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Direct Evidence for the Preferential Binding of *Escherichia coli* RNA Polymerase Holoenzyme to the Ends of Deoxyribonucleic Acid Restriction Fragments†

Paul Melançon,‡ Richard R. Burgess, and M. Thomas Record, Jr.*

ABSTRACT: *Escherichia coli* RNA polymerase holoenzyme has been observed to form a variety of nonpromoter complexes with DNA restriction fragments in experiments performed with the nitrocellulose filter assay [Melançon, P., Burgess, R. R., & Record, M. T., Jr. (1982) *Biochemistry 21*, 4318–4331]. Here we report the use of this assay to investigate aspects of the weak (heparin-sensitive) interactions of RNA polymerase core and holoenzyme with a 1600 base pair (bp) fragment of T7 DNA which contains no promoters or TB (tight binding; heparin-resistant) sites. Under the ionic conditions investigated [50 mM NaCl/10 mM $MgCl_2$/10 mM sodium *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (pH 7.7)], both core and holoenzyme bind to the linear DNA fragment and cause comparable levels of filter retention. When the DNA fragment is self-ligated into a circular molecule (nonsupercoiled), the extent of binding of holoenzyme (but not that of core) is dramatically reduced. This directly proves our previous hypotheses that holoenzyme recognizes and preferentially binds to the ends of DNA fragments and that this mode of binding is responsible for most of the heparin-sensitive filter retention of nonpromoter fragments. The residual mode of binding of holoenzyme detected with the circular DNAs was considered in determining the amount of protein bound at ends only. To calculate end-binding constants (K_E), the amount of protein bound nonspecifically (which does not appear to cause efficient filter retention) was also taken into consideration. At 0 °C, we obtain a value for K_E of $(2.1 \pm 0.5) \times 10^8$ M^{-1}, in good agreement with that determined earlier. This value of K_E is relatively constant over the temperature range 0–37 °C. The magnitude of K_E indicates that ends can effectively compete with some promoters for RNA polymerase. Therefore, for in vitro promoter binding studies where enzyme is not in excess, end binding (like nonspecific binding) must be considered in the analysis of the promoter binding data, as discussed earlier [Shaner, S. L., Melançon, P., Lee, K. S., Burgess, R. R., & Record, M. T., Jr. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 463–472]. The apparent greater specificity for DNA ends of holoenzyme relative to core polymerase is discussed in terms of a steric model in which the σ subunit helps to reduce the affinity of holoenzyme for interior DNA sites through unfavorable steric contacts that are absent in an end complex.

Escherichia coli RNA polymerase (RNAP),[1] the multisubunit enzyme responsible for the synthesis of RNA in *E. coli*, exists in two major forms: core (subunit structure $\alpha_2\beta\beta'$) and holoenzyme ($\alpha_2\beta\beta'\sigma$). The additional presence of the σ subunit allows the holoenzyme to recognize specific (promoter) regions on the DNA, from which RNA synthesis is correctly and efficiently initiated. Both forms of the enzyme exhibit general affinities for DNA. Some nonpromoter interactions of holoenzyme may play a role in the promoter search mechanism [see von Hippel et al. (1982)]. In addition, they reduce significantly the solution concentration of holoenzyme under most in vitro conditions used to investigate binding to promoters. When the nitrocellulose filter binding assay is used, the potential for retention of DNA by RNAP bound at nonpromoter sites must be considered in addition to the reduction of free enzyme concentration. A wide range of techniques has been used to study such nonspecific interactions. A survey of the results is provided by Shaner et al. (1983).

We have recently studied the interactions between RNAP holoenzyme and an unfractionated *Hae*III digest of T7 DNA by using the nitrocellulose filter binding assay (Melançon et al., 1982). Two major classes of nonpromoter interactions were detected by this assay. [Other assays can detect and quantify a third weaker class of nonpromoter complexes [cf. Shaner et al. (1983) and Kadesch et al. (1981a, 1982)], but that class is not likely to give rise to efficient filter retention.] The two classes detected by filter binding consist of (i) complexes which form instantaneously on the time scale of mixing and are sensitive to a 10-s challenge with the polyanion heparin and (ii) complexes which form more slowly ($t_{1/2}$ = 2–3 min), are resistant to a heparin challenge, and are designated TB (for tight binding; Kadesch et al., 1981b). At 0 °C, only the first class is detected. Whereas the first class of complexes was observed on all *Hae*III fragments investigated, TB complexes appear to be located on only a subset of the fragments. This last property allowed the isolation of DNA restriction fragments that did not appear to carry either promoter or significant TB sites and that were therefore suitable to study the heparin-sensitive binding detected by the filter assay. The comparison of binding constants determined by using DNA molecules of different lengths (800 and 2000 bp) provided strong but indirect evidence that the ends of DNA fragments show a markedly higher affinity for RNAP holoenzyme than do random interior sites and that end binding could be responsible for most of the filter retention of promoter-free DNA fragments (Melançon et al., 1982). Using the same end-binding hypothesis but taking into consideration the reduction

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[1] Abbreviations: RNAP, *Escherichia coli* RNA polymerase; TB, tight binding; BSA, bovine serum albumin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); L, linear; CC, closed circular; NC, nicked circular.

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in the solution concentration of RNAP caused by nonspecific binding, Shaner et al. (1983) reanalyzed those data and calculated values for the end-binding constant (K_E) for *Hae*III cut, blunt-ended fragments. Values for K_E, measured in the presence of 0.01 M $MgCl_2$, ranged from 10^7 M^{-1} (in 0.2 M NaCl) to 10^9 M^{-1} (in 0.05 M NaCl).

Previous studies performed either with DNA polymerase and exonuclease competition assays (Berg et al., 1965) or by electron microscopy (Lescure et al., 1976; Hirsh & Schleif, 1976; Williams, 1977; Williams & Chamberlin, 1977; Kingston et al., 1981) indicated that RNAP binds to the ends of DNA fragments. However, these studies did not provide a quantitative analysis of end binding and therefore could not discuss the preference of RNAP holoenzyme for ends.

