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Quantitation of the Interaction of *Escherichia coli* RNA Polymerase Holoenzyme with Double-Helical DNA Using a Thermodynamically Rigorous Centrifugation Method†

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ABSTRACT: The nonspecific interaction of *E. coli* RNA polymerase holoenzyme with native bacteriophage P22 DNA was characterized by means of a rigorous centrifugation technique. A bound polymerase molecule was found to cover 42 base pairs, which implies that the configuration of the protein on DNA is similar for both specific and nonspecific binding. The data at each ionic strength studied can be fit well with single average association constants, $K = 4.7 \times 10^5$ M^{-1} at 0.15 M Na^+ and $K = 7.8 \times 10^4$ M^{-1} at 0.20 M Na^+. (These values for K are for DNA concentrations in moles of base pairs per liter.) Binding to phage T7 DNA was found to be similar. About seven ionic interactions are involved in the binding. At least 500 polymerase molecules can bind simultaneously to P22 DNA (or to T7 DNA) at 0.15 M Na^+; these 500 sites appear to have approximately the same affinity for holoenzyme. The sedimentation approach indicates that the G-C-rich DNA from *M. luteus* has fewer nonspecific binding sites for RNA polymerase. The notion that holoenzyme binds preferentially to A-T-rich regions was investigated by using synthetic DNAs. Very tight binding was seen to both poly[d(A-T)] and poly(dA)·poly(dT). However, a considerable (and unexpected) interaction was also observed with poly(dG)·poly(dC) at 0.20 M Na^+, indicating that variations in the affinity of polymerase for different "natural" DNA sequences are not trivially related to G-C content. The results presented here agree very well with those obtained previously by deHaseth et al. [deHaseth, P. L., Lohman, T. M., Burgess, R. R., & Record, M. T., Jr. (1978) *Biochemistry 17*, 1612], using DNA–cellulose chromatography. The magnitudes and salt dependences of K are similar, and we too find little effect on nonspecific binding of temperature or pH. The binding characterized here resembles the "tight-binding" complexes of Kadesch et al. [Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980) *J. Mol. Biol. 136*, 79] in some but not all respects. The large number of relatively high-affinity nonspecific binding sites must be accounted for in proposed mechanisms of promoter site selection by RNA polymerase.

RNA polymerase binds tightly to its specific functional sites on DNA (promoters) and displays a lesser but marked affinity for other DNA regions as well. Such "nonspecific" binding apparently modulates transcription in vivo; studies with an *E. coli* minicell producing strain show that there is no active RNA polymerase in the cytoplasm, implying that all enzyme molecules are chromosome bound in the bacterium (Cohen et al., 1968; Rünzi & Matzura, 1976). Nonspecific binding thus plays a role in the mechanism whereby polymerase seeks and finds promoter sites at which to initiate transcription, and characterization of this binding is essential for interpretation

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of both in vivo and in vitro experiments on polymerase function. Physical studies of the nonspecific association of RNA polymerase with DNA yield information about the gross features of the interaction such as how tight the binding is or whether there is base sequence or base composition specificity. The results of such studies will also be relevant to elucidation of polymerase–*promoter* interactions, since both nonspecific and specific binding surely share common features.

By means of band sedimentation in sucrose gradients, Richardson (1966) and Pettijohn & Kamiya (1967) established that the number of RNA polymerase molecules which can bind to DNA far exceeds the number of true promoter regions. Hinkle & Chamberlin (1972a,b) performed extensive studies by using a nitrocellulose filter assay to characterize "A" (high-affinity) and "B" (nonspecific) sites for holoenzyme on T7 DNA. Record and co-workers (deHaseth et al., 1978) used DNA-cellulose chromatography to quantify the nonspecific interactions of both core and holoenzyme with calf thymus DNA. They later verified their conclusions by applying difference boundary sedimentation to the polymerase–T7 DNA system (Lohman et al., 1980). Williams & Chamberlin (1977) have developed a technique for measuring DNA–protein interactions by electron microscopy. In addition to identifying true promoter regions, they reported two types of nonspecific holoenzyme–T7 DNA binding sites having widely different association constants. They initially attributed the higher affinity complexes to enzyme which had somehow been damaged in handling. Kadesch et al. (1980a,b) later showed that these complexes are not artifacts and that T7 DNA has about 100 such tight-binding (TB) sites measured by electron microscopy, in addition to "random" nonspecific sites of much lower affinity. The TB complexes have similarities to those described by de Haseth et al. (1978); the measured association constants are of the same order of magnitude, and both groups of workers show that the binding diminishes sharply at higher salt concentrations.

At ionic strengths typically used in in vitro studies of holoenzyme–promoter interactions, the association constant for polymerase binding to TB sites is greater than 10^8 M^{-1}. Kadesch et al. (1980a) point out, however, that such a large nonspecific affinity is inconsistent with a "free-diffusion" model for the process by which RNA polymerase seeks and finds promoters. In this model, for which they have some preliminary support, complexes of polymerase with nonpromoter DNA are transient, with rapid rates of dissociation. They therefore propose that the bulk of nonspecific binding under typical solution conditions is of the low-affinity "random" type so that the nonspecific association constants derived from DNA–cellulose chromatography may not be directly revelant to the mechanism of polymerase–promoter binding.

