×10^{-9} M; ^{3}H-labeled nonpromoter fragments, 1.01×10^{-9} M. Buffer: 0.01 M Hepes, 7.35 (at 37 °C) and 7.51 (at 0 °C), 10^{-3} M DTT, 10^{-3} M Na_3EDTA, and 50 μg/mL BSA, plus the indicated concentrations of NaCl. Background was 1% of maximum dpm. Maximum dpm were 580 for ^{32}P and 2820 for ^{3}H. Symbols: (○) ^{32}P-labeled promoter, 37 °C; (●) ^{3}H-labeled nonpromoter, 37 °C; (◇) ^{32}P-labeled promoter, 0 °C; (◆) ^{3}H-labeled nonpromoter, 0 °C. The curves drawn through the data are to facilitate visual inspection only.

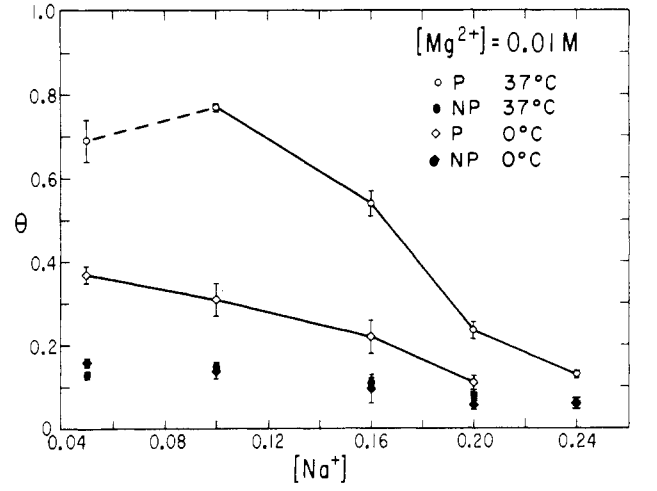


FIGURE 4: Fraction of DNA retained on the filter as a function of added sodium ion at 0 and 37 °C in the presence of Mg^{2+}. Protocol A. All concentrations are the same as in Figure 3 except [EDTA] was reduced to 10^{-4} M and 0.01 M $MgCl_2$ was included. Background was 3% of total dpm. Symbols: (○) ^{32}P-labeled promoter, 37 °C; (●) ^{3}H-nonpromoter, 37 °C; (◇) ^{32}P-labeled promoter, 0 °C; (◆) ^{3}H-labeled nonpromoter, 0 °C.

weight from the A1/D promoter fragment by 10–20%. We have used the groups of fragments labeled a, b, and c in Figure 1 as sources of nonspecific DNA for the double-label filter binding experiments described in this paper. In justification of this, we note that the C promoter is present on a 60-bp fragment in the HaeIII digest (F. W. Studier, personal communication), leaving only the E promoter unaccounted for. The HaeIII restriction sites near the right end of T7 DNA have not been determined, and consequently the size of the E promoter fragment is not known. However, the similar response of the various nonpromoter fragments to changes in solution conditions rules out the presence of a strong-binding site on any of these fragments. [This similarity of response was determined in filter binding assays where the retained DNA was eluted from the filter and displayed on a polyacrylamide gel. These assays also show enhanced retention of the fragment labeled P (H. S. Strauss, R. Boston, R. R. Burgess, and M. T. Record, Jr., unpublished experiments).]

The occurrence of both the A1 and D promoters on the fragment investigated here introduces an additional complexity into the interpretation of our experimental results. In discussing the data, we assume that the interactions of these two promoters with RNA polymerase are affected similarly by temperature, pH, and ion concentrations.

(3) Selectivity of RNA Polymerase Holoenzyme for the A1/D Promoter Fragment at Equilibrium Exists over a Wide Range of Temperatures, pH, and Ionic Conditions. (a) Temperature. Figure 2 shows the effects of temperature at two ionic conditions (0.1 M NaCl and 0.01 M $MgCl_2$; 0.2 M NaCl) on the selectivity of interactions of RNA polymerase with the A1/D promoter fragment. Selectivity ($\theta_P > \theta_D$) is present at all temperatures examined, in the presence or absence of Mg^{2+}. At both ionic conditions, θ_P increases gradually with increasing temperature in the ranges 0–17 and 21–37 °C; a relatively abrupt increase in θ_P occurs between 17 and 21 °C. On the other hand, both the data in Figure 2 for θ_D and the results of deHaseth et al. (1978) indicate that nonpromoter binding of RNA polymerase is essentially independent of temperature over this range. Consequently, one may conclude that the extent of selectivity of polymerase for these promoters increases with increasing temperature.