In the work presented here, we have compared the binding of RNAP holoenzyme and core polymerase to linear and circular DNA molecules which appear to be free of TB and promoter sites. Our results provide direct and quantitative evidence for the preferential binding of RNAP holoenzyme to DNA ends and demonstrate that end binding is responsible for most but not all of the observed heparin-sensitive filter retention of nonpromoter DNA by holoenzyme. This therefore confirms the validity of the end-binding hypothesis used in our previous analysis of data on the heparin-sensitive interactions detected by filter binding (Melançon et al., 1982; Shaner et al., 1983). The value for K_E that we calculate in this work by using a single 1600 bp fragment is in very good agreement with the one obtained earlier under similar conditions for mixed populations of 800 bp fragments, and of 2000 bp fragments (Shaner et al., 1983). Those values of K_E, determined as a function of [Na^+] (Shaner et al., 1983), can therefore be used to estimate the extent of end binding under a variety of incubation conditions.

Materials and Methods

Chemicals and Buffers. Tris and Na^+-Hepes were from Sigma Biochemical. BSA was from Miles (Pentex), dithiothreitol from Eastman Kodak Co., and agarose from Bio-Rad Labs. Liquified phenol (Mallinckrodt) was distilled and stored at −20 °C until use. Salts were reagent grade. Na^+-Hepes and $MgCl_2$ were dried and kept in a desiccator. All solutions were prepared with distilled water which was filtered and deionized through a Milli Q-3 Millipore system. TE buffer consists of 5 mM Tris-HCl (pH 7.9) and 0.5 mM EDTA. The binding reactions were performed in binding buffer (BB) containing 50 mM NaCl/10 mM $MgCl_2$/10 mM Na^+-Hepes/0.1 mM EDTA/0.1 mM dithiothreitol and 20 μg/mL BSA. The pH of BB was adjusted with HCl such that the pH was 7.7 at the temperature investigated, using a value for $\Delta pK_a/\Delta T$ of −0.015 (Good et al., 1966). After filtration, the filters were rinsed with a solution containing 10 mM Tris-HCl (pH 7.9) and 0.1 M NaCl. Preparative ligation was carried out in a buffer (LB) consisting of 10 mM Tris (pH 7.7)/50 mM NaCl/10 mM $MgCl_2$/5 mM dithiothreitol/0.25 mM ATP and 50 μg/mL BSA. Gel electrophoresis was done in 40 mM Tris base/20 mM acetic acid/2 mM EDTA (pH 8.1).

RNA Polymerase. The enzyme preparation used in this work is the same one we used previously (Melançon et al., 1982). RNA polymerase from the K-12 strain of *E. coli* was prepared by the method of Burgess & Jendrisak (1975) as modified by Lowe et al. (1979). The σ subunit and core enzyme were prepared from purified holoenzyme on a Bio-Rex 70 column (Lowe et al., 1979). The holoenzyme (87% σ saturated) was found to be 66 ± 10% active, by using the assay of Chamberlin et al. (1979). For the filter binding experiments, holoenzyme was supplemented with purified σ to yield a σ content of 110 ± 10%. The determination of protein concentration and the dilution of protein for the binding experiments were performed as described by Melançon et al. (1982).

Plasmid Construction. Three DNA fragments with an average length of ~800 bp were coisolated from the *Hae*III digest of T7DIII DNA by polyacrylamide gel electrophoresis as described by Melançon et al. (1982). From the recently determined sequence of T7 DNA, we infer that the fragments are actually 806, 782, and 781 bp in length and start at positions 3600, 24 180, and 30 780 bp, respectively, from the genetic left end of T7 (Dunn & Studier, 1983). This mixture of fragments was cloned directly by blunt-end ligation at a ratio of 10 insert fragments to 1 cloning vector. The vector was the self-ligated 2243 bp *Rsa*1 fragment of pBR322 (replicated as a plasmid), cut with *Eco*RI and filled in with the Klenow fragment of DNA polymerase I (a generous gift of P. Olins). First, clones were selected in which the insertion of *Hae*III fragments had regenerated *Eco*RI sites at the junctions on both sides of the insert. Two of those with a 1600 bp T7 DNA insert (dimers of the original ~800 bp fragments with a new *Hae*III site in the middle) were selected for further study. The nature of the dimer inserts was unambiguously established by restriction mapping. The insert of pPM103 is made up of the sequence from 24 962 to 24 180 bp linked to that from 3600 to 4406 bp as numbered in Dunn & Studier (1983) (see also Figure 1). The insert of pPM104 contains sequences of 30 780–31 561 bp linked with those from 24 180 to 24 962 bp.

Isolation of Linear and Circular DNAs. Plasmids were grown, labeled in vivo with 5 mCi/L ^3H-labeled thymidine, and treated to the cleared lysate stage as described by Bolivar & Backman (1979). RNase A was used during lysis. After phenol extraction of the cleared lysate, the crude plasmid DNA was separated from RNA fragments by chromatography on a 300-mL Sephacryl S-300 column (Norgard, 1981). The T7 insert DNA fragments were separated from vector DNA by digestion with *Eco*RI (New England BioLabs and Biotec) and preparative agarose gel electrophoresis in the presence of 0.1 μg/mL ethidium bromide. After electroelution, the fragments were purified by filtration through a nitrocellulose filter (0.45 μm) and two successive extractions with freshly saturated phenol followed by four extractions with ether. The DNA was precipitated with 2.5 volumes of ethanol, rinsed with 70% ethanol, and resuspended in TE buffer. The DNA concentration was measured spectrophotometrically by using an extinction coefficient per mole of base pairs of E_{260} = 13 000. The specific activity was 5.8×10^4 cpm/μg. The DNA was diluted to 3 μg/mL in 15 mL of LB and preincubated at 16 °C. Covalent joining was initiated by the addition of a 32 unit/μg sample of DNA of T4 DNA ligase (New England BioLabs, 1400 units/μL). After 4 h, the DNA was precipitated by the addition of spermine to 10 mM as described by Hoopes & McClure (1981). Monomeric circles were separated from other ligation products by gel electrophoresis (1.75% agarose gel containing 0.1 μg/mL ethidium bromide) and purified as above. DNA solutions were stored at 4 °C in TE buffer. Linear blunt-ended fragments were prepared prior to a binding experiment by restriction of covalently closed circles with *Hae*III (1 unit/μg for 1 h).