Both de Haseth et al. (1978) and Kadesch et al. (1980a,b) discuss and deal with a number of possible artifacts in their respective experimental approaches. We report here studies of nonspecific RNA polymerase–DNA interactions using a third technique—a sedimentation method which circumvents some problems in the column chromatography and electron microscopy experiments and which yields new information about the characteristics of holoenzyme binding to a variety of double-helical DNAs under various reaction conditions.

Materials and Methods

Poly[d(A-T)] was obtained from P.-L. Laboratories; poly(dA)·poly(dT), poly(dG)·poly(dC), and *Micrococcus luteus* DNA (ML DNA) were from Sigma Chemical Co. These DNAs were routinely phenol extracted before use. Their sizes were characterized by centrifugation; $s_{20,w}$ values were 5.4 S for poly[d(A-T)], 11.4 S for poly(dA)·poly(dT), 9.2 S for poly(dG)·poly(dC), and 18.5 S for ML DNA.

Salmonella typhimurium and bacteriophage P22 were gifts of Dr. Jonathan King. Phage P22 containing a lysis mutation was grown in a nonpermissive host. The bacteria were lysed with chloroform, and the phages were then purified by CsCl centrifugation. The DNA was recovered after thrice phenol extracting the phage. This DNA sedimented with a very sharp boundary, having $s_{20,w}$ = 25.1 S.

Phage T7 and an *E. coli* B host were provided by Dr. Loren Snyder. Lysates from 100 agar plates were combined, and the phages were banded in CsCl. The DNA was purified by three phenol extractions. Native T7 DNA sedimented as a very sharp boundary with $s_{20,w}$ = 28.3 S.

RNA polymerase was isolated from *E. coli* K-12 strain PR7 (Reiner, 1969), which was obtained from the *E. coli* Genetic Stock Center, Yale University (stock center no. 4948). Holoenzyme was prepared by the method of Burgess & Jendrisak (1975) as modified by Lowe et al. (1979) to include a single-stranded DNA–agarose column (Nüsslein & Heyden, 1972). The holoenzyme thus obtained was determined to be about 70% σ saturated by scanning sodium dodecyl sulfate–polyacrylamide gels which had been stained with Coomassie blue. This is a typical level of σ saturation to be expected from this purification procedure (Lowe et al., 1979).

A part of the holoenzyme preparation was used for isolation of σ factor by the method of Lowe et al. (1979), with tandem columns of Bio-Rex 70 and Whatman DE-52 DEAE-cellulose. The σ factor obtained was >95% pure by sodium dodecyl sulfate–polyacrylamide gels. Holoenzyme saturated with σ was prepared by adding some of this pure σ to the 70% σ-saturated material described above. This resulted in greater than 100% saturation as deduced by scanning of sodium dodecyl sulfate–polyacrylamide gels and also by centrifugation experiments which revealed the presence of a small amount of slower moving excess σ factor.

The enzyme was further characterized by the assay of Chamberlin et al. (1979). The protocol described therein was followed exactly except that [^{14}C]ATP was used instead of [α-^{32}P]CTP. Holoenzyme was stored in 50% glycerol at -20° C in the storage buffer of Burgess & Jendrisak (1975).

Williams & Chamberlin (1977) suggested that rapid dilution of RNA polymerase could produce some damaged enzyme molecules with altered DNA affinity. We tested this possibility by preparing polymerase–DNA solutions both by direct addition of enzyme to the DNA solution and by the slow dilution technique suggested by Gonzalez et al. (1977). We compared these solutions by using our centrifugation technique and found that they behaved identically. Therefore, we made holoenzyme–DNA mixtures for binding studies by carefully adding enzyme from the storage bottle to a DNA solution at an appropriate salt concentration, and then gently vortexing to assure mixing.

The following extinction coefficients were used to evaluate concentrations of components in our experiments. For RNA polymerase, ϵ_{280} was taken to be 3.0×10^5/mol [using $\epsilon_{280}^{1\%}$ = 6.5 (Burgess, 1976) and a molecular weight of 460 000 (Lowe et al., 1979)]; values of $\epsilon_{260} = 1.68 \times 10^5$ and $\epsilon_{230} = 2.63 \times 10^6$ were derived from the measured ultraviolet absorbance spectrum of the protein corrected for a small nonzero optical density at 340 nm. Extinction coefficients for the double-helical DNAs per mole of base pairs are the following: P22 DNA, ϵ_{260} = 13 000 (Felsenfeld & Hirschman, 1965) and ϵ_{230} = 5300 (from spectral measurements); T7 DNA, ϵ_{260} = 13 000; ML DNA, ϵ_{260} = 13 930 (Felsenfeld & Hirschman, 1965);