(b) NaCl Concentration. Figures 3 and 4 show the effect of NaCl concentration on the binding of holoenzyme to the A1/D promoter fragment and a nonpromoter fragment. In all cases, the extent of binding decreases with increasing NaCl concentration, indicating the importance of electrostatic interactions and counterion release in driving complex formation (Record et al., 1978). In Figure 3, the binding and filtration were performed in the absence of Mg^{2+} at 0 and 37 °C. Selectivity of polymerase for the promoter fragment is observed over the entire salt range examined, at both 0 and 37 °C. At

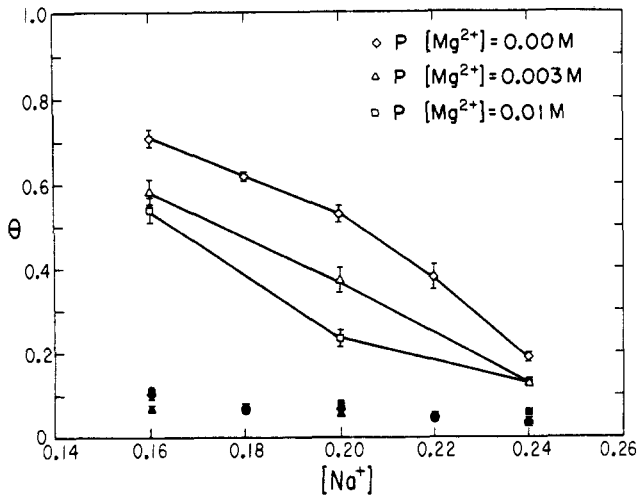


FIGURE 5: Fraction of DNA retained on the filter as a function of added sodium ion at different Mg^{2+} concentrations at 37 °C. Protocol A. The $[Mg^{2+}] = 0$ and $[Mg^{2+}] = 0.01$ M curves are taken from Figures 3 and 4. Other than $MgCl_2$, the concentrations are those described in Figures 3 and 4. Symbols: (◇) ^{32}P-labeled promoter, $[Mg^{2+}] = 0$; (◆) ^3H-labeled nonpromoter, $[Mg^{2+}] = 0$; (△) ^{32}P-labeled promoter, $[Mg^{2+}] = 0.003$ M; (▲) ^3H-labeled nonpromoter, $[Mg^{2+}] = 0.003$ M; (□) ^{32}P-labeled promoter, $[Mg^{2+}] = 0.01$ M; (■) ^3H-labeled nonpromoter, $[Mg^{2+}] = 0.01$ M.

every salt concentration, the value of θ_P is larger at 37 °C than at 0 °C, indicating (as in Figure 2) a higher affinity of polymerase for promoter DNA at the higher temperature. In the experiments shown in Figure 4, the binding and filtration were performed in the presence of 0.01 M $MgCl_2$. Again selectivity is observed over the entire range of Na^+ concentrations examined, and the binding of polymerase to the promoter sequence is stronger at 37 °C than at 0 °C, at any concentration of Na^+.

We tested core polymerase for selective binding by performing experiments similar to those in Figures 3 and 4 (at 37 °C only). The extent of retention of ^{32}P- and ^3H-labeled fragments was the same, within experimental error, under each solution condition tested (data not shown). These results indicate that, if equilibrium selectivity exists with core polymerase, the extent of selectivity is less than can be detected in this assay (see above). [The extent of fragment retention was also higher than that obtained for nonpromoter fragments under the same conditions, in agreement with the results of deHaseth et al. (1978).]