Determination of End-Binding Constants (K_E). The nitrocellulose filter binding assay is basically the one described in Melançon et al. (1982). The extent of DNA retention caused by RNAP was determined before and after a short heparin challenge for both linear (L), nicked circular (NC),

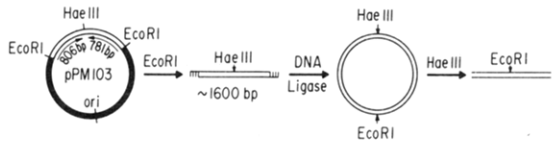

FIGURE 1: Schematic representation of the preparation of circular and linear DNA molecules. ^{3}H-Labeled plasmid DNA (pPM103 or pPM104) containing a 1600 bp dimer insert of T7 DNA is cut with *Eco*RI, and the 1600 bp fragment is isolated. After self-ligation with T4 DNA ligase, closed circles are obtained that have both an *Eco*RI and a *Hae*III site. Blunt-ended linear 1600 bp molecules are prepared by restriction with *Hae*III. All the experimental details are given under Materials and Methods. The length and orientation relative to the genetic left end of T7 of the fragments in the dimer insert are given below the plasmid. The four-base 5′-protruding *Eco*RI cohesive ends are represented by hatch marks.

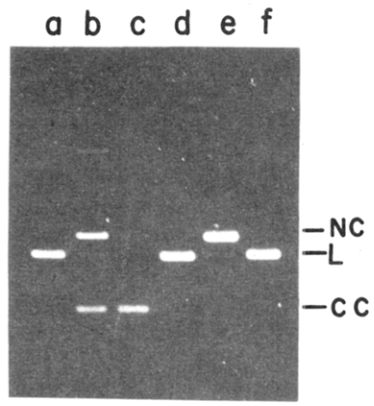

FIGURE 2: Products of preparative ligation of the insert of pPM103. Products obtained by following the scheme shown in Figure 1 were analyzed on a 1.5% agarose gel run in the presence of 0.1 μg/mL ethidium bromide. (Lane a) 1600 bp linear *Eco*RI fragment (L) obtained from ^{3}H-labeled pPM103; (lane b) same DNA as in (a) but after incubation with DNA ligase (NC, nicked circular; C, closed circular); (lanes c and e) CC and NC DNA, respectively, after isolation by preparative gel electrophoresis; (lanes d and f) material from lanes c and e, respectively, after digestion with 1 unit of *Hae*III for 30 min. The amount of DNA loaded was (lane a) 20, (lane b) 60, or (lanes c–f) 30 ng. The gel was stained for 10 min with 0.5 μg/mL ethidium bromide and destained in water for 1 h. The gel was photographed with Polaroid type 57 film.

and closed circular (CC) DNA. The background (the amount of DNA retained on the filter in the absence of RNAP) was the same for the three kinds of DNA (<1% of the amount filtered). The filter binding results were corrected for background retention and expressed as the fraction of the total amount of DNA initially present in the filtered aliquot (θ_L, θ_{NC}, and θ_{CC}, respectively). To obtain the fraction of filter retained DNA molecules having one or more heparin-sensitive (weak) complexes, the data were analyzed in either of two ways by a procedure similar to that outlined by Strauss et al. (1980b). First, the fraction containing any weak complexes, θ_W, can be obtained as $\theta_W = 1 - [(1 - \theta_L)/(1 - \theta_+)]$, where θ_+ is the fraction of molecules forming heparin-resistant complexes. For θ_+, the average of the values obtained for the linear and circular molecules was used. The *per molecule* binding density, r_W, is then given as $r_W = 2[1 - (1 - \theta_W)^{1/2}]$. On the other hand, θ_E, the fraction with weak complexes that can be formed only on linear molecules, is obtained as $\theta_E = 1 - [(1 - \theta_L)/(1 - \theta_{CC})]$ and, then, $r_E = 2[1 - (1 - \theta_E)^{1/2}]$. Those calculations assume that the efficiency of the assay for end complexes is 100% under our experimental conditions. We have obtained evidence that strongly supports this assumption (P. Melançon et al., unpublished results). For the determination of K_E, the amount of protein bound at sites other than ends was taken into consideration to get the free protein concentration ($[RNAP]_f$): (i) the value of θ_+ was used to estimate the concentration of tightly bound enzyme; (ii) the concentration of nonspecific (NS) interior complexes was calculated by using the value of K_{NS} appropriate for these ionic conditions (3.3×10^5 M^{-1}; Shaner et al., 1983). Binding constants were then calculated for individual pairs of θ values at a given RNAP concentration and also obtained from the slopes of double-reciprocal $[1/(0.5r_E)]$ vs. $1/[RNAP]_f$ plots of the whole titration data set.

Results

Preparation of Circular and Linear DNAs. (a) Approach. In Figure 1, we show schematically the approach that was used to produce linear and covalently closed molecules that are otherwise identical. The T7 800 bp *Hae*III (blunt-ended) fragments appeared ideal to study the nature and properties of the heparin-sensitive complexes because (i) they do not readily form TB complexes with holoenzyme and (ii) quantitative information about their interaction with holoenzyme is already available under various incubation conditions (Melançon et al., 1982). The problem of inefficient ligation of the *Hae*III blunt ends was overcome by cloning those *Hae*III fragments into a plasmid with filled-in *Eco*RI sites, a procedure that provides new cohesive ends to the inserted DNA fragment. In about one-third of the plasmids in which T7 DNA had been successfully inserted, the inserts turned out to be 1600 bp dimers created during the cloning. In most cases, a *Hae*III site was regenerated at the junction, as expected. With such dimers, circular molecules can be easily prepared with DNA ligase. As depicted in Figure 1, these circles can later be recut with *Hae*III to produce blunt-ended 1600 bp fragments. The binding properties of circular molecules can then be compared with those of linear fragments similar to the ones previously investigated (Melançon et al., 1982).