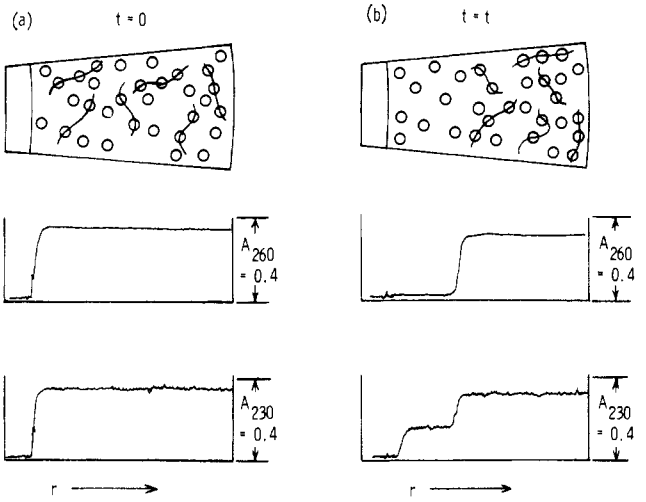

FIGURE 1: Schematic diagram of centrifugation experiment, showing scans at two wavelengths (a) at the start of an experiment and (b) at a later time ($t = t$) at which the faster moving DNA–protein complexes have moved about halfway down the cell. The complexes can be characterized by using the sedimentation coefficient and the absorbances at 260 and 230 nm of the rapidly moving section of the sedimenting boundary. The slower moving material is identified as RNA polymerase from its sedimentation and absorption characteristics.

poly[d(A-T)], ϵ_{262} = 13 300 (Inman & Baldwin, 1962); poly(dA)·poly(dT), ϵ_{260} = 12 000; poly(dG)·poly(dC), ϵ_{253} = 14 800 (Wells et al., 1970).

The protocol of Laemmli (1970) was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gel tubes (5-mm diameter) were silanized before use. The 8.75%, 10-cm-long gels were stained after electrophoresis with Coomassie blue, destained, and scanned at 550 nm by using a Gilford spectrophotometer equipped with a gel scanner device and a chart recorder.

Boundary sedimentation in alkaline solution, 0.9 M NaCl and 0.1 M NaOH (Studier, 1965), was performed in the analytical ultracentrifuge by using cells with Kel-F centerpieces.

Binding experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with absorption optics and a photoelectric scanner. The method is a modification of that of Jensen & von Hippel (1977) and has been successfully used to determine thermodynamic parameters for the nonspecific binding to DNA of *lac* repressor (Revzin & von Hippel, 1977) and of the catabolite activator protein (Saxe & Revzin, 1979). A typical experiment is illustrated schematically in Figure 1. The DNA–protein solution is uniformly distributed in the cell at the start of the run. As sedimentation occurs, the polymerase–DNA complexes move rapidly down the cell, leaving behind the slower moving unbound protein. The "scanner" device of the instrument yields the absorbance (concentration) distribution throughout the cell at any time during the run. The concentrations of DNA and RNA polymerase at each point in the centrifuge cell can then be evaluated by analysis of the absorbance readings at two wavelengths (260 and 230 nm were routinely used) since changes in absorbance when the enzyme binds to DNA are negligible. The complexes are always in equilibrium with free protein. Each run permits evaluation of the concentrations of free and bound protein to yield one point on a Scatchard (1949) plot. A set of experiments with various input concentrations of the macromolecules generates a complete plot, which can be analyzed by using the theory of McGhee & von Hippel (1974) for the noncooperatve binding of large ligands to DNA.

The following protocol was used to determine values of ν (binding density, moles of RNA polymerase bound/mole of DNA base pairs) and L_f (concentration of unbound protein). Prior to centrifugation, the absorbance spectrum of each DNA–protein solution was read in the Gilford spectrophotometer, and the concentrations of polymerase and DNA were determined by a two-wavelength analysis. These concentrations always agreed closely with the expected values, based on the amounts of DNA and enzyme pipetted into the solution. The concentration of free protein (L_f) was determined following centrifugation by using the absorbance of the slower moving protein. Due to the wide spectral band-pass in the model E optical system, it was necessary to determine calibration factors to convert absorbance readings from the centrifuge runs to correspond to readings in the Gilford (Saxe & Revzin, 1979). Finally, by use of the value for L_f and the input polymerase and DNA concentrations, the value of ν was computed. A check on these results comes from values of ν determined from analysis of the absorbances at 260 and 230 nm of the faster moving DNA–protein complexes. The two approaches to evaluating ν gave good agreement.

