Mirabelli, C. K., Huang, C.-H., & Crooke, S. T. (1980) *Cancer Res. 40*, 4173-4177.
Mirabelli, C. K., Huang, C.-H., Prestayko, A. W., & Crooke, S. T. (1981) *Proc. Am. Assoc. Cancer Res. 22*, 251.
Muller, W. E. G., Yamazaki, Z., Breler, H. J., & Zahn, R. K. (1972) *Eur. J. Biochem. 31*, 518.
Povirk, L. F., Wubker, W., Kohnlein, W., & Hutchinson, F. (1977) *Nucleic Acids Res. 4*, 3573-3580.
Povirk, L. F., Hogan, M., & Dattagupta, N. (1979) *Biochemistry 18*, 96-101.
Povirk, L. F., Hogan, M., Dattagupta, N., & Buecher, M. (1981) *Biochemistry 20*, 665-670.
Sanger, F., & Coulson, A. R. (1975) *J. Mol. Biol. 94*, 441-448.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73*, 814-822.
Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978a) *Biochemistry 17*, 2746-2754.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1978b) *Biochemistry 17*, 2740-2746.
Strong, J. E., & Crooke, S. T. (1978) *Cancer Res. 38*, 3322-3326.
Suguira, Y. (1980) *J. Am. Chem. Soc. 102*, 5208-5215.
Suzuki, H., Nagai, K., Yamaki, J., Tanaka, N., & Umezawa, H. (1969) *J. Antibiot. 22*, 446-450.
Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983-5987.
Takeshita, M., Kappen, L. S., Grollman, A. P., Eisenberg, M., & Goldberg, I. H. (1981) *Biochemistry 20*, 7599-7606.
Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Umezawa, Y., Naganawa, H., & Umezawa, H. (1978) *J. Antibiot. 31*, 801-804.
Tanaka, T., & Weisblum, B. (1974) *J. Bacteriol. 121*, 354-362.
Umezawa, H. (1978) in *Bleomycin: Current Status and New Developments* (Carter, S. K., Crooke, S. T., & Umezawa, H., Eds.) pp 15-19, Academic Press, New York.
Umezawa, H., Maeda, K., Takeuchi, T., & Okanai, Y. (1966) *J. Antibiot. 19*, 200-209.

Nitrocellulose Filter Binding Studies of the Interactions of *Escherichia coli* RNA Polymerase Holoenzyme with Deoxyribonucleic Acid Restriction Fragments: Evidence for Multiple Classes of Nonpromoter Interactions, Some of Which Display Promoter-like Properties†

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ABSTRACT: The variety of detectable interactions between *Escherichia coli* RNA polymerase holoenzyme and an unfractionated *Hae*III digest of T7D111 DNA has been examined by using a filter binding gel electrophoresis assay, appropriately modified to study nonpromoter interactions. Filter-retainable complexes form rapidly on all fragments in the digest, either at 0 °C or at 37 °C. These complexes differ from the fast-forming specific complexes observed on promoter-containing fragments in that they are sensitive to competition by the polyanion heparin. From binding studies on two isolated sets of fragments of average size 800 and 2000 base pairs, we infer that the fast-forming, heparin-sensitive complexes occur at the ends of fragments. Binding constants for this class of interactions increase modestly with increasing temperature (yielding a van't Hoff $\Delta H°$ of 4 ± 1 kcal) and decrease strongly with increasing salt concentration; the large salt dependence is qualitatively similar to those observed previously for promoter and nonpromoter complexes. In addition, complexes which exhibit a slow, strongly temperature-dependent rate of formation are found on a subset of T7D111 fragments, as well as on some *Hae*III fragments of λCI47, P22, and SV40 DNA. These complexes are stable to a competition with heparin and are similar to the tight-binding (TB) complexes recently described by Kadesch et al. [Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980) *J. Mol. Biol. 136*, 79-93]. In addition to their stability, these complexes share with promoter complexes the ability to initiate transcription. In both cases, initiation is sensitive to rifampicin and displays specific nucleoside triphosphate requirements. We find that these TB complexes are formed in general at interior sites rather than at the ends of fragments. The extent of formation of TB complexes is very sensitive to salt concentration. Neither the kinetic nor thermodynamic aspects of this reaction have yielded to simple interpretation. Because (i) weak complexes can be eliminated by a short challenge with a competitor and (ii) RNAP forms tight complexes at TB sites much more slowly than at strong promoters, we conclude that filter binding can be used to quantify the interactions of polymerase with strong promoters carried on restriction fragments.

Escherichia coli RNA polymerase holoenzyme (RNAP),[1] like other genome regulatory proteins that act at specific target sites on DNA, exhibits a significant general affinity for other regions of DNA. These nonpromoter[2] interactions are im-

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[1] Abbreviations: RNAP, *E. coli* RNA polymerase holoenzyme; NTP, ribonucleoside 5'-triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Bis, *N*,*N*'-methylenebis(acrylamide); BAC, diacrylylcystamine; Me_2SO, dimethyl sulfoxide; bp, base pair.

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portant for a variety of reasons. (i) The equilibrium distribution of the protein depends on the affinities and numbers of available specific and nonspecific sites on the DNA. In the case of RNAP, there is a 2-fold excess of enzyme over the amount required to maintain a full level of transcription in exponentially growing cells (Oeschger & Berlyn, 1975). However, studies with minicell producing strains have indicated that there is little active RNAP in the cytoplasm (Cohen et al., 1968; Rünzi & Matzura, 1976). Therefore nonpromoter interactions may indeed play a role in the intracellular distribution of the enzyme [both its free concentration and its localization in space; for a discussion, see von Hippel (1979)]. (ii) Nonpromoter interactions may also be involved in the kinetic mechanism leading to promoter binding. By analogy to the models initially developed to analyze *lac* repressor–operator binding kinetics [for a recent discussion, see Berg et al. (1981)], it has been suggested that RNAP first binds randomly and is then redistributed on the DNA lattice by a one-dimensional diffusion mechanism involving nonpromoter interactions (Belintsev et al., 1980). (iii) Furthermore, nonpromoter binding has practical implications for in vitro thermodynamic and kinetic studies (Hinkle & Chamberlin, 1972a,b; Strauss et al., 1980a,b). For example, since the formation of nonpromoter complexes reduces the concentration of free enzyme, an understanding of nonpromoter binding is essential to interpret quantitative in vitro studies of the interaction of RNAP with promoters. For experiments in which 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. Finally, it is probable that all RNAP–DNA interactions share some common characteristics.

Several studies of nonpromoter interactions of RNAP have appeared. deHaseth et al. (1978) determined the affinity of RNAP for double-stranded calf thymus DNA as a function of solution conditions using quantitative DNA–cellulose chromatography. Lohman et al. (1980) extended the range of ionic conditions investigated to higher salt concentrations using intact T7 DNA and a difference boundary sedimentation technique. Though complete binding isotherms could not be determined in these studies, approximate binding constants were calculated from data obtained at low to moderate binding densities. These binding constants (K_{obsd}^{R}) showed an extraordinary sensitivity to ionic conditions ($-d \log K_{obsd}^{R}/d \log [Na^+] = 10.5$ for holoenzyme in the absence of Mg^{2+}), but little dependence on pH or temperature. Recently Revzin & Woychik (1981) have obtained binding isotherms for the interaction of RNAP with P22 DNA by a thermodynamically rigorous centrifugation technique. Only a single class of complexes was detected, characterized by a binding constant whose magnitude and dependence on solution conditions were in reasonable agreement with the observations of deHaseth et al. (1978) and Lohman et al. (1980). [At 0.2 M NaCl, pH 7.9, and 5 °C, K_{obsd}^{R} is approximately $(0.5–1.0) \times 10^5$ M^{-1} (nucleotides)].