(b) Characterization of the Products. Samples of products prepared at various steps during the isolation precedure are depicted on an agarose gel (containing ethidium bromide) shown in Figure 2. Treatment of the isolated 1600 bp *Eco*RI fragment (L, lane a) with T4 DNA ligase at low DNA concentration gives rise to two major new products (lane b), which can be separated by preparative electrophoresis on agarose gels. Direct determination of the ratio of DNA present in each band by scintillation counting indicates that as much as 30% of the DNA is not converted to the fast-moving form. We have also established that (i) once isolated both new forms of DNA still migrate as single species (lanes c and e) (there is a small amount of NC contaminant in the isolated CC DNA), (ii) further treatment of both of these isolated DNAs with excess ligase does not lead to any observable change in mobility (data not shown), (iii) both DNAs migrate with a mobility identical with that of the original linear 1600 bp molecule (lane a) when recut with *Hae*III (lanes d and f) or *Eco*RI (data not shown), (iv) restriction of the fast-moving material, but not the slow-moving band, leads to a significant enhancement of ethidium bromide staining (compare lanes c and d with lanes e and f), and (v) when electrophoresis is carried out in the absence of ethidium bromide the mobility of all DNA species is increased except for that of the faster moving material, which now migrates as two slower topoisomers (data not shown). Based on the above observations, with particular consideration given to the relative levels of dye binding, we conclude that the two ligation products shown in lanes c and e are, respectively, the closed (CC) and nicked (NC; due to a single-strand break) forms of a self-ligated, 1600 bp fragment.

We expect a very small amount of superhelicity in the closed molecules in BB. As mentioned above, the population of CC

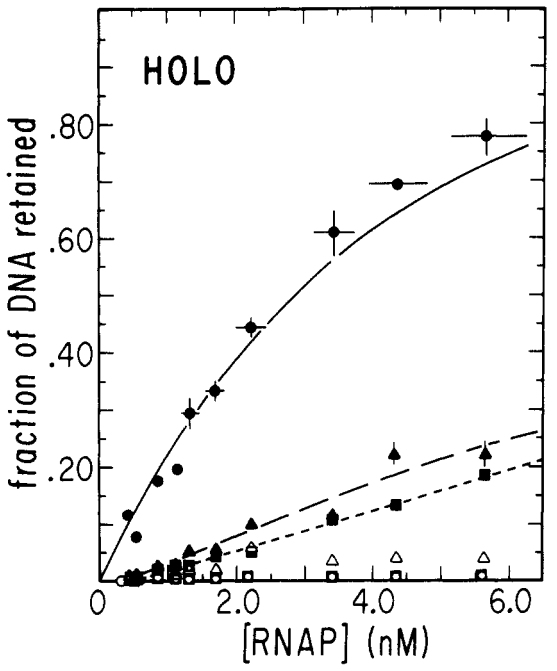

FIGURE 3: Titration of the linear and circular 1600 bp fragments (pPM103) with holo RNAP. Individual dilutions (0.45–0.5 nM) of ^{3}H-labeled blunt-ended linear (○, ●), closed circular (□, ■), or nicked circular (△, ▲) DNA fragments were prepared in BB, preincubated at 0 °C and titrated with RNAP. After a 2.5-min incubation, multiple samples were withdrawn and filtered. The filled symbols correspond to data obtained with samples (usually averages of triplicates) that were filtered directly. The data for the open symbols (averages of duplicates) were obtained after the 10-s challenge with heparin (5 μg/mL). σ-Saturated holoenzyme was used. The line through the filled circles was drawn by using theoretical values for θ_L calculated by assuming a value for K_E of 2.1×10^8 M^{-1}. The other lines are quadratic fits through the data points. The error in the θ values contains the error for the determination of DNA concentration and corresponds to 1 standard deviation from the mean of triplicates. The error in protein concentration, a 10% random error in the protein dilution, was indicated only for the θ_L values but was the same for the three sets of data.

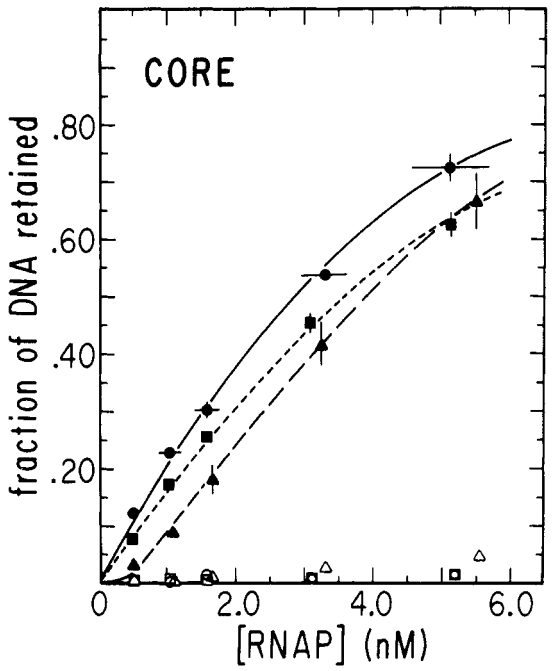

FIGURE 4: Titrations of the linear and circular 1600 bp fragments (pPM103) with core polymerase. Experiments were carried out as described in the legend to Figure 3 except that core polymerase was used instead of holoenzyme. The symbols are the same as those used for Figure 3. All the lines are quadratic fits through the data points.

molecules can be resolved into at least two topoisomers. Because ligation was performed in a buffer which was virtually identical with BB, the distribution of writhe should also be centered near zero in BB. The change in temperature from 16 to 0 °C (from ligation to binding conditions) should result in the creation of at most 0.7 positive supertwist based on a value for the temperature coefficient of the average rotation angle of $(-11 \pm 2) \times 10^{-3}$ deg bp^{-1} K^{-1} (Depew & Wang, 1975).