The centrifugation technique can conveniently be used to survey holoenzyme-DNA interactions under a variety of circumstances. This does not require generation of complete Scatchard plots but can be done by comparative experiments. For example, to determine the temperature dependence of RNA polymerase–DNA binding, 1.5 mL of a DNA–protein solution was prepared and maintained at 4 °C. A 0.4-mL aliquot was removed, placed in a cold centrifuge cell, and centrifuged at 5 °C. A second aliquot was incubated 2 h later at 37 °C for 10 min, then transferred to a prewarmed centrifuge cell at 37 °C by using prewarmed syringes, and immediately centrifuged at 35 °C. The remaining solution was brought to 20 °C 2 h after this procedure, its absorbance spectrum was read in the Gilford, and it was then centrifuged at 20 °C. Thus, measurements were made on the same DNA–protein solution at different temperatures. The fact that this solution was kept at 4 °C for several hours between the first and last runs should not affect the results; we found no variation in nonspecific binding behavior even after a polymerase–DNA solution was kept at 20 °C for 6 h. The amount of unbound polymerase in each solution is a measure of any changes in binding affinity over the 5–35 °C temperature range. For each temperature, the quantities ν and ν/L_f were evaluated; these were used, along with the value $n = 42$ (see below), to determine the association constant from the equation of McGhee & von Hippel (1974).

Similar comparative experiments were done with holoenzyme–DNA solutions at different pH values. In this case, nominally identical solutions were prepared separately at pHs 7.4, 7.9, and 8.4, and the actual DNA and polymerase concentrations were evaluated from spectral data.

Unless otherwise noted, buffer solutions contained 10 mM Tris-HCl, 0.1 mM Na_2EDTA, and 5% glycerol, plus NaCl to give the desired $[Na^+]$, pH 7.9 at 20 °C. For experiments which involved variation of temperature, the buffers contained 0.01 M Na_2HPO_4 (pH 7.9), 0.1 mM Na_2EDTA, and 5% glycerol, plus NaCl to give the desired total concentration of Na^+.

Results

Characterization of Macromolecules. Since RNA polymerase is known to bind strongly to nicks and gaps in DNA (Hinkle et al., 1972), we used sedimentation in alkaline solution to show that the P22 DNA with which most of our experiments were done contained on average no more than one

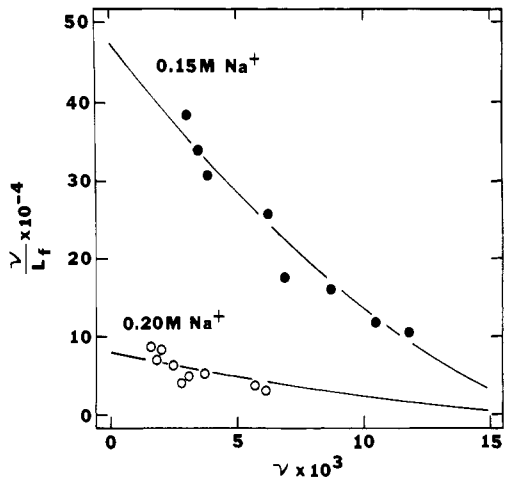

FIGURE 2: Scatchard plot for RNA polymerase–DNA binding at T = 20 °C, pH 7.9: [Na^+] = 0.15 M (●); [Na^+] = 0.20 M (○). The binding density, ν, is in moles of holoenzyme bound per mole of DNA base pairs; L_f is the molar concentration of free (unbound) protein. The theoretical curves were derived by a method of least-squares fit of the data to the equation of McGhee & von Hippel (1974).

nick per whole DNA molecule. The T7 DNA used in some centrifugation experiments and in the assay of Chamberlin et al. (1979) to evaluate polymerase activity had even fewer nicks (about one per three DNA molecules). Possible problems in data interpretation due to imperfections in the synthetic DNAs will be touched on below.

Critical evaluation of many results in the extensive literature on RNA polymerase is hindered by difficulties in comparing data from different laboratories, which may have prepared the enzyme by different protocols. We have thus taken pains to characterize our enzyme as completely as was practical. We showed by gel electrophoresis and centrifugation studies that our polymerase was saturated with σ factor, so we are truly working with pure holoenzyme uncontaminated with core polymerase. Our holoenzyme was found to have a specific activity of 150 milliunits/mg of protein, which corresponds to about 28% active polymerase molecules according to the assay of Chamberlin et al. (1979); this is a typical value for RNA polymerase preparations. We emphasize that our Scatchard plots show no evidence for two classes of holoenzyme behavior in nonspecific binding (see below). That is, the 72% of our molecules which are enzymatically inactive apparently display the same nonspecific binding affinity as the 28% which are active. Furthermore, in a solution at low salt, [Na^+] = 0.10 M, having a DNA to polymerase ratio of 200 base pairs per enzyme molecule (DNA/RP = 200), a centrifugation experiment showed that all polymerase was DNA bound. Since we could have detected as little as 5% of the input protein as free protein, we conclude that all RNA polymerase molecules in our preparation are capable of nonspecific binding.

Pressure Effects on Nonspecific Binding Are Negligible. Harrington & Kegeles (1973) observed that effects of pressure on interactions between macromolecules can, in theory, be enormous. We tested for this possibility in our system by first bringing a DNA–protein solution to 16 000 rpm and observing the amount of unbound protein after the complexes had sedimented about one-third of the way down the cell. The speed was then changed to 36 000 rpm. No change at all was observed in the amount of free polymerase; hence, the increased pressure at higher speed does not affect the equilibrium. Furthermore, as will be seen below, association constants determined from the sedimentation method are in quantitative agreement with those derived from column chromatography experiments at atmospheric pressure (deHaseth et al., 1978).