On the other hand, two distinct classes of nonpromoter interactions were observed by electron microscopy (Kadesch et al., 1980a,b). In the first mode, the enzyme binds in a temperature-independent way to random nonpromoter sites. The value of the association constant was estimated to be $\sim 10^4$ M^{-1} and found not to be very sensitive to salt concentration or temperature. In the other mode, called TB for tight binding, the enzyme bound at discrete, apparently nonrandom sites at a rather slow rate ($k_a \sim 10^5$ M^{-1} s^{-1}) at 37 °C. The rate of dissociation ($t_{1/2}$ = 30 min in 50 mM NaCl/10 mM $MgCl_2$) increased by 30-fold when the added [NaCl] was increased from 50 to 100 mM. No such binding was observed at 0 °C. An earlier study with the kinetic formaldehyde method had indicated the existence of a type of interaction similar to the tight binding reported by Kadesch et al. (Zarudnaya et al., 1976; Cherny et al., 1977). RNAP was found to form stable complexes and to destabilize the DNA helix at a limited number of sites on T7 and T2 DNA; the effect was sensitive to the temperature and the salt concentration. The kinetics were not studied.

The membrane filter binding assay (Jones & Berg, 1966; Riggs et al., 1970; Hinkle & Chamberlin, 1972a,b) has been widely used to study specific protein–DNA interactions. Filter retention by RNAP of DNA fragments not containing promoters has also been observed [Jones et al., 1977; Maquat & Reznikoff, 1978; Taylor & Burgess, 1979; for discussion, see Strauss et al. (1981)]. However there has been no systematic study of the nature and properties of the nonpromoter interactions giving rise to such filter retention.

We report here our investigation of the range and properties of RNAP–DNA interactions detected by the filter binding assay. We have chosen the *Hae*III digest of T7 DNA as a mixture of blunt-end fragments (~60), only three of which are known to contain promoters (see Materials and Methods), since it had the potential to display a wide variety of nonpromoter binding sites. For quantitative measurements, individual *Hae*III fragments of ^{3}H-labeled T7 DNA were isolated. A pair of 2000-bp fragments [previously employed by Strauss et al. (1980a,b)] and a set of three fragments of about 800 bp were used as examples of promoter-free DNA. Using the filter assay, we are able to distinguish two classes (weak and tight) of nonpromoter interactions on the basis of the time and temperature dependence of their formation. Both types of interactions are extremely sensitive to salt concentration. Tight nonpromoter complexes and promoter complexes are similar in that they (i) can survive a short challenge with the competitor heparin and (ii) are able to initiate transcription, but can be distinguished on the basis of their rate of formation. Since weak binding can be eliminated by competition with heparin and since tight nonpromoter binding occurs slowly, we conclude that it is possible to study the binding of RNAP at strong promoters with minimal interference from other sites.

Materials and Methods

E. coli RNA Polymerase. *E. coli* RNA polymerase was purified from K12 cells 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 enzyme were prepared from purified holoenzyme on a Bio-Rex 70 column (Burgess, 1976; Lowe et al., 1979). σ-saturated enzyme was prepared by adding purified σ subunit to the purified holoenzyme (0.2 mol of σ/mol of holoenzyme) to obtain 110 ± 10% saturated holoenzyme (R. R. Burgess, unpublished result). The holoenzyme (4.3 mg/mL) and saturated holoenzyme (1 mg/mL) were stored at −20 °C in a 50% (v/v) glycerol storage buffer (Burgess & Jendrisak, 1975). Concentrations were determined by absorbance (corrected for light scattering) at 280 nm (Burgess, 1976). The holoenzyme was characterized with respect to its ability to initiate transcription in a rifampicin challenge assay (Mangel & Chamberlin, 1974), as described in Strauss et al. (1980a). We observed that 85% of the rapidly starting complexes were resistant to the rifampicin challenge. The fraction of active

[2] We have chosen to use nonpromoter in place of nonspecific because some of the interactions of RNAP with promoter-free DNA are sequence specific.

enzyme was determined as described by Chamberlin et al. (1979). Using the internally determined elongation rate, we find that 60–70% of the σ-saturated holoenzyme is active.

Buffers. Tris, Hepes, and $NaDodSO_4$ were from Sigma Biochemicals. BSA was from Miles (Pentex). Dithiothreitol was from Eastman Kodak Co. Salts were reagent grade. DNA/T buffer contained 0.1 M NaCl, 0.1 mM EDTA, and 0.01 M Tris-HCl (pH 7.9). The binding reactions were performed in binding buffer (BB) containing 10 mM Hepes (pH 7.2 at 25 °C; also contains 10 mM Na^+), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 μg/mL BSA, and, unless otherwise indicated, 100 mM NaCl (0.1 M BB). After sample filtration, the nitrocellulose filters were rinsed with washing buffer (WB) containing 10 mM Tris-HCl, pH 7.9, and either 0.1 M NaCl (0.1 M WB) or 0.6 M NaCl (0.6 M WB). Filter-bound DNA was eluted with 0.2% $NaDodSO_4$, 0.3 M sodium acetate, and 10 mM Tris-HCl (pH 7.9) (elution buffer). The electrophoresis buffer was 90 mM Tris/90 mM boric acid (pH 8.3) with 2.5 mM Na_2EDTA (Peacock & Dingman, 1968).

DNA. The T7 deletion mutant D111 lacks three of the promoters for RNAP (the A2, A3, and B promoters; Studier, 1975) out of the seven identified by electron microscopy (Koller et al., 1978) and transcription analysis (Stahl & Chamberlin, 1977) on wild-type T7 DNA. Unlabeled T7D111 phage was grown on *E. coli* C. ^{3}H-Labeled phage was grown on a thy$^-$ mutant of *E. coli* C, using [*methyl*-^{3}H]thymidine (New England Nuclear) in the growth medium at 2.5 mCi/L. The intact phage DNA was obtained by four gentle extractions with redistilled phenol. Pure phage DNA from P22, SV40, and λCI47 (a nonlysogenic deletion mutant of λ^+; Kaiser, 1957), as well as pBR322 plasmid DNA, were generous gifts from Arnold Revzin, Janet Mertz, Grace Roe, and Glen Staffeld, respectively. DNA was stored in DNA/T buffer at 4 °C. The DNA concentrations were determined spectrophotometrically by using an extinction coefficient per mole of base pairs of E_{260} = 13 000. The specific activity of the [^{3}H]DNA was 6.33×10^4 dpm/μg.