The nature and position of the single-strand break(s) in the NC DNA could not be clearly identified. Whatever damage was incurred by the DNA (e.g., loss of terminal phosphate, degradation by endo- or exonuclease, photochemical damage, etc.) must have resulted from treatment with *Eco*RI or subsequent isolation since most of the original plasmid was supercoiled (data not shown).

RNA Polymerase Holoenzyme Shows a Preference for Linear over Circular Forms of the Nonpromoter DNA Fragment. Titrations of linear (solid line) and of circular DNAs in the covalently closed (small dashes) or nicked (large dashes) state were performed with RNAP. The results for holoenzyme and core polymerase are shown in Figures 3 and 4, respectively. The extent of filter retention was determined both before and after a short challenge with the polyanionic competitor heparin. The binding measurements were all carried out at 0 °C in order to minimize the formation of tight (TB) complexes.

After a heparin challenge (open symbols, Figures 3 and 4), very little filter retention is detected even at the highest concentration of protein. The small amount that is detected is always higher for the nicked circles. This is true for both holoenzyme and core polymerase. When the formation of weak (heparin-sensitive but filter-retainable) complexes is measured (filled symbols), the two forms of RNAP show different binding behavior. As shown in Figure 3, RNAP holoenzyme binds preferentially to the linear DNA molecules at all concentrations examined and exhibits a similar reduced binding affinity for both forms of circular DNA (nicked and closed). Core polymerase causes much higher levels of filter retention of the circular DNAs than those observed with holoenzyme, such that almost no difference between the affinities of core for the three forms of DNA is detectable (Figure 4). These differences in the behavior of core and holoenzyme do not result from the breakdown of the circular DNAs by an endonuclease contaminant in the core preparation, because incubation of closed circular DNA (2 nM) with holo or core RNAP (8 nM) in BB at 37 °C for 15 min does not lead to the appearance of any detectable amount of nicked circular or linear molecules (data not shown). We conclude that the different levels of retention of linear and circular DNAs caused by holoenzyme are not due to a contamination of the holoenzyme with core polymerase. Similar discrimination between circular and linear molecules by holoenzme was observed with DNA prepared with a different 1600 bp fragment (insert of pPM104; Scott Cayley, unpublished results), and we thus conclude that it is a general property of RNAP holoenzyme.

The Higher Affinity of Holoenzyme for Linear DNA Than for Circular DNA Is Due to End Binding. Except for the presence or absence of ends, there is minimal chemical or physical difference between the linear and closed circular DNAs. The linear DNA was obtained by restriction of CC DNA with *Hae*III immediately prior to the binding measurement. In addition, as discussed above, topological differences between the linear and circular molecules were

minimized by the proper choice of ligation conditions. Even if a small number of supertwists were present, they could not affect the binding of holoenzyme to the CC molecule since it has been shown that RNAP does not unwind DNA at 0 °C, even when bound to promoters (Gamper & Hearst, 1982). Furthermore, both the nicked and closed circles give similar (lower) levels of retention. Finally, examination of the DNA sequence at the ends and at the new junction of the two 800 bp fragments demonstrated that no promoter-like sequences were present. The results presented in Figure 3 therefore demonstrate that holoenzyme recognizes DNA ends as different from nonspecific sites in the interior of the DNA molecule.

Determination of End-Binding Constants (K_E). The low but significant levels of filter retention observed with the circular molecules (θ_{CC}) clearly show that the retention of linear molecules cannot be caused solely by end binding. For estimation of the level of retention due to end binding only, the θ_L values must be corrected for this residual mode of binding. Values of θ_{CC} provide an empirical measure of the extent of filter retention from this source (see Materials and Methods). Not only the formation of end complexes but also the binding of RNAP at random nonspecific sites throughout the DNA will decrease the free RNAP concentration. This effect, the importance of which has been previously recognized by Strauss et al. (1980a,b) and Stefano & Gralla (1982a), has been recently discussed by Shaner et al. (1983). Contributions from these modes of binding must therefore be ascertained in order to properly determine equilibrium constants for the binding of holoenzyme to DNA ends (K_E). It is possible that filter retention of the circular DNA is caused by nonspecific binding, but it is unlikely that such complexes are efficiently detected, indicating that the values of θ_{CC} are lower-bound estimates of the level of nonspecific binding in solution (see below). We have therefore calculated the extent of nonspecific binding by using the value of the affinity constant (K_{NS}) appropriate to these ionic conditions (3.3×10^5 M^{-1}; Shaner et al., 1983).

A double-reciprocal plot of the titration data corrected in this manner gave a value for K_E of 2.1×10^8 M^{-1}. This single value provides a good fit to the data throughout most of the protein concentration range when it is used to calculate theoretical θ_L values (cf. solid line in Figure 3). This K_E value translates into a specificity ratio for end binding (K_E/K_{NS}) of about 600 at these solution conditions. Since this number is smaller than the ratio of nonspecific sites to ends ($\sim$1600), our ability to detect preferential binding to DNA ends depends on the lower efficiency of detection of nonspecific complexes. The magnitude of K_E is mildly sensitive to the choice of the value of K_{NS}. If a lower estimate (Kadesch et al., 1981a; $K_{NS} = 2 \times 10^4$ M^{-1}) is used, the value of K_E is reduced to 1.3×10^8 M^{-1} and the end-specificity ratio is increased to 6500.