Table I: Binding of RNA Polymerase Holoenzyme to Double-Helical (P22) DNA[a]

[Na^+] = 0.15 M			[Na^+] = 0.20 M		
T (°C)	pH	K (M^{-1})	T (°C)	pH	K (M^{-1})
20	7.9	4.7×10^5	20	7.9	7.8×10^4
5	7.9	6.3×10^5	5	7.9	10.6×10^4
35	7.9	2.7×10^5	35	7.9	5.5×10^4
20	7.4	12.0×10^5	20	7.4	10.6×10^4
20	8.4	3.7×10^5	20	8.4	5.9×10^4

[a] The binding constants in the first row, at T = 20 °C, pH 7.9, are accurately known from Scatchard plots (Figure 2). The other binding constants in this table were each determined from duplicate experiments as described in the text and are thus likely to be less precise. Units of K involve moles of DNA base pairs per liter.

Binding of Holoenzyme to P22 DNA. It was possible to accurately analyze a series of polymerase–DNA solutions at both 0.15 and 0.20 M Na^+, T = 20 °C, pH 7.9. The Scatchard plots obtained are shown in Figure 2. The data were fit by a method of least-squares approach, using the equation of McGhee & von Hippel (1974) and varying the parameters K (association constant) and n (binding site size, the number of base pairs covered by a bound protein molecule). Excellent fits are obtained over the entire range of data; it is found that $n = 42 \pm 2$ base pairs at both ionic strengths while $K = (4.7 \pm 0.4) \times 10^5$ M^{-1} at 0.15 M Na^+ and $K = (7.8 \pm 0.5) \times 10^4$ M^{-1} at 0.20 M Na^+. (These values of K are computed by using DNA concentrations in moles of DNA *base pairs* per liter.) For our purposes, we may consider P22 DNA to have a random nucleotide sequence, so the values of K are averages over any variations in nonspecific binding affinity of holoenzyme for particular DNA sequences. We emphasize, however, that the data can be fit quite well by assuming that a single value for K is applicable to all nonspecific polymerase–DNA interactions at a given ionic strength.

Our confidence in the results in Figure 2 is heightened by data (not shown) obtained with our original polymerase preparation, which was only 70% σ saturated. In this case, the Scatchard curves resembled those in Figure 2, but at higher values of ν, ν/L_f did not approach zero quite as one would have expected. Since DNA–cellulose chromatography had showed that core polymerase binds more tightly to native DNA than does holoenzyme at these salt concentrations (deHaseth et al., 1978; Lohman et al., 1980), we reevaluated our data by assuming that 30% of the input enzyme was firmly bound to DNA in all solutions. The recalculated Scatchard plots agreed exactly with the results in Figure 2. We are thus confident that Figure 2 accurately reflects the behavior of holoenzyme in its interactions with nonpromoter-containing DNAs.

Polymerase–DNA Binding under Other Conditions. Results of the experiments at different temperatures and pH values are shown in Table I. The association constants for conditions other than T = 20 °C, pH 7.9, are not expected to be as accurate as those determined by full Scatchard analysis, but they do indicate that there is not a strong dependence of K on either temperature or pH.

The effect of Mg^{2+} on the polymerase–DNA interaction was assessed by comparing the amount of unbound enzyme in solutions containing the same concentrations of DNA and protein, at either 0.15 M Na^+, 0.15 M Na^+ plus 0.01 M Mg^{2+}, or 0.20 M Na^+. Approximately the same levels of unbound enzyme were found in the Mg^{2+}-containing and the 0.20 M Na^+ solutions. Thus, Mg^{2+} seems to act as a competitor (with Na^+ and RNA polymerase) for DNA binding sites, and its effect on the interaction primarily reflects its contribution to

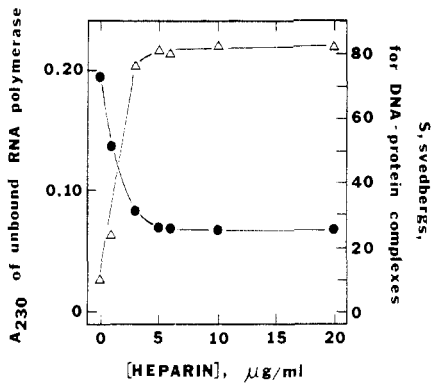

FIGURE 3: Effects of heparin on RNA polymerase–DNA binding (see text) monitored by measurement of the sedimentation coefficient of the faster moving part of the boundary (●) and the absorbance at 230 nm of unbound protein (△). P22 DNA alone was found to have $s_{20,w}$ = 25 S. The total contribution to A_{230} from RNA polymerase in the DNA–protein solutions was about 0.230. Thus, there is no detectable holoenzyme binding at heparin concentrations above 5 μg/mL. These solutions were at 0.15 M Na^+, T = 20 °C, pH 7.9.

the ionic strength of the solution. This is consistent with the conclusions reached by deHaseth et al. (1978) from DNA–cellulose chromatography data.