Figures 3 and 4 appear to demonstrate that the selectivity of polymerase for promoter sequences decreases with increasing Na^+ concentration. Such a conclusion is unwarranted, however, without a quantitative analysis of the data. The inequality $\theta_P > \theta_D$ demonstrates the existence of selectivity under all salt conditions examined; however, the difference $\theta_P - \theta_D$ is, in general, *not* an accurate measure of the extent of selectivity. If θ_D is known to be independent of a particular variable (see the discussion of temperature effects above), then differences in θ_P can be correlated directly with differences in the extent of selectivity. However, in the present case it is known that θ_D is a strong function of Na^+ concentration (deHaseth et al., 1978). Evidence for this can be seen in Figures 3 and 4, though here the decrease in θ_D with increasing Na^+ concentration appears small because the magnitudes of θ_D are small. Consequently, no conclusion can be drawn about the effect of Na^+ concentration on the extent of selectivity, without recourse to binding constants (Strauss et al., 1980).

(*c*) *Effects of Mg^{2+} Concentration.* Figure 5 compares the effects of Mg^{2+} and Na^+ concentrations on θ_P and θ_D at 37 °C. The curves at 0.01 M Mg^{2+} and in the absence of Mg^{2+} are reproduced from Figures 4 and 3, respectively. Data at an intermediate Mg^{2+} concentration (0.003 M) are also included. In all cases, $\theta_P > \theta_D$, and selective binding is observed. Also, θ_P decreases with increasing Mg^{2+} concentration at constant Na^+ concentration and decreases with increasing Na^+ concentration at each Mg^{2+} concentration examined. Both these observations demonstrate that the *dominant* role of Mg^{2+} is as a competitor with polymerase for DNA sites (deHaseth et al., 1977a,b; Record et al., 1977). Similar effects are seen in the nonspecific binding of holoenzyme to nonpromoter DNA (deHaseth et al., 1978).

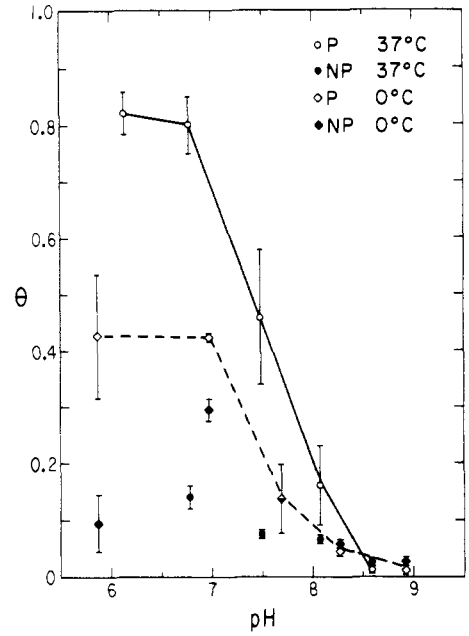


FIGURE 6: The fraction of DNA retained on filters as a function of pH at 37 and 0 °C. Protocol B. Buffer: 10^{-3} M Na_2EDTA, 10^{-3} M DTT, 50 μg/mL BSA, and 0.20 M NaCl; also, as appropriate, 0.01 M cacodylate, 0.01 M Hepes, and 0.01 M Bicine at the indicated pH values. (pH values were measured at the temperature of incubation.) Concentrations: RNA polymerase, 7.35×10^{-10} M; ^{32}P-labeled promoter fragment, 1.5×10^{-10} M; ^3H-labeled nonpromoter, 1.07×10^{-10} M. Background ^3H was 3% of total and ^{32}P was 6% of total. Maximum dpm were 1145 for ^3H and 508 for ^{32}P. Symbols: (○) ^{32}P-labeled promoter, 37 °C; (●) ^3H-labeled nonpromoter, 37 °C; (◇) ^{32}P-promoter, 0 °C; (◆) ^3H-labeled nonpromoter, 0 °C.

(*d*) *Effects of pH.* Figure 6 illustrates the pH dependences of θ_P and θ_D at 0 and 37 °C in the absence of Mg^{2+} by using protocol B. Similar results were obtained by using protocol A (data not shown). At both temperatures, θ_P decreases rapidly with increasing pH in the range 6.5–8.5. Because nonspecific binding has been shown to have only a weak dependence on pH (deHaseth et al., 1978), and because θ_P has decreased to the level of θ_D above pH 8, it appears that the equilibrium selectivity ratio of polymerase for the promoters investigated here is substantially decreased by an increase in pH.