Our previous study of mixtures of *Hae*III fragments of either 800 or 2000 bp provided values of K_E over a wide range of [Na^+]. We have now confirmed the validity of the analysis of those data in terms of end-binding constants. Also, under identical ionic conditions, the value of K_E that can now be calculated from the data of Figure 3 is in good agreement with the value of K_E reported at that time.[2] The previously published set of values of K_E can therefore be used to estimate the levels of end binding over a wide range of electrolyte concentrations for in vitro studies. We do not expect that the lack of correction for the mode of binding shown by the circular DNA can cause an overestimation of more than 20% in the magnitude of the end-binding constant.[3] A more serious complication for the generalized use of our values of K_E may come from their potential dependence on the nature of the end (as discussed below).

End Binding Is Relatively Independent of Temperature. Binding experiments with RNAP holoenzyme and linear and circular DNA molecules were performed at various temperatures between 0 and 37 °C. The level of filter retention of both types of molecules increased with temperature. This increase was predominantly heparin resistant and was approximately the same for the circular and linear DNAs. As a result, the values of K_E obtained [$(2.2 \pm 0.2) \times 10^8$ M^{-1}] are relatively independent of temperature and are in agreement with the value given above.

Previous measurements with the three 800 bp *Hae*III fragments of T7 DNA had given a value for the van't Hoff enthalpy of 4 ± 1 kcal mol^{-1} (Melançon et al., 1982). In that study, no adjustment was made for the temperature-dependent pK_a of the buffer, and consequently, the pH decreased by 0.6 pH unit from 0 to 37 °C. We have not yet investigated the dependence of end binding on pH. However, Strauss et al., (1981; their Figure 3) have shown that increasing the pH of both the incubation and wash solutions results in a significant decrease in the amount of filter retention caused by these nonpromoter interactions. Since variations in pH at the wash step alone do not affect the retention of RNAP–DNA complexes (P. Melançon et al., unpublished results), this observation in fact demonstrates that the *formation* of these complexes must be sensitive to pH. [Note that the value of d log K_E/d pH will have the same sign as that measured for promoter complexes (Strauss et al., 1980b).] We conclude that the temperature dependence of K_E (corresponding to a $\Delta H°$ of less than 4 kcal mol^{-1}) is very small, especially when compared with values of $\Delta H°$ of up to 50 kcal mol^{-1} reported for the formation of open promoter complexes (Mangel & Chamberlin, 1974; Strauss et al., 1980b).

Other RNAP–DNA Interactions. At 0 °C, holoenzyme forms filter-retainable complexes to a small but significant extent with the circular DNA (Figure 3). Even though the retention of NC DNA is always slightly higher, the difference between NC and CC DNA is almost within experimental error. Since neither molecule has ends, filter retention must be caused by binding to interior sites. A quantitative analysis of these data is limited by the fact that we have no direct information regarding the nature and numbers of binding sites or the efficiency with which the complexes can be detected. If one assumes that the number of sites is equal to the number of phosphates and that the efficiency of filter retention is 100%, a value for K_{NS} of 1.1×10^4 M^{-1} is obtained. This is clearly smaller than the value that is used here to obtain the free protein concentration ($K_{NS} = 3.3 \times 10^5$ M^{-1}; Shaner et al., 1983) and is more comparable to the values obtained by Kadesch et al. (1981a, 1982). Since such nonspecific complexes are likely to be formed at diffusion-limited rates (k_a

[2] Previously, the analysis of the data collected with the 800 and 2000 bp fragments gave a temperature-corrected value for K_E of 2.6×10^8 M^{-1} (Shaner et al., 1983). If one assumes, as was done in that case, that all the filter retention observed with linear molecules (θ_L) is caused by end binding, an average value for K_E of 2.4×10^8 M^{-1} is calculated from the θ_L values shown in Figure 3.

[3] Those values of K_E were all obtained in the presence of Mg^{2+}, and it is most likely that the error introduced by having to correct for the competitive effect of Mg^{2+} will be more important. At this point, it is also difficult to predict what effect an altered ion distribution near the end of the polyelectrolyte (Record & Lohman, 1978) may have on the analysis of the salt dependence of K_E.

$\sim 10^8$ M^{-1} s^{-1}), we would expect their lifetimes to be at most in the millisecond range; therefore, their efficiency of filter retention should be very low. Independent quantitative information on the nonpromoter interaction of RNAP with circular DNA is being obtained by using a competition filter assay with a promoter fragment (K. S. Lee, unpublished results).

The levels of filter retention of the two circular DNAs are much higher with core polymerase than those observed with holoenzyme (Figures 3 and 4). (There is a possible sigmoidicity in the NC DNA titration curve, suggestive of a cooperative effect for which we have no independent corroboration.) Use of the same assumptions for core polymerase as those described above for holoenzyme to analyze the θ_{CC} values yields a value of K_{NS} of 6×10^4 M^{-1}, which provides a good fit to the titration data with core polymerase (Figure 4). If we assume that the only effect of Mg^{2+} is as a competitive ligand for DNA phosphates, then extrapolation of the data of de Haseth et al. (1978) yields an estimate for K_{NS} in BB in excess of 10^8 M^{-1}. Clearly, an unambiguous interpretation of the nonspecific data for core polymerase must also await further work. Whatever the quantitative interpretation of these data may be, it appears that core polymerase binds with a higher affinity to nonpromoter DNA than holoenzyme does, as judged by the filter retention data. This is in qualitative agreement with previous measurements of the nonspecific binding of RNAP (de Haseth et al., 1978; Lohman et al., 1980) that predict that at the low electrolyte concentration we are using, core polymerase has a higher affinity for DNA than holoenzyme does.

One may ask if preferential binding to ends by core polymerase (comparable to that observed with holoenzyme) is masked by the generally higher binding affinity of core for DNA. Values of K_E were obtained by assuming that the small differences in θ_{CC} and θ_L observed with core polymerase were significant. If the ratio of values of K_{NS} for core and holoenzyme is assumed to be 6, as estimated above from the filter retention data, we obtain for core polymerase a preferential end-binding ratio (K_E/K_{NS}) at least 10-fold smaller than the one measured for holoenzyme. If the value of K_{NS} for core were actually higher, the ratio of K_E/K_{NS} for core would be even smaller.