Nonspecific Binding Is Weakened at Low [Na^+]. While the above results show that RNA polymerase–DNA interactions diminish at higher salt concentrations, we note here that the DNA–protein affinity is decreased at *low* ionic strengths (<0.05 M Na^+) as well. We have not investigated this effect in detail, although we have found it to be more pronounced with ML DNA. Thus, in solutions at 0.01 M Na^+, with a DNA/RP ratio of 250, about 15% of the protein is not bound in a P22 DNA solution while 34% of the polymerase is free if ML DNA is used.

There Are a Large Number of Nonspecific Binding Sites on P22 DNA. It is evident from Figure 2 that at 0.15 M Na^+ a binding density of at least ν = 0.012 can be achieved. Since a bound polymerase molecule covers 42 base pairs, this corresponds to 50% saturation, or roughly 500 binding sites per P22 DNA molecule.

Heparin Sensitivity. Kadesch et al. (1980a) reported that the dissociation rates for tight-binding nonspecific complexes were unaffected by excess heparin. Although all our work involves equilibrium, not kinetic, studies, we felt it worthwhile to check the effects of heparin on holoenzyme–P22 DNA binding. The results are shown in Figure 3, which also illustrates the wealth of data whch can be derived from the centrifugation technique. The extent of the protein-DNA interaction was monitored by measurement of the amount of unbound polymerase (which increases with heparin concentration) and the sedimentation coefficient of the complexes (which decreases toward that of free DNA, since the protein binds less well at higher heparin concentrations). The heparin-induced dissociation is half completed in these solutions at about 2 μg/mL polyanion. If the average molecular weight of heparin is 12000 (Merck Index, 9th ed.), this corresponds to about 1.67×10^{-7} M heparin molecules. Since the concentration of enzyme in these solutions was 1.34×10^{-7} M, it appears that heparin competes very well for RNA polymerase and that only a few molecules of heparin per holoenzyme in the solution can eliminate the interaction with DNA.

Base Sequence Specificity. While the bulk of our studies were performed with P22 DNA, we also studied the binding of RNA polymerase to two other natural DNAs. The nonspecific binding to T7 DNA was identical with that to P22. This was checked at 0.15 Na^+ and 0.20 M Na^+, at DNA/RP ratios of 55 and 42, respectively. The (average) association constants for polymerase interacting with the two DNAs are the same, and the numbers of binding sites per DNA molecule are equal.

The situation is different for the G-C-rich ML DNA. We assayed the binding at 0.10 M Na^+ at a ML DNA/RP ratio of 234 and found 10% of the polymerase molecules free in solution. This contrasts with the result reported above that in a similar solution containing P22 DNA all polymerase molecules were DNA bound. Thus, ML DNA appears to have fewer nonspecific binding sites than does P22 DNA. Furthermore, at 0.10 M Na^+ and at a ML DNA/RP ratio of 102, 30% of the polymerase molecules were not complexed to DNA. These results are not quantitatively consistent with a single association constant for all nonspecific binding sites on ML DNA, so further studies were done with synthetic DNAs.

The affinity of RNA polymerase for the alternating copolymer poly[d(A-T)] is very high. Indeed, at a d(A-T)/RP ratio of 125, all holoenzyme is complexed at ionic strengths up to 0.25 M Na^+, and there is some interaction even up to about 0.45 M Na^+. (In polymerase–P22 DNA solutions, there is little binding at 0.25 M Na^+, and no binding was seen above [Na^+] = 0.30 M.) Complexes of RNA polymerase with poly[d(A–T)] and with all the natural DNAs we tested are soluble over a wide range of ionic strengths, 0.01–0.50 M Na^+. This is not the case with poly(dA)·poly(dT) or poly(dG)·poly(dC). For the former, aggregation occurs at a (dA)·(dT)/RP ratio of 125 at 0.10 M Na^+. At this (dA)·(dT)/RP ratio, there was a small amount of precipitation at 0.20 M Na^+, but it was possible to do a centrifugation experiment, which showed that all polymerase was DNA bound under these conditions. The aggregation is more severe with poly(dG)·poly(dC), occurring to such an extent at 0.20 M Na^+ to render meaningless sedimentation of the solution.

The foregoing results imply that the effects of base sequence on holoenzyme–DNA interactions may not be simple. A generalization which accounts for most of the data is that A-T-rich DNA regions bind polymerase more tightly than do G-C-rich sequences. However, the poly(dG)·poly(dC) data do not support this conclusion. Since holoenzyme–P22 DNA and holoenzyme–ML DNA solutions at 0.20 M Na^+ contain much protein which is not DNA bound, one might expect little if any binding to poly(dG)·poly(dC); nevertheless, there clearly are DNA–protein interactions in this system which lead to precipitation of virtually all the complexes even at 0.20 M Na^+. Interpretation of data for the synthetic DNAs is further complicated by the fact that these are not completely double-helical molecules; the poly[d(A-T)] presumably contains hairpins while the poly(dA)·poly(dT) and poly(dG)·poly(dC) will have nicks and gaps.