Discussion

(*1*) *Filter Binding Assay.* In using the filter assay to obtain information about the affinity of RNA polymerase for promoter fragments and/or nonspecific DNA fragments as a function of solution conditions, it is necessary to consider numerous potential artifacts [cf. Hinkle & Chamberlin (1972a)]. Ideally, all DNA fragments (promoter and nonspecific) having one or more bound polymerase molecules at the time of filtration will be retained on the filter. In practice, the situation may be complicated by possible changes in the

extent of retention by the filter of preexisting polymerase–DNA complexes as a function of solution conditions, by possible displacement of the DNA from the polymerase by the nitrocellulose (or any other) polyanion (Chamberlin, 1976), or by the dissociation of weak complexes during dilution, filtration, and the subsequent wash (H. S. Strauss, R. Boston, R. R. Burgess, and M. T. Record, Jr., unpublished experiments). We believe that substantial information about the effect of solution variables on the RNA polymerase–promoter interaction and the selectivity of polymerase for promoters over nonspecific DNA can be obtained from the filter binding method, subject to three assumptions. First, we assume that the retention on the filter of preexisting RNA polymerase–promoter complexes is independent of solution conditions. We have shown that the retention of RNA polymerase on the filter is independent of the solution conditions used in these experiments (H. S. Strauss, R. Boston, R. R. Burgess, and M. T. Record, Jr., unpublished experiments), but we cannot test this for polymerase–DNA complexes. Second, we assume that RNA polymerase–promoter complexes are not affected by displacement and dissociation during the dilution, filtration, and wash steps. This assumption is discussed by H. S. Strauss, R. Boston, R. R. Burgess, and M. T. Record, Jr. (unpublished experiments.) (RNA polymerase–nonspecific DNA complexes have much shorter half-times of dissociation from individual sites than do polymerase–promoter complexes. We estimate that these half-times for local dissociation are substantially less than the time it takes to perform the filtration steps. Consequently, it is not possible to interpret the nonspecific binding data in absolute terms. However, the observed level of nonspecific binding can be considered a lower limit on the amount of nonspecific complex formation.) The final assumption is that retention of promoter fragments by nonspecific binding of RNA polymerase can be corrected for by using nonspecific DNA fragments of a comparable size as an internal control. Since the number of nonspecific binding sites on the promoter and nonpromoter fragments are approximately the same (±10%), this assumption appears justified.

(*2*) *Maximum Extent of Retention.* In experiments performed by protocol A (dilution protocol; Figures 2–5), the ratio of RNA polymerase molecules to promoters was less than 1, assuming two promoters per promoter-containing fragment. At low salt concentrations, in the presence or absence of Mg^{2+}, the binding of polymerase to DNA is sufficiently strong that virtually all (>90%) the polymerase is bound, either specifically or nonspecifically. Quantitative calculations (using the two-promoter model) discussed in the following paper show that at least 70 ± 5% of all polymerase molecules in the solution are bound to promoters under these conditions (Strauss et al., 1980). More than 90% of the RNA polymerase is bound to DNA when both promoter and nonspecifically bound RNA polymerase are considered. It is also reasonable to conclude that, were it not for competition between promoter sites and nonspecific sites for polymerase, essentially all polymerase molecules would be bound at promoters. Therefore, the assumption that the polymerase is 100% active in binding to promoters appears reasonable. [If only one promoter site were utilized per fragment (a less likely model, in our opinion), then at least 55 ± 5% of the polymerase molecules in the solution are bound to promoter sites (Strauss et al., 1980).] Also, analysis of the distribution of polymerase between the promoter and nonpromoter fragments under conditions of low salt concentration and limiting amounts of polymerase verifies the assumption that only one molecule of polymerase is required to retain a 10^6-dalton DNA fragment on a nitrocellulose filter. In addition, it confirms that holoenzyme binds to specific sites on DNA as the protomer [cf. Krakow et al. (1976)].

(*3*) *Salt Effects on Promoter Binding.* The increase in the level of binding of RNA polymerase to promoter sites with decreasing NaCl concentration is characteristic of many protein–DNA interactions. It indicates that complex formation is accompanied by the net release of thermodynamically bound Na^+ (and possible Cl^-) ions from the DNA (and protein). In general, counterion release occurs when polyelectrolyte charges are neutralized by ionic interactions during complex formation. The extent of counterion release, conveniently measured by the sensitivity of the equilibrium association constant to NaCl concentration, provides a measure of the maximum number of ionic interactions in the protein–DNA complex. Moreover, counterion release is a driving force for association as the counterion concentration is reduced (Record et al., 1976, 1977, 1978; deHaseth et al., 1977a,b, 1978).