We have also performed some experiments at 37 °C with holoenzyme and the various DNA forms (data not shown). As mentioned above, we observe a significant increase in the levels of filter retention with temperature for all three forms of DNA. Most of the increase is the result of an increase in the level of heparin-resistant binding, which may indicate the presence of weak TB sites that were not detected previously under competitive binding conditions (Melançon et al., 1982). Titrations of NC DNA with core at 37 °C also showed increased binding over the levels of core binding observed at 0 °C, but the amount of heparin-resistant binding was significantly less than that observed with holo RNAP. This agrees with our earlier observation that core does not form TB complexes (Melançon et al., 1982).

Hinkle et al. (1972) found that single-strand nicks are high-affinity sites for RNAP at 37 °C. The NC DNA contains a single-strand break in one of the strands, and we therefore expected NC DNA to form heparin-resistant complexes to a much greater extent than the CC DNA. At the single RNAP holoenzyme concentration for which we have data, however, we do not observe a significant difference between the behavior of the two circular DNAs, even at 37 °C (data not shown). As mentioned above, core polymerase does not form large amounts of heparin-resistant complexes ($\theta_{NC} < 0.15$ at 37 °C, [RNAP] = 5.5 nM and [DNA] = 0.5 nM). Within the limits of our assay, neither core polymerase nor holoenzyme seems to form tight complexes at the sites of the single-strand breaks present in the NC DNA.

Discussion

We previously reported binding data obtained with RNAP holoenzyme and two sets of DNA fragments of 800 and 2000 bp. Measurements were carried out in the presence of $MgCl_2$ (10 mM) over a wide range of Na^+ concentrations (pH 7.0). The analysis of these data was most internally consistent if one assumed that all the heparin-sensitive binding occurred at the ends of the fragments (Melançon et al., 1982). Further analysis of the same data, incorporating a correction for the protein bound nonspecifically (which we assumed does not cause filter retention but does decrease the free RNAP concentration), brought the two sets of data into complete agreement such that they could be fit by using a single curve. Values of K_E were obtained as a function of [Na^+] (Shaner et al., 1983). In the present work, we demonstrate directly that holo RNAP recognizes and binds preferentially to DNA ends, and thereby confirm the validity of the above analysis.

These values of K_E are large, ranging from 10^7 M^{-1} (0.2 M NaCl) to 10^9 M^{-1} (0.05 M NaCl) in a buffer (pH 7.0) containing 0.01 M $MgCl_2$. Such binding constants are comparable to those observed for the interaction of RNAP holoenzyme with some promoters, which means that ends can effectively compete for RNAP. Therefore, we anticipate that in studies performed with promoters on small DNA fragments, site overlap will occur if the promoter is close to the end (de Haseth et al., 1983). More generally, if the concentration of protein is not in excess over that of DNA sites, the concentration of free RNAP may be significantly reduced by the formation of end complexes. On the basis of the present work, one may feel confident in using the previously published values of K_E to quantify such effects for fragments with *Hae*III blunt ends [cf. Shaner et al. (1983)].

The general applicability of such a calculation using our data on the *Hae*III ends remains to be demonstrated since the dependence of K_E on the nature of the end has not been examined. On this point, Hirsh & Schleif (1976) noted that such binding did not appear to be strongly dependent upon the DNA sequence near the end. As reported above, we have seen a discrimination between circular and linear molecules with a second 1600 bp fragment, and our present estimate for K_E agrees with the value obtained previously with different fragments under identical ionic conditions. Therefore, our data, though obtained only with *Hae*III blunt-ended fragments, support their statement. This does not rule out the possibility that the immediate sequence at the end has an influence on the affinity of RNAP.[4] Some preliminary work on the binding of holoenzyme to self-ligated circles recut with *Eco*RI or *Hae*III suggests that end binding might be different for these two linear DNAs (P. Melançon, unpublished results). In particular, much higher levels of heparin-resistant binding are observed for the staggered-end *Eco*RI fragment. In addition, it is often observed in in vitro transcription experiments that the nature of the ends (e.g., blunt vs. staggered) can have an influence on the amount of end to end transcription (Wayne

[4] One should keep in mind that special DNA sequences close to an end may favor the formation of end complexes. In phosphatase protection experiments, we have indeed observed that a few fragments from the *Hae*III digestion of T7 DNA can rapidly form heparin-resistant complexes at or near the end (Melançon et al., 1982).

Taylor, personal communication). A more detailed, systematic study of the effects of the structure and sequence of the ends on end binding will be required to resolve this question.

Binding to Holoenzyme to Interior DNA Sites May Be Sterically Unfavorable: A Model. We have found that the ratio of the binding affinities of holoenzyme for ends and for interior (random) sites, K_E/K_{NS}, is in excess of 600. This specificity for ends is much less pronounced for core polymerase. It is therefore possible that the preference of holoenzyme for ends over interior sites may provide insight into the promoter-specific binding interactions of holoenzyme. Models to explain this preferential binding to ends may in principle be based on the two distinctive characteristics of ends: their relatively lower stability toward denaturation and their unique steric properties. [In addition, the polyelectrolyte properties of ends are different from those of interior regions (Record & Lohman, 1978).]