Discussion

We have applied a thermodynamically rigorous centrifugation technique to characterize the nonspecific DNA binding of RNA polymerase holoenzyme. Scatchard analysis of the data reveals that 42 base pairs of P22 DNA are covered by a bound polymerase molecule. This is in excellent agreement with results from RNA polymerase–*lac* promoter studies which showed that bound enzyme protects about 43 base pairs from nuclease digestion (Gilbert, 1976) and that bound polymerase affects the methylation of a 38 base-pair section of the promoter (Johnsrud, 1978). Our approach yields a somewhat larger site size than the n = 28 reported by Reisbig et al. (1979) based on studies of optical changes accompanying polymerase–poly[d(A–T)] binding. Our results are consistent with the statement that holoenzyme has the same configuration

on DNA whether bound specificially or nonspecifically. This is particularly interesting in view of low-angle X-ray data which show that polymerase has an elongated shape, with a maximal dimension of about 150 Å (Pilz et al., 1972), a length which corresponds to about 44 DNA base pairs.

Our quantitative conclusions are based on measurements made by using well-characterized macromolecules; the polymerase was truly σ saturated, and the extent of double-helix imperfections in P22 DNA was shown to be negligible. All RNA polymerase molecules were found to be capable of nonspecific binding, and there was no evidence for grossly different binding behavior for the 28% of the molecules which were "active" as defined by the enzymatic assay of Chamberlin et al. (1979). Interpretation of the centrifugation data requires only one assumption, namely, that pressure effects can be neglected. We have provided data to justify this assumption. It is gratifying that our results are in excellent agreement with those obtained by deHaseth et al. (1978) with calf thymus DNA–cellulose chromatography at normal pressure. The association constant reported by those workers at 0.20 M Na^+ is about 2×10^5 M^{-1} (with DNA concentration in base pairs) while we find $K = 0.8 \times 10^5$ M^{-1}. These values are remarkably close, considering the differences in the techniques used to obtain them. We observe, as did deHaseth et al. (1978), that pH and temperature have little effect on the binding. On the other hand, there is a marked ionic strength dependence. Applying the theory of Record et al. (1976) to our binding constants at 0.15 and 0.20 M Na^+ yields the result that 7 ± 1 ionic interactions accompany the nonspecific binding of RNA polymerase to P22 DNA. This is in fair agreement with, though somewhat lower than, the values of 11 and 9 ionic interactions derived from column chromatography (deHaseth et al., 1978) and difference boundary sedimentation (Lohman et al., 1980), respectively. The techniques of Record and co-workers are conveniently used for measurements under a variety of solution conditions. Their values for the number of ionic interactions are derived from a series of binding constants while our value arises from data only at 0.15 and 0.20 M Na^+. However, confidence in our result is enhanced by the fact that independent measurements with the 70% σ-saturated polymerase preparation gave exactly the same answer.

The studies on base sequence specificity are interesting though not definitive. While it is reasonable to expect some effects of base sequence on the binding, the data in Figure 2 can nevertheless be fit by single average values for the association constants. Thus, there do not seem to be large variations in the nonspecific binding affinity along P22 DNA or T7 DNA. The situation is different for the G-C-rich DNA from *M. luteus* which, by our technique, does not show as many binding sites as the phage DNAs. It has been proposed that RNA polymerase "melts into" promoters at A-T-rich sequences (Dickson et al., 1975; Siebenlist, 1979). This leads to the notion that holoenzyme will have a higher nonspecific affinity for A-T-rich regions, which is supported by the high-affinity complexes formed with poly[d(A-T)] and poly(dA)·poly(dT), but which is contradicted by the interaction seen with poly(dG)·poly(dC) even at 0.20 M Na^+. Further studies with additional polynucleotides will be needed to clarify this situation.

With respect to the results of Kadesch et al. (1980a,b), we note that the interactions we have characterized here clearly differ from the "random" nonspecific binding complexes seen by electron microscopy. The association constants we have measured are orders of magnitude higher than those reported for "random" complexes (Kadesch et al., 1980a). In fact, the sedimentation technique as used here would not even detect the weak "random" interactions. Furthermore, we find a pronounced salt dependence which is not characteristic of the "random" binding.