Magnesium ions compete with proteins for DNA sites. This nonspecific effect of Mg^{2+} tends to reduce the extent of protein–DNA complex formation (Record et al., 1976, 1977, 1978). In Figure 5, the decrease in RNA polymerase binding with increasing [Mg^{2+}] and, at 0.24 M Na^+, the insensitivity of binding to Mg^{2+} concentration are reflections of the general competitive effect of Mg^{2+} (i.e., the competition between Na^+, Mg^{2+}, and RNA polymerase for DNA phosphates).

(*4*) *Temperature and pH Effects.* The temperature dependence of θ_P shown in Figure 2 suggests that a conformational change or other cooperative process occurs near 20 °C, resulting in an abrupt though modest increase in the affinity of polymerase for promoter sequences. This effect could be due to RNA polymerase and/or the DNA. Wu et al. (1976) have described a temperature-dependent conformation transition in holoenzyme. Wang et al. (1977) have demonstrated a temperature-dependent increase in the unwinding of DNA at a fixed ratio of RNA polymerase to DNA.

Temperature effects have been noticed in many assays which examine different aspects of the transcription process. Experiments which measure the temperature dependence of the initiation of transcription on T7 DNA show sharp transitions near 20 °C (Mangel & Chamberlin, 1974; Chamberlin et al., 1976; Miller & Burgess, 1978; Dausse et al., 1972), as do experiments which measure the production of discrete RNA transcripts (Stahl & Chamberlin, 1977; Dausse et al., 1976).

The sensitivity of different transcription assays to solution conditions such as temperature and monovalent and divalent cation concentrations has led to a model of the selective initiation of transcription (Walter et al., 1967; Travers, 1974; Chamberlin, 1974, 1976). The model postulates the existence of two distinct RNA polymerase–promoter binary complexes before the formation of an RNA polymerase–promoter–nucleoside triphosphate ternary complex. The initial binary complex is formed between the polymerase and double-stranded promoter (closed complex) and is followed by the formation of a second, more stable complex between the polymerase and locally separated strands of DNA at or near the promoter (open complex). The model proposes that the transition between the closed and open complexes is affected by solution conditions.

The selectivity observed between 0 and 17 °C demonstrates the existence of a complex of intermediate stability between general nonspecific and high-temperature promoter complexes. This observation complements the electron microscope data of Williams & Chamberlin (1977) which showed that RNA polymerase is preferentially bound at or near the A1 and A3 promoters of T7 at 0 °C. The direct demonstration of low-

temperature complexes of intermediate affinity supports the notion of two distinct stable RNA polymerase–promoter complexes. The abrupt temperature transition is consistent with the model of DNA melting although the data can also be interpreted in terms of a conformational change in the protein.

The steep pH dependence of RNA polymerase–promoter binding may be interpreted in terms of a requirement for protonation of the RNA polymerase in the formation of the promoter complex. This appears to be true at both 0 and 37 °C although the details of the process may be different at the two temperatures. The quantitative analysis of this data is presented in the following paper (Strauss et al., 1980).

The RNA polymerase–DNA interactions studied here are very sensitive to changes in pH in the physiological range. This has important experimental and perhaps physiological consequences. Most in vitro RNA polymerase binding and initiation studies have been performed near pH 8 using Tris as the buffer (Seeburg & Schaller, 1975; Seeburg et al., 1977; Hinkle & Chamberlin, 1972a,b; Mangel & Chamberlin, 1974; von Gabain & Bujard, 1977). Because the pK of Tris is a sensitive function of temperature and because of the steep pH dependence of the RNA polymerase–promoter interaction, we have concluded that Tris is not a satisfactory buffer for studies involving promoter binding. To reduce the problems of maintaining (or correcting the binding data to) a constant pH over a range of temperatures, we have used Hepes buffer for which the temperature coefficient of the pK is substantially smaller. In the determination of the temperature dependence of promoter binding (Figure 2), the pH of Hepes buffer only decreased from 7.50 at 0 °C to 7.35 at 37 °C whereas in Tris, the corresponding change would have been from pH 8.30 (at 0 °C) to 7.35 (at 37 °C).