Restriction Fragments. DNA was digested overnight (usually 1–2 mg in 4–5 mL) in *Hae*III buffer (10 mM Tris, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, and 50 μg/mL BSA) with enough *Hae*III restriction enzyme (New England Biolab) to give complete digestion in 12 h. The digest of the unlabeled DNA was extracted 4 times with redistilled phenol, concentrated, and stored in 0.5-mL aliquots in DNA/T buffer at −20 °C. Digestion of the ^{3}H-labeled DNA was stopped by the addition of 1 mL of electrophoresis buffer containing 50% (v/v) glycerol and 0.02% bromphenol blue. The reaction mixture was layered onto a 4% polyacrylamide–25% glycerol slab gel (Blakesley & Wells, 1975) in which the Bis was substituted with an equimolar amount of BAC (Hansen, 1976). The gel was run, and the bands were visualized as described by Strauss et al. (1980a). The gel slices containing the fragments of interest were dissolved by the additions of 0.2 mL of 2-mercaptoethanol per g of gel. The resulting slurries were diluted with 4 volumes of DNA/T buffer and loaded on 1-mL DEAE-cellulose (Whatman DE-52) columns (Hirsch & Schleif, 1976). The columns were first washed with 10 mL of DNA/T buffer followed by 5 mL of DNA/T buffer adjusted to 0.35 M NaCl, and then the DNA was eluted at 0.6 M NaCl. The eluate was adjusted to a final NaCl concentration of 0.3 M. The ethidium bromide was removed by four extractions with 1-butanol at 0 °C. The fragments were precipitated with 3 volumes of 100% ethanol and resuspended in DNA/T buffer. Yields were dependent on the fragments size but were always in excess of 80%. The purity of the isolated fragments was determined by analytical gel electrophoresis. The fragment molar concentrations were determined by scintillation counting, using the specific activity determined previously. The fragment sizes were obtained from Figure 2 of Studier et al. (1979).

An unfractionated *Hae*III digest of T7D111 was end labeled with [γ-^{32}P]ATP by Sarah Holder using the method of Maxam & Gilbert (1980). The fragments were separated from the unreacted nucleotides by passage over a 10-mL Sephadex G-50 column. The purity of the DNA was checked by thin-layer chromatography on a poly(ethylenimine) plate (Brinkmann) followed by autoradiography (Miller & Burgess, 1978). The specific activity of the end-labeled DNA fragments was 9×10^5 dpm/μg.

Determination of the Number of Single-Stranded Nicks. The integrity of the [^{3}H]DNA fragments after isolation was determined by using gel electrophoresis to quantify the fraction of the DNA that migrated as full-length molecules after strand separation. Aliquots of various isolated fragments were treated for 1 h at 50 °C in 1 M glyoxal/50% (v/v) Me_2SO as described by McMaster & Carmichael (1977). Several aliquots were layered on a 4% polyacrylamide slab gel and electrophoresed for 12 h at 110 V. The gel was stained with methylene blue (0.02% in 20 mM sodium acetate/20 mM acetic acid) for 1 h and destained in H_2O for 2 h. The full-length DNA bands were cut out and dissolved by incubating overnight at 50 °C with 0.2 mL of $HClO_4$ and 0.5 mL of H_2O_2 in a closed scintillation vial. After the addition of cocktail (Aquasol; New England Nuclear), the samples were subjected to scintillation counting. Chemiluminescence was reduced to background levels by cooling the vials to −20 °C before counting. The total amount of DNA present per sample was determined directly by subjecting 20-μL aliquots of the glyoxal/Me_2SO mixture to scintillation counting. We found that all of the DNA present in solution (some DNA was lost by adsorption or precipitation) migrates in the denatured full-length band and therefore conclude that the isolated DNA fragments do not contain a detectable number of single-stranded nicks (<0.05 nick per fragment).

Nitrocellulose Filter Binding. Nitrocellulose filters (Schleicher & Schuell; BA 85; 24 mm-circles) were treated with 0.4 M KOH (Lin & Riggs, 1972) and were then washed extensively with distilled water prior to filtration. The samples were filtered at constant suction (4–5 mmHg lower than atmospheric pressure; this corresponds to a flow rate of 8–9 mL/min) on a 10-place filter holder equipped with individual Teflon valves (Hoeffer Model FH 224V).

(*i*) *Gel Electrophoresis Assays.* We have used two basic filter binding assays involving gel electrophoresis to characterize RNAP–DNA interactions. In the first protocol 1-mL portions of the RNAP–DNA mixture (3 μg/mL each) were filtered through nitrocellulose membranes either directly or immediately after a 10-s challenge with heparin (Sigma Biochemicals). The heparin challenge is performed by transferring the sample to a separate tube containing 20 μL of heparin solution for a final heparin concentration of either 5 or 10 μg/mL. Under those conditions, any free or rapidly dissociated RNAP will bind tightly to the heparin. After filtration, the filters are rinsed with 0.5 mL of 0.1 M WB. In the other protocol, an initiation assay (Taylor & Burgess, 1979), some of the samples are transferred to separate tubes containing nucleoside triphosphates (final concentration 0.1 mM each) and incubated at 37 °C for 1 min prior to filtration. The filters are then rinsed with 2 mL of 0.6 M WB. The

details of the length and temperature of the incubation, the kind of triphosphates, the nature of controls, etc., are given under Results and in the figure legends.

The filter-bound material was eluted for 1 h in 400 μL of elution buffer, and the filters were rinsed with another 100-μL aliquot. The pooled eluates were precipitated by the addition of 1.2 mL of 100% ethanol, and the DNA fragments were then separated by electrophoresis on polyacrylamide gels (cf. Strauss et al., 1981). Because we wished to demonstrate the effect of variations in the incubation condition on the type of DNA fragments retained on the filter, it was important to minimize variations occurring in the steps subsequent to elution from the filter. For estimation of the efficiency of DNA recovery, first 40 ng of easily identifiable DNA fragments was directly added to the elution buffer to serve as recovery markers; these fragments of 3250, 650, and 430 bp (*Hin*cII/*Eco*RI digest of pBR322) are indicated by an asterisk in Figure 1. Second, an identical amount of those marker fragments and 0.1 μg of the complete T7 digest were loaded directly on the gel for use as an internal calibration standard. Random variations associated with ethanol precipitation and redissolution of the eluted DNA, as well as with the loading and staining of the gel, can thus be controlled. After electrophoresis, the gels were stained for 5 min with ethidium bromide (5 μg/mL in 100 mM NaCl/10 mM Tris, pH 7.9) and destained for 1 h in water. The gel was illuminated from the bottom with a transilluminator (U.V. Products Model C-63) and photographed by using either positive (Polaroid, type 57) or negative (Kodak, Tri-X pan professional) film.

The DNA recovery efficiency was estimated by comparing the amount of marker DNA in a given lane to that found in the standard lane. Once corrected for partial recovery, a similar comparison with the T7 DNA yields the amount of DNA initially retained on the filter. In the experiments reported here, the recovery efficiency was approximately 50%. [Experiments with labeled DNAs under identical conditions showed that a higher efficiency (>80%) could be achieved if the DNA pellets were resuspended in 1 mL, using an ultrasonic bath.] Although our system allows a quantitative analysis of the gel results, we have limited our interpretation in general to visual estimates using the markers and standards. Quantitative measurements were more readily performed with individual radioactively labeled DNA fragments.