The possibility that the relative instability of the ends of a fragment to denaturation is the source of the preferential interactions of RNAP holoenzyme with ends is worthy of consideration, since RNAP is known to be a melting protein, both in its higher binding affinity for single-stranded DNA than for nonpromoter double-stranded DNA (de Haseth et al., 1978) and in its ability to form transcriptionally competent open complexes at promoters. However, there are several counterarguments that weigh against this proposal. Most important are the observations that (i) whereas large temperature dependences have been reported for the formation of open-promoter complexes (Mangel & Chamberlin, 1974; Strauss et al., 1980b; J.-H. Roe et al., unpublished results) and TB complexes (Melançon et al., 1982), the enthalpy change accompanying end binding is close to zero and (ii) the rates of formation and dissociation of end complexes appear to be faster than those observed for many open-promoter complexes (Melançon et al., 1982). Both the small enthalpy change and the fast kinetics would argue against a mode of end-binding interaction in which preexisting base pairs are denatured, unless the enthalpy of denaturation is compensated by enthalpically favorable RNAP–DNA contacts which can be formed and broken sequentially, so as to avoid a kinetic intermediate of high activation enthalpy in either direction of reaction. Alternatively, one might propose that sufficient regions of open bases exist at the ends of fragments under the reaction conditions used here (0 °C, 0.01 M Mg^{2+}/0.06 M Na^+, pH 7.7) to enable RNAP to interact with the single-stranded ends. (In this case, the enthalpy of melting would not contribute to the binding thermodynamics.) However, we expect little fraying of the G·C-rich ends of the *Hae*III restriction fragments under the conditions of our experiments. For short oligonucleotides with A·T sequences at their ends, high-resolution NMR studies indicate that the terminal and possibly the penultimate positions are significantly frayed at temperatures 20–30 °C below the melting temperature (Patel & Hilbers, 1975; Early et al., 1977). Essentially no fraying is observed for terminal G·C base pairs under comparable conditions (Patel & Canuel, 1979; Early et al., 1977). Consequently, we do not favor a model involving the interaction of RNAP with denatured regions at the fragment ends but propose instead models in which the preference of RNAP for fragment ends is steric in origin.

One such model is illustrated schematically in Figure 5, wherein we postulate that, under the conditions of the experiments reported here, holoenzyme exists predominantly in a conformation designated R which is able to bind to ends of DNA molecules (Figure 5a) but is unable to bind to interior

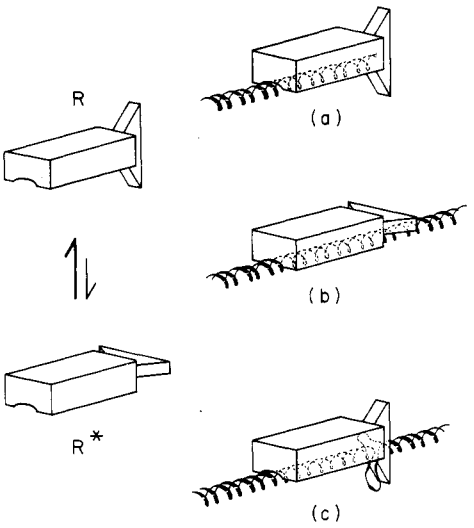

FIGURE 5: Schematic steric model for the observed preference of RNAP holoenzyme for the ends of DNA fragments. The two putative R and R* conformations are shown. The use of geometric forms is arbitrary and solely for the purpose of illustration. Also represented are (a) an end complex with species R, (b) a random interior complex or a closed-promoter complex with species R*, and (c) an open-promoter complex with its DNA partially melted such that holoenzyme can take up the R conformation. See text for details.

DNA sites without concomitant distortion of either the DNA or the enzyme. [Since no pronounced preference of core RNAP for ends is observed, it is possible that the presence of the σ subunit in the DNA binding region of holoenzyme (Simpson, 1979) is at least in part responsible for this steric constraint. However, Figure 5 is intended to be completely schematic and not to represent the subunits of RNAP holoenzyme.] Binding of holoenzyme to interior sites, which appears to involve the same number of ionic contacts as the binding of holoenzyme to ends (Melançon et al., 1982; Shaner et al., 1983), must then involve either conformational distortion of the DNA or a conformationally altered form of the polymerase. Figure 5b illustrates the latter alternative, in which a less stable conformation (R*) (which in this model would exist in dynamic equilibrium with R) binds to interior regions of DNA to form either random complexes or closed-promoter complexes without unfavorable steric effects. In this model, the preference of RNAP for ends is a measure of its preference for the R conformation. The model predicts that allosteric effectors of RNAP conformation (e.g., ppGpp, rifampicin, anions, etc.), which might shift the R $\rightleftharpoons$ R* equilibrium, would have a predictable effect on the preference of holoenzyme for end sites.[5] This steric model involving two conformations of protein is analogous to that proposed to explain the end-binding mode of the gene 32 protein of phage T4 (Kowalczykowski et al., 1981).

If closed-promoter complexes as well as random interior complexes are characterized by this unfavorable conformational free energy, it is plausible to propose that the transition to the more stable open-promoter complex may be accompanied by a transition of the protein to the more stable R form,

[5] A variety of techniques have provided evidence for the conformational variability of RNAP and how it is affected by the binding of various ligands such as anions, nucleotides, and drugs. In particular, an apparent heterogeneity in holoenzyme, detected by sedimentation velocity, was correlated with template preference, binding to tRNA, and regulation by ppGpp (Travers et al., 1980). Also, a linkage between differential anion binding and a conformational equilibrium was proposed by Shaner et al. (1982) to explain their data on the aggregation of RNAP.

which is permitted by the increased conformational flexibility of the open DNA single strands (Figure 5c). In this case, the free energy of the protein conformational transition would contribute to the stabilization of the open-promoter complex. One could further infer from this proposal that a conformational change from R* to R could be a mechanistically significant step on the pathway to open-promoter complex formation [cf. Hawley & McClure (1982), Rosenberg et al. (1982), and J.-H. Roe et al. (unpublished results)]. This conformational change might act as a nucleation event for DNA unwinding, particularly if it involves rotation of one domain of the protein relative to another about the axis of the DNA binding site. As such, this proposal is compatible with the "untwist and melt" model suggested by Stefano & Gralla (1982b) to explain the effect of altering the spacing between the two consensus regions of promoters.

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