A comparison of the steep pH dependence of RNA polymerase–promoter complexes presented here with the slight to moderate dependence found for RNA polymerase–nonpromoter complexes (deHaseth et al., 1978) indicates that the equilibrium selectivity ratio decreases with increasing pH. It is possible that selective binding at 0 °C has not been observed previously by filter binding [see Seeburg et al. (1977)] because the experiments were performed at too high a pH.

Conclusion

We have shown that *E. coli* RNA polymerase can bind selectively to a promoter DNA fragment (in preference to nonpromoter DNA fragments) over a wide variety of solution conditions. This selectivity does not require Mg^{2+} or any other divalent cation and clearly exists even at 0 °C. We also find a large dependence of RNA polymerase binding and selectively on the pH of the solution. Finally, we note agreement between our results on the selectivity of RNA polymerase binding as a function of temperature and the models which include two distinct binary complexes of RNA polymerase–promoter binding proposed by Chamberlin (1974, 1976), Travers (1974), and Walter et al. (1967).

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Binding of *Escherichia coli* Ribonucleic Acid Polymerase Holoenzyme to a Bacteriophage T7 Promoter-Containing Fragment: Evaluation of Promoter Binding Constants as a Function of Solution Conditions†

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ABSTRACT: In this paper we obtain thermodynamic and molecular information about the specific complexes formed between *Escherichia coli* RNA polymerase holoenzyme and a restriction fragment of T7 D111 DNA carrying the A1 and D promoters. Specific binding was observed at both 0 and 37 °C over a wide range of pH values and ion concentrations [Strauss, H. S., Burgess, R. R., & Record, M. T., Jr. (1980) *Biochemistry* (first paper of four in this issue)]. The specific complexes formed at these two temperatures may correspond to the closed and open promoter complexes discussed by Chamberlin [Chamberlin, M. J. (1976) *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 159-161, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY]. Promoter binding constants K_{obsd}^{RP} are obtained from competition filter binding data by using a statistical analysis and previously determined values of the nonspecific holoenzyme-DNA binding constant K_{obsd}^{RD}. From the magnitudes of K_{obsd}^{RP} at 0 and 37 °C, and the dependences of these binding constants on pH and ion concentrations, we conclude that, under physiological ionic conditions, both the 0 and the 37 °C complexes are stabilized to a large extent by the formation of ionic interactions and the accompanying release of counterions and that one or two protonation events ($pK \sim 7.4$) are required for complex formation in both cases. However, the 0 and 37 °C complexes differ in their sensitivity to ion concentrations as well as in the magnitude of K_{obsd}^{RP}, and we conclude that the two complexes are distinct. (More counterion release accompanies formation of the 37 °C complex). Comparisons of the two complexes with one another and with nonspecific holoenzyme-DNA complexes are drawn from the binding data. We have also examined the equilibrium selectivity ratio ($K_{obsd}^{RP}/K_{obsd}^{RD}$) and find it to be a sensitive function of temperature and ionic conditions. Selectivity of holoenzyme for promoter sites on the promoter-containing fragment is higher at 37 °C than at 0 °C under the conditions investigated. Selectivity at either temperature is increased by reducing the pH (in the range 6.1-8.6). At 37 °C, selectivity is increased by reducing the salt concentration. Under approximately physiological conditions (0.2 M NaCl and 0.003 M $MgCl_2$, pH 7.4, 37 °C), the equilibrium selectivity ratio is found to be of order of magnitude 10^4.

In the preceding paper (Strauss et al., 1980), the selective binding of *Escherichia coli* RNA polymerase holoenzyme to a 1550 base pair HaeIII restriction fragment of T7 D111 DNA was demonstrated under a variety of solution conditions by using a double-label nitrocellulose filter binding assay. In particular, selectivity could be shown to exist at 0 °C (at or below neutral pH) over a wide range of NaCl concentrations, in the presence or absence of $MgCl_2$. This evidence for a low temperature specific complex between RNA polymerase and promoter regions confirms and extends the results of Williams & Chamberlin (1977), who found specificity of binding of holoenzyme to a T7 promoter fragment at 0 °C by electron microscopy. This species may represent the specific but

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