(*ii*) *Binding Assays with Isolated Fragments.* ^{3}H-Labeled purified DNA fragments were incubated at 37 °C in BB. The total fragment molar concentration was 10^{-9} M. The reaction was started by adding σ-saturated holoenzyme [3–10 μL of a dilution (4.4×10^{-7} M) of the stock solution]. After 2 min, portions of the incubation mixture were withdrawn and filtered as described above for the gel electrophoresis assay. Aliquots were also filtered after a heparin challenge in order to determine the level of binding. The filters were prewashed with 0.35 mL of BB and, after filtration, rinsed with 0.5 mL of 0.1 M WB. Aliquots of 40 μL of the same incubation mixture were spotted separately on dry filters to determine the amount of DNA present. The filters were dried, dissolved with 0.6 mL of Cellosolve (ethylene glycol monoethyl ether; Fisher Scientific Co.), and subjected to scintillation counting in a toluene-based cocktail. The results are corrected for the retention of DNA observed in the absence of RNAP (<1% of the amount filtered) and expressed as the fraction of the total amount of DNA initially present in the filtered aliquot ($>5 \times 10^3$ cpm).

Determination of Binding Constants. The fraction of DNA retention due to weakly bound RNAP (θ_w) was determined as the difference in retention measured before and after a heparin challenge. The fraction of DNA with a tight complex that also had a weak complex was determined by iteration. Binding constants were calculated assuming either (i) a large number of sites equal to the number of phosphate groups per fragment (random binding, K_{obsd}^R) or (ii) only two sites (end binding, K_{obsd}^E). Since in both cases there are more than one site per fragment and since binding can occur at one or more of those sites, the retention data were statistically corrected for the random-binding (i) and end-binding (ii) models as follows: (i) $r = \ln([1 - \theta_w]^{-1})$ and (ii) $r = 2[1 - (1 - \theta_w)^{1/2}]$, respectively, where r is the binding density on a per molecule basis (Strauss et al., 1980b). The concentrations of both weakly and tightly bound enzyme were used to obtain the free RNAP concentration; any reduction in RNAP concentration from possible additional modes of binding which are not filter retainable was neglected.

End-Protection Experiments. Calf intestine phosphatase (grade I, Boehringer Mannheim) was dialyzed against 10 mM Tris (pH 7.9)/0.1 M NaCl and stored in aliquots at −20 °C. The phosphatase activity was determined by incubating the enzyme with the ^{32}P-labeled *Hae*III digest at 37 °C and quenching with 5% CCl_3COOH after various lengths of time. The precipitated DNA was collected on filters (Whatman, GF/C) and subjected to liquid scintillation counting. In the absence of RNAP we could obtain complete hydrolysis of the labeled ends (3 μg/mL DNA) in less than 30 s in 0.1 M BB, even in the presence of 3 μg/mL heparin. Using these hydrolysis conditions, we have determined the extent of end protection caused by RNAP (6 μg/mL) after either a 2-min or 15-min incubation with DNA fragments (3 μg/mL). Hydrolysis was done both in the presence and in the absence of heparin (3 μg/mL). The phosphatase digestion was stopped after 30 s by the addition of inorganic phosphate to a final concentration of 30 mM (Chaconas & van de Sande, 1980). In a parallel experiment aliquots of the same RNAP–DNA mixture were subjected to filter binding. Equivalent amounts of the DNA from the two treatments were loaded on a 4–16% polyacrylamide gradient gel. After electrophoresis, autoradiography allowed a direct comparison of the extent of binding and the extent of end protection.

Results

(*1*) *E. coli RNAP Forms at Least Three Types of Complexes with DNA Fragments.* A general goal of our experiments, such as the one shown in Figure 1, was to determine conditions under which various types of interactions occurring between RNAP and DNA are detected by the nitrocellulose filter binding assay. T7D111 *Hae*III restriction fragments were mixed with RNAP at a weight ratio of 1:1 (an average of two enzyme molecules per DNA fragment). The incubation conditions are described in the legend to Figure 1. Under these conditions it has already been shown that most *Hae*III fragments of T7D111 DNA can be retained on nitrocellulose filters if the mixture is filtered without a heparin challenge (Strauss et al., 1981). In order to determine if the interactions so detected are temperature and/or time dependent, we performed the incubation at 0 or 37 °C for either 0.5 or 15 min. The stability of the complexes formed under these various conditions was tested by competing half of each sample for 10 s with heparin (10 μg/mL) prior to filtration. The pattern of DNA fragments retained for each of these cases is shown in Figure 1A.

In the absence of a heparin competition, all the T7D111 fragments present in the standard lane (Figure 1, lane 1) were retained on the filter (lanes 2, 4, 6, and 8). Separate exper-

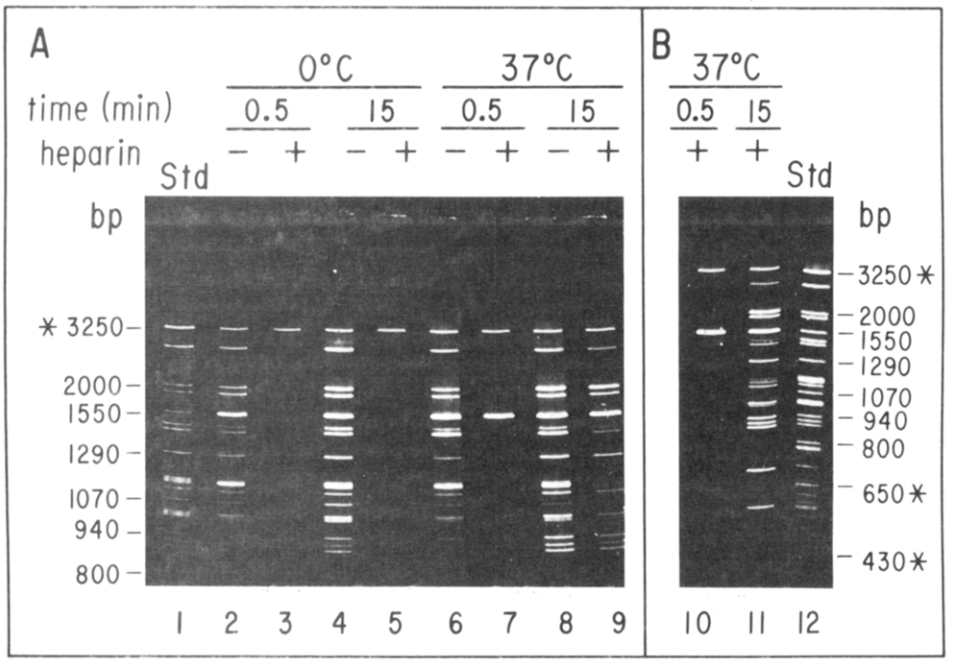

FIGURE 1: Temperature and time dependence of formation of filter-retainable complexes. (A) *Hae*III-digested T7D111 DNA (3 μg/mL) was preincubated at either 0 °C (lanes 2–5) or 37 °C (lanes 6–9) in 0.1 M BB. RNAP was added to a final concentration of 3 μg/mL at time zero. Two 1-mL portions were withdrawn either 0.5 or 15 min after mixing. In each case, one aliquot was filtered directly, and the other was challenged with heparin as described under Materials and Methods. (B) RNAP was preincubated at a concentration of 3 μg/mL for 15 min at 37 °C in 0.1 M BB. At time zero, *Hae*III-digested T7D111 DNA was added to a concentration of 3 μg/mL. One-milliliter portions were withdrawn 0.5 and 15 min later, challenged with heparin and filtered as described above. The temperature and length of the incubation (min) and whether a heparin challenge was done are all indicated above the gel. The size (in base pairs) has been indicated for some of the fragments. Lanes 1 and 12 were loaded with an unfractionated *Hae*III digest of T7D111 (Std). Fragments of pBR322 which were added to the elution buffer to serve as recovery markers are indicated by asterisks.

iments demonstrated that this observation was strictly dependent on the addition of enzyme and that the amount of DNA retained depended on the ratio of protein to DNA. It is clear that filter-retainable complexes are formed on all fragments in less than 0.5 min even at 0 °C.

When the complexes formed at 0 °C are subjected to a heparin challenge, no T7 DNA fragments are observed on the gel (Figure 1, lanes 3 and 5). The possibility of DNA loss during elution can be eliminated since the same amount of recovery marker (top band) is present in those two lanes as in other lanes. Also, even if the length of the heparin challenge is shortened to a few seconds, no T7 D111 DNA fragments are detected on the filter. Most of the complexes formed at 37 °C in less than 30 s (lane 6) are also heparin sensitive since very few fragments are present after a heparin challenge (lane 7).

RNAP can form stable complexes that survive the heparin challenge if the incubation is done at 37 °C, although the retention pattern depends dramatically on the length of the incubation (Figure 1, lanes 7 and 9). Only the 1550-bp fragment containing the A1 and D promoters was detected with a short incubation time (lane 7). Under these same conditions the 360-bp fragment containing the C promoter (McConnell, 1979) has also been observed in gels that were electrophoresed for a shorter time. Therefore, under the incubation conditions of Figure 1, stable complexes are formed in less than 30 s on fragments known to contain promoters. Other T7D111 fragments form heparin-resistant complexes with RNAP if the incubation is carried out for 15 min (lane 9), and therefore, the formation of tight complexes is not limited to promoter-containing fragments. This observation has been found to be independent of the length of the heparin challenge (from 5 s to 1 min) and of heparin concentration (over the range 5–100 μg/mL).

Williams & Chamberlin (1977) have observed that enzyme inactivation was responsible for generating a form of RNAP exhibiting extensive, stable, random binding. We have therefore tested whether or not the slow rate of formation of the tight nonpromoter complexes could result from a slow denaturation of the enzyme that was occurring during incubation. The enzyme was preincubated in the absence of DNA for 15 min at 37 °C under conditions identical with those of Figure 1A; DNA was then added, and 1-mL portions were filtered after a heparin challenge as before. As shown in Figure 1B, preincubation of the enzyme does not change the observation that very little heparin-resistant binding occurs in 0.5 min. We may therefore conclude that native RNAP forms tight complexes at nonpromoter sites on T7D111 DNA.

We have so far described three broad classes of interactions detected with the filter assay: (i) a weak interaction observed on all fragments, at either 0 or 37 °C, in less than 30 s, (ii) a strong interaction also observed in less than 30 s, but only on fragments containing promoters and only at 37 °C, and (iii) another strong interaction observed on more fragments but only at longer incubation times at 37 °C. This division is likely an oversimplification. This information and additional properties that will be described below are summarized in Table I.

(*2*) *All Detectable Interactions Are Salt Sensitive.* Previous studies of the nonpromoter interaction of the enzyme with DNA by various techniques have not provided entirely consistent results. One point of disagreement has concerned the dependence of the interactions on salt concentration. To further characterize the nonpromoter interactions detected by the filter binding assay, we asked if these interactions were affected by changes in the salt concentration of the incubation solution (see Figure 2). RNAP and DNA fragments were incubated at 37 °C at various salt concentrations and filtered either before or after a heparin challenge as described in the

Table I: Properties of the Interactions Detected by Filter Binding

type of site	fragment length (bp)	retained after heparin challenge	temp dependent	retained at [NaCl] = 0.2 M	time of formation (s)	initiation competent
promoter (A/D)	1550	+	+	+	<30	+
nonpromoter (tight)	2000	+	+	–	>30	+
nonpromoter (weak)	1040, 800	–	–	+	<30	–

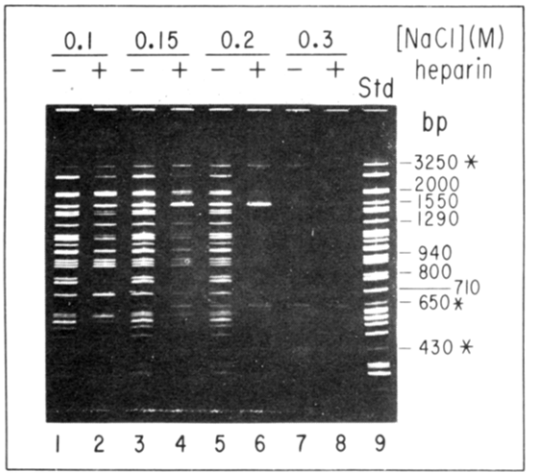

FIGURE 2: Effect of variations in the salt concentration on the formation of slow- and fast-forming filter retainable complexes. RNAP and *Hae*III-digested T7D111 DNA were incubated at 37 °C in BB containing either 0.1 (lanes 1 and 2), 0.15 (lanes 3 and 4), 0.2 (lanes 5 and 6), or 0.3 M NaCl (lanes 7 and 8). The RNAP and DNA concentrations are the same as for Figure 1A. 0.5 min after mixing, half of the mixture was directly filtered (–heparin). After a 15-min incubation, the other half was briefly challenged with heparin (5 μg/mL) prior to filtration (+heparin).

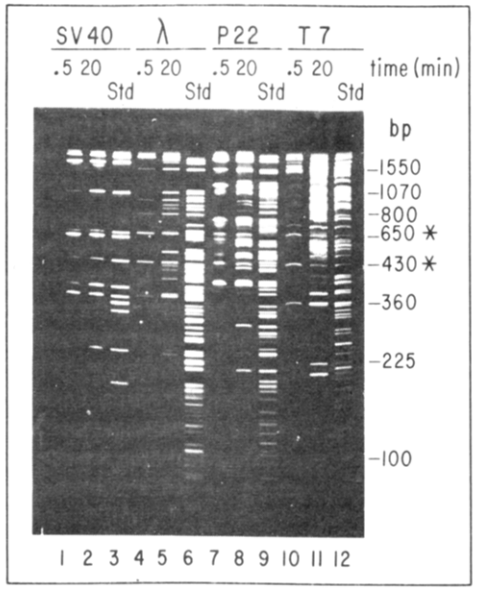

FIGURE 3: Formation of tight complexes between RNAP and DNA fragments from restriction digests of various viral DNAs. *Hae*III digests of SV40 DNA (lanes 1–3), λCI47 DNA (lanes 4–6), P22 DNA (lanes 7–9), and T7D111 DNA (lanes 10–12) were incubated with RNAP at 37 °C. Except for the concentration of enzyme which was 9 μg/mL, all conditions were as described for Figure 1A. One-milliliter portions (λ, P22, T7) or 250-μL portions (SV40) were withdrawn and challenged with heparin (5 μg/mL) either 0.5 or 20 min after mixing. The filtration and wash were done as described in Figure 1A. The amounts of viral DNA loaded in the standard lanes (lanes 3, 6, 9, and 12) were respectively 0.1, 0.4, 0.4, and 0.4 μg. The amount of marker plasmid DNA was 100 ng.

legend to Figure 2; the incubation conditions are indicated above each lane. A comparison of lanes 3 and 5 indicates that there is less of the weak binding (0.5-min incubation) at 0.2 M NaCl than at 0.15 M NaCl. Only faint DNA bands can be detected at 0.3 M NaCl (lane 7). The slow but heparin-resistant binding (15-min incubation) is obviously reduced in going from 0.1 M to 0.15 M NaCl (lanes 2 and 4), and except for the promoter-containing fragment, no T7 DNA is detected after an incubation with 0.2 M NaCl. We have shown, using isolated ^{3}H-labeled fragments, that varying the filtration conditions per se does not affect the level of retention of tight complexes initially formed at low salt (see Discussion). Therefore our observation cannot be solely due to a reduced affinity of the complexes for the filter and must reflect a true variation in the concentration of complexes in solution. Although a quantitative analysis of the salt effect using the gel assay is not possible, it is quite clear that all of the interactions detected by this technique are sensitive to salt concentration.

(*3*) *Formation of Filter-Retainable Nonpromoter Complexes Is Not Limited to T7D111 DNA.* We have first verified that our results are the same in experiments carried out with restriction digests made with different *Hae*III and T7 DNA preparations. An experiment similar to that shown in Figure 1 was repeated with a *Hae*III digest of λCI47 DNA to test whether heparin-sensitive complexes were formed on DNAs other than T7. We found that heparin-sensitive complexes were also formed rapidly on all DNA fragments (data not shown). Figure 3 shows the DNA fragments that can form heparin-resistant complexes when *Hae*III digests of SV40, λCI47, P22, and T7D111 DNA are incubated with RNAP; the incubation was done for either 0.5 or 20 min in 0.1 M BB at 37 °C as described in the figure legend. Each of the digests contains fragments that form heparin-resistant complexes after a long incubation (20 min), but the fraction of the fragments that can do so varies for the different digests. Most of the high molecular weight fragments of the P22 and T7D111 digests form tight complexes (see upper half of lanes 8 and 11). On the other hand, fewer fragments are observed in the lower half of the gel. This decrease in the probability of finding a tight binding site on a given fragment as its size decreases is more apparent for P22 than for T7. In the λCI47 digest, even in the high molecular weight region, a significant fraction of the fragments does not form heparin-resistant complexes (lanes 4 and 5). Lanes 1 and 2 show that most of the fragments in the SV40 digest form tight complexes, including all the large ones. In contrast with those of λ, P22, and T7, the majority of the tight complexes formed on SV40, a eukaryotic viral genome, are already present after the 0.5-min incubation. This is not surprising since several "promoters" recognized by the *E. coli* holoenzyme have been identified on SV40 (Zain et al., 1974; Lescure et al., 1976; Saragosti et al., 1980). There are, however, three fragments that are present in higher amounts after the 20-min incubation (lane 2). The slow formation of heparin-resistant complexes thus occurs on a wide range of sizes and types of DNA fragments.

(*4*) *Properties of the Heparin-Sensitive Interaction.* When ^{3}H-labeled isolated *Hae*III fragments were used, the properties of the heparin-sensitive (weak) interaction described above were investigated with a more quantitative filter assay. Most of the work was done with a group of three ~800-base-pair

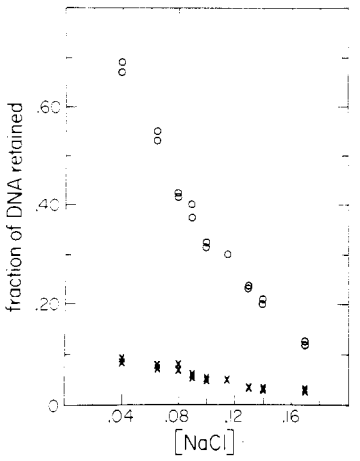

FIGURE 4: Effect of [NaCl] on the formation of heparin-sensitive complexes on the 800-bp DNA fragments. Purified *Hae*III restriction fragments of DNA (1 nM) were incubated with σ-saturated holoenzyme (2 nM) at 37 °C in BB containing various concentrations of NaCl. After a 2-min incubation, five separate aliquots were filtered as described under Materials and Methods, two of which were subjected to a heparin challenge. The results are expressed as the fraction of DNA retained. The values obtained before (O) or after (×) a heparin challenge are plotted as a function of [NaCl] added to BB. Each point represents a separate experiment and is the average of triplicate determinations. Measurements were also done at an enzyme:DNA ratio of 1:1. The ionic strength of the solutions was tested by measuring their conductivity with a Radiometer (Model CDM2e) conductivity meter.

fragments since they did not form tight complexes to a significant extent under the conditions of our assay (see Figure 1B). A pair of 2000-bp fragments used by Strauss et al. (1980a,b) as promoter-free DNA was also studied; as seen in Figure 1 this set is capable of forming TB complexes to a significant extent. The fraction of filter-retainable DNA either before (θ_-) or after (θ_+) the short heparin challenge was determined as described under Materials and Methods. Values measured with the 800-bp fragments at a fixed protein and DNA concentration but with varying NaCl concentrations are shown in Figure 4. Results were also obtained at other protein or DNA concentrations for the 800- and 2000-bp fragments (not shown).

At least 70% of the DNA molecules can form filter-retainable complexes, the majority of which are sensitive to the heparin challenge. It is also clear that the level of retention is extremely sensitive to the NaCl concentration. There is a small but significant amount of filter retention observed after the heparin challenge. This is not likely to be due to contamination of our preparation by fragments containing TB sites, since no such contamination was observed by analytical gel electrophoresis. The retention could result in part from the binding of RNAP at single-strand breaks [as suggested by Kadesch et al. (1980b), although we could not detect a significant amount of such sites in our preparation] or be due to damaged enzyme as proposed by Williams & Chamberlin (1977). Similar measurements with the 2000-bp fragments have given comparable results, except for the expected higher level of time-dependent retention that was observed after the heparin challenge.

As described under Materials and Methods, one can calculate binding constants from the retention data if one assumes that the reaction has reached equilibrium. For the calculation we also assume that the efficiency of detection of the complexes is 100%, independent of solution conditions, and that all enzyme molecules are active in forming such complexes. Our values are therefore lower estimates. Since the nature of the

Table II: Binding Constants for the Heparin-Sensitive Interaction as a Function of Protein and DNA (800-bp Fragments) Concentrations

Part A[a]

[RNAP] (nM)	[DNA] (nM)	θ_-[b]	θ_+[b]	K_{obsd}^R[c] (10^5 M^{-1})	K_{obsd}^E[c] (10^8 M^{-1})
1.0	1.0	0.29 ± 0.03	0.04 ± 0.02	3.9 ± 0.9	2.9 ± 0.6
2.0	1.0	0.45 ± 0.03	0.05 ± 0.02	2.9 ± 0.6	2.5 ± 0.5
4.0	1.0	0.63 ± 0.01	0.07 ± 0.02	2.3 ± 0.2	2.1 ± 0.2
2.0	2.0	0.30 ± 0.03	0.05 ± 0.02	1.8 ± 0.5	1.5 ± 0.5
6.0	2.0	0.58 ± 0.01	0.08 ± 0.02	1.4 ± 0.1	1.2 ± 0.1

Part B[d]

T (°C)	θ_-	θ_+	K_{obsd}^R (10^5 M^{-1})	K_{obsd}^E (10^8 M^{-1})
37	0.45 ± 0.03	0.05 ± 0.02	2.9 ± 0.6	2.5 ± 0.5
22	0.34 ± 0.04	0.035 ± 0.02	1.8 ± 0.5	1.5 ± 0.5
0	0.26 ± 0.02	0.02 ± 0.02	1.2 ± 0.2	1.0 ± 0.2

[a] The temperature was 37 °C and the reaction was done in 0.05 M BB. [b] θ_- and θ_+ are respectively the fraction of DNA retained on the filter before and after a short heparin challenge. Each value is the average of two parallel single determinations done with the same protein and DNA dilutions. [c] K_{obsd}^R and K_{obsd}^E are the binding constants calculated by using the random- and end-binding models, respectively. [d] The concentration of RNAP was 2 nM and that of DNA fragment 1 nM. The reaction was done in 0.05 M BB.

interaction is unknown, the data are analyzed by assuming either of two extreme models: (i) the binding occurs randomly and the number of sites is equal to the number of bases (K_{obsd}^R) or (ii) binding occurs at ends and there are thus only two sites per fragment (K_{obsd}^R).[3] The retention values and binding constants obtained from experiments in which the protein and DNA (800-bp fragments) concentrations were varied under a fixed set of incubation conditions are presented in Table IIA. Although the product of protein and DNA concentrations varies by a factor of 12, the binding constant K_{obsd} varies only by factors of 2.8 and 2.4 for the random- and end-binding models, respectively. If our measurements reflected a single class of complexes at equilibrium, one would expect the value of K_{obsd} to remain constant. The observed variation appears to be small enough to justify an equilibrium analysis. Most of our measurements were done at a DNA concentration of 1 nM and at an E:D molar ratio of 2:1 or 1:1.

Figure 1 demonstrated that the formation of heparin-sensitive complexes was much less affected by temperature than was the case for the heparin-resistant complexes. However, the data reported in Table IIB indicate that the formation of heparin-sensitive complexes is somewhat temperature dependent. Using the van't Hoff equation we calculate a $\Delta H°$ for the reaction of approximately 4 ± 1 kcal.

Figure 5 summarizes the effect of NaCl on the observed binding constants calculated from the filter retention data measured with the 800-bp and 2000-bp fragments by using either the random-binding model (Figure 5A) or the end-binding model (Figure 5B). (In the end-binding calculations, we assumed that there was no contribution from random interior binding. This point is addressed in more detail under Discussion.) As expected, competition by Mg^{2+} produces curved log–log plots. The equations for the solid lines were

[3] K_{obsd} will be used when both the random- and end-binding models are referred to.

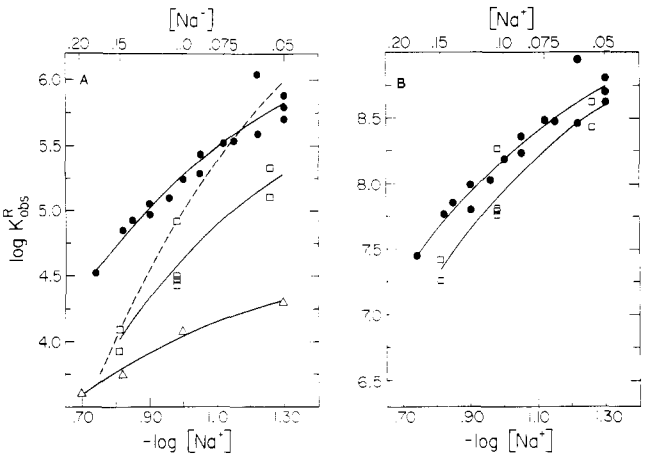

FIGURE 5: Salt dependence of the observed binding constant for the RNAP–DNA heparin-sensitive interaction: log–log plots. All the data used for this plot were obtained in the presence of 10 mM $MgCl_2$. (A) The calculations were done by assuming a random-binding model in which the number of sites is equal to the number of phosphates. (B) The calculations were done by assuming an end-binding model. The solid lines through the data points correspond to nonlinear least-squares fits to the equation described in Record et al. (1977; eq 5), assuming that log K_{obsd}^{Mg} = −1.75 log [Na^+] + 0.32 (Strauss et al., 1980b). (●) Binding constants for the interaction with the 800-bp fragments. The data shown in Figure 4 and values measured at other proteins and DNA concentrations were used. Each point is the average of two separate experiments; triplicate determinations were made in each experiment. (□) Binding constants for the interactions with the 2000-bp fragments. Since these fragments form a significant number of heparin-resistant complexes that varies with time, the extent of filter retention, before and after a heparin challenge, was measured as a function of time. In each experiment, values of K_{obsd} were calculated at two time points. At 0.1 M NaCl two separate experiments were done. For comparison (A) also contains results from other published work. (△) Binding constants reported by Kadesch et al. (1980b; Figure 4) for a 3800-bp *Mbo*I fragment of T7 DNA at 0 °C, in 10 mM Tris/pH 8.0. The curve fitting was done by us. (---) Theoretical line obtained by deHaseth et al. (1978).

obtained by nonlinear least-squares regression analysis of the data for each fragment using the competitive binding theory of Record et al. (1977; eq 5). We have used the relation log $K_{obsd}^{Mg^{2+}}$ = −1.75 log [Na^+] + 0.32 obtained by Strauss et al. (1980b) (note that there is an incorrect sign in the B term of their equation) to describe the competitive effect of Mg^{2+} on the binding of RNAP to DNA. For comparison we have included in Figure 5A the data points reported by Kadesch et al. (1980a) for the binding of RNAP to a 3800-bp *Mbo*I fragment of T7 DNA and the results of deHaseth et al. (1978) for the binding of RNAP to high molecular weight calf thymus DNA.

Table III gives the values of K_{obsd} (0.1 M), the predicted value of K_{obsd} in 0.1 M NaCl/10 mM $MgCl_2$, and of −(d log K_{obsd}/d log [Na^+])$_{[Mg^{2+}]=0}$, the predicted number of monovalent ions that would be released upon complex formation in the absence of Mg^{2+}, that were obtained from the fits to the data of Figure 5. For each fragment, the values of −(d log K_{obsd}/d log [Na^+])$_{[Mg^{2+}]=0}$ clearly do not depend on the model chosen. The values obtained for the 800-bp and 2000-bp fragments are also similar (within error). The average value for −(d log K_{obsd}/d log [Na^+])$_{[Mg^{2+}]=0}$ of 6 ± 1 is significantly larger than the value of 2.8 obtained from our fits of the data reported by Kadesch et al. (1980a).

Figure 5A demonstrates that calculated values of K_{obsd}^{R} are significantly smaller for the 2000-bp fragments than for the 800-bp fragments. This result rules out a random-binding model. For the two fragment sets we studied, Figure 5B indicates that this disparity is substantially reduced by using

Table III: Analysis of the Salt Dependence of the Binding Constants for the Heparin-Sensitive Interaction

fragment size (bp)	model[a]	K_{obsd} (0.1 M)[b] (M^{-1})	−(d log K_{obsd}/d log [Na^+])$_{[Mg^{2+}]=0}$ [b]
800	R	1.9×10^5	5.5
800	E	1.5×10^8	5.5
2000	R	4×10^4	6.6
2000	E	9×10^7	6.6
3800[c]	R	1.1×10^4	2.8
d	R	(1.2×10^5)[d]	(10.8)[d]

[a] As in the text, models R and E refer to the random- and end-binding models, respectively. [b] K_{obsd} (0.1 M) is the predicted value of the binding constant in 0.1 M NaCl/10 mM $MgCl_2$. The quantity −(d log K_{obsd}/d log [Na^+])$_{[Mg^{2+}]=0}$ is the theoretically predicted slope of the linear plot of log K_{obsd} vs. −log [Na^+] in the absence of Mg^{2+}. The values of K_{obsd} (0.1 M) and −(d log K_{obsd}/d log [Na^+])$_{[Mg^{2+}]=0}$ were obtained from the nonlinear fits of the data of Figure 5 using the competitive binding theory of Record et al. (1977; eq 5). [c] The data were taken from Kadesch et al. (1980b). [d] The values of the parameters are those reported by deHaseth et al. (1978) for the nonpromoter binding of RNAP to high molecular weight calf thymus DNA.

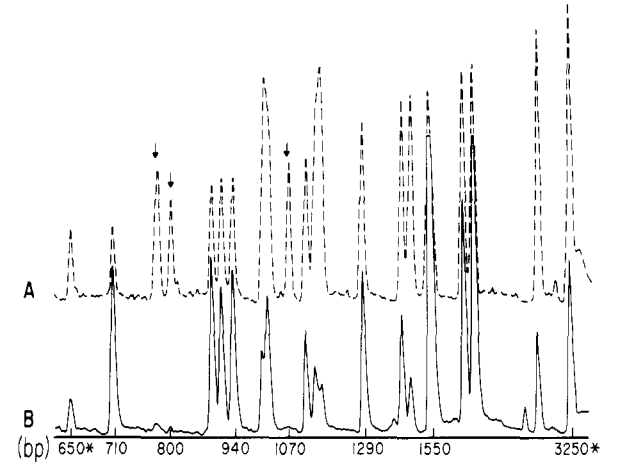

FIGURE 6: Densitometer tracings of lanes 11 and 12 of Figure 1B. A photographic negative of the gel shown in Figure 1B was prepared by using Tri-X film (Kodak). The negative was scanned with a Joyce-Loebl microdensitometer. (A) Scan of lane 12; (B) scan of line 11. The size in base pairs for some of the fragments has been indicated under the corresponding peaks. Arrows indicate three bands present in (A) but missing in (B).

an end-binding model. The approximate independence of K_{obsd}^{E} on the length of the fragment strongly suggests that a model with a relatively small and constant number of sites per fragment (e.g., ends) is the appropriate one to use to analyze our heparin-sensitive filter retention data. (Further interpretation will be presented under Discussion.)

(*5*) *Properties of the Heparin-Resistant Interaction.* (*a*) *Not All T7D111 Fragments Form Tight Nonpromoter Complexes.* A visual inspection of lane 9 or 11 of Figure 1 suggests that heparin-resistant complexes are not formed equally well on all T7 DNA fragments. To demonstrate this point more clearly, we scanned negatives of photographs of lanes 11 and 12 with a microdensitometer. The results are shown in Figure 6. Lane 11 (Figure 6B) contains DNA fragments that are able to form heparin-resistant complexes whereas lane 12 (Figure 6A) contains an equimolar amount of all the fragments produced by the restriction enzyme. A comparison of the two scans shows that three peaks are absent in lane 11. These fragments, indicated by the arrows, must be missing the sites recognized by the enzyme where the stable complexes are formed. In addition, the relative heights of some of the other

peaks in lane 11 do not correspond to those of lane 12. These differences are the result of fragments having either different numbers of such sites or sites with different binding affinities or different kinetics of formation. This fragment-specific behavior suggests that the stable nonpromoter complexes do not form randomly. In addition, this specificity of tight binding makes it seem unlikely that fragment ends are the sites where tight binding occurs, although in principle this is an attractive model because of the lower stability of these sites to denaturation ("melting in"). Phosphatase protection experiments (data not shown) reinforce this conclusion by demonstrating that after a heparin challenge, the level of end protection provided by RNAP to phosphatase digestion is much lower than the level of fragment retention.[4]

(*b*) *Formation of Tight Nonpromoter Complexes Is Not Observed with Core Polymerase.* In order to ascertain that tight binding is a property of holoenzyme, we have verified that incubation of core enzyme with T7 DNA fragments did not lead to time-dependent retention of DNA on the filter (data not shown). Heparin-resistant retention with the characteristic time dependence was, however, observed when an equimolar quantity of pure σ subunit was added to the core–DNA mixture (data not shown). We have also verified that tight binding is detected with RNAP isolated in different laboratories (R. R. Burgess, McArdle Laboratory; W. R. McClure, Carnegie-Mellon) or from different *E. coli* strains (data not shown).

(*c*) *Tight Nonpromoter Complexes Are Competent to Initiate Transcription.* Since the slow-forming nonpromoter complexes have been shown to be resistant to a short competition with heparin, as are open promoter complexes, it is interesting to ask whether their stability is also associated with an opening of the DNA double helix. Taylor & Burgess (1979) have demonstrated that ternary RNAP–DNA–RNA promoter complexes (formed upon addition of NTPs to the incubation mixture) can be retained on the filter even *after* a 0.6 M NaCl wash provided that the new RNA chain is more than two to four bases long. Only background levels are observed when triphosphates are omitted from the incubation. It is thus possible to separate fragments that contain sites at which the enzyme can initiate transcription from those fragments that do not.

Experiments similar to those depicted in Figure 1A were performed to determine whether ternary initiation complexes could be formed on nonpromoter fragments and, if so, if they exhibited the same time and temperature dependence as that observed for the tight nonpromoter binding. The results are shown in Figure 7. Lanes 2, 4, 7, and 8 are control experiments performed in the absence of NTPs at 0 or 37 °C. These

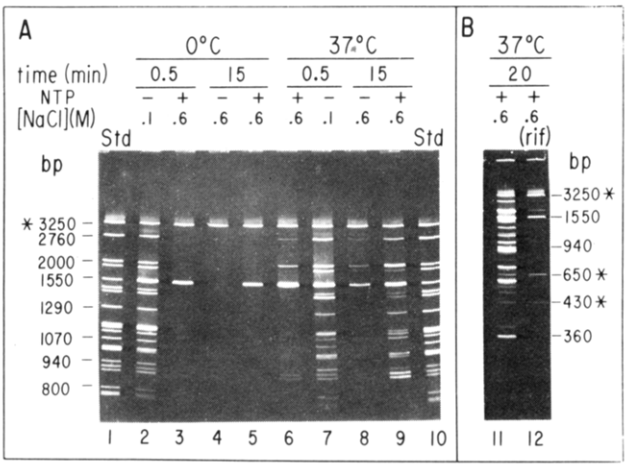

FIGURE 7: Formation of initiation-competent complexes between RNAP and *Hae*III fragments of T7D111 DNA: time, temperature dependence, and effect of rifampicin. (A) RNAP and *Hae*III-digested T7D111 DNA were incubated either at 0 °C (lanes 2–5) or at 37 °C (lanes 