The polymerase–P22 DNA interactions we have studied do resemble the higher affinity "TB" complexes of Kadesch et al. (1980b). Extrapolation of our data to 0.05 M NaCl yields $K \sim 5 \times 10^8$ M^{-1}, which is in agreement with the association constant deduced by Kadesch et al. (1980b), and the binding is strongly salt dependent. However, we find at least 500 strong binding sites for RNA polymerase on P22 DNA (or T7 DNA) while Kadesch et al. estimate that only about 100 TB complexes can form. Furthermore, we find that equilibrium is reached in a holoenzyme–P22 DNA solution at 5 °C in less than 30 min. (This is the time required to mix solutions, fill cells, bring the centrifuge to speed, and allow the complex to sediment away from the meniscus; in fact, perhaps much less than 30 min is required to reach equilibrium.) In contrast, Kadesch et al. (1980b) report no TB complexes formed after incubation for 8 min at 0 °C. We find that the polymerase–nonspecific DNA binding equilibrium is heparin sensitive, but, to date, we have not studied the effects of heparin on dissociation rates, as did Kadesch et al. (1980b). Experiments along this line are under way.

To conclude then, we observe that under solution conditions normally used for in vitro RNA polymerase assays (and which may reasonably approximate the physiological situation) there are a large number of relatively high affinity nonspecific binding sites for holoenzyme on typical DNAs. These must be taken into consideration when postulating models of promoter site selection.

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Mechanism of Action of Moloney Murine Leukemia Virus RNA-Directed DNA Polymerase Associated RNase H (RNase H I)†

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ABSTRACT: The mechanism of action of the ribonuclease H (RNase H) activity associated with Moloney murine leukemia virus RNA-directed DNA polymerase (RNase H I) and the two-subunit ($\alpha\beta$) form of avian myeloblastosis virus DNA polymerase were compared by utilizing the model substrate $(A)_n \cdot (dT)_n$ and polyacrylamide gel electrophoresis in 7 M urea to analyze digestion products. Examination on 25% polyacrylamide gels revealed that a larger proportion of the RNase H I oligonucleotide products generated by limited digestion of $[^3H](A)_{\langle 1100 \rangle} \cdot (dT)_n$ were acid insoluble (15–26 nucleotides long) than acid soluble (less than 15 nucleotides long), while the opposite was true for products generated by $\alpha\beta$ RNase H. RNase H I was capable of attacking RNA in RNA·DNA in the 5′ to 3′ and 3′ to 5′ directions, as demonstrated by the use of $[^3H,3'\text{- or } 5'\text{-}^{32}P](A)_{\langle 380 \rangle} \cdot (dT)_n$ and cellulose–$[^3H](A)_n \cdot (dT)_n$. Both RNase H I and $\alpha\beta$ RNase H degraded $[^3H](A)_n \cdot (dT)_n$ with a partially processive mechanism, based upon classical substrate competition experiments and analyses of the kinetics of degradation of $[^3H,3'\text{- or } 5'\text{-}^{32}P](A)_{\langle 380 \rangle} \cdot (dT)_n$. That is, both enzymes remain bound to a RNA·DNA substrate through a finite number of hydrolytic events but dissociate before the RNA is completely degraded. Both RNase H I and $\alpha\beta$ RNase H were capable of degrading $[^{14}C](A)_n$ in $[^3H](C)_n$–$[^{14}C](A)_n$–$[^{32}P](dA)_n \cdot (dT)_n$, suggesting that retroviral RNase H is capable of removing the tRNA primer at the 5′ terminus of minus strand DNA at the appropriate time during retroviral DNA synthesis in vitro.

Retrovirus RNA-directed DNA polymerase (reverse transcriptase) has been studied extensively in recent years (Temin & Baltimore, 1972; Green & Gerard, 1974; Wu & Gallo, 1977; Verma, 1977; Gerard & Grandgenett, 1980). Such effort is warranted since the reverse transcriptase molecule is extremely complex. The prototype for the avian enzyme, avian myeloblastosis virus (AMV)[1] $\alpha\beta$ reverse transcriptase, has multiple enzymatic activities, i.e., DNA polymerase (Baltimore, 1970; Mizutani & Temin, 1970), RNase H (Moelling et al., 1971), and DNA endonuclease (Golomb & Grandgenett, 1979; Grandgenett et al., 1980), as well as distinct nucleic acid binding activities, i.e., ability to selectively bind to $tRNA^{Trp}$ (Panet et al., 1975) and to unwind RNA·DNA and duplex DNA (Collett et al., 1978). The single-subunit α form of AMV reverse transcriptase lacks at least two of these activities, DNA endonuclease (Golomb & Grandgenett, 1979) and ability to selectively bind to $tRNA^{Trp}$ (Grandgenett et al., 1976). Murine retrovirus reverse transcriptase resembles AMV α in being a single-subunit enzyme (Moelling, 1976; Verma, 1975) that lacks DNA endonuclease activity (Moelling, 1974; Verma, 1975) and ability to selectively bind to its putative primer, $tRNA^{Pro}$ (Haseltine et al., 1977).

The RNase H activity of the avian enzymes has been thoroughly characterized. Both AMV α and $\alpha\beta$ RNase H are exonucleases that attack RNA·DNA in either the 5′ to 3′ or

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[1] Abbreviations used: AMV, avian myeloblastosis virus; M-MLV, Moloney murine leukemia virus; the symbols ⟨⟩ indicate average values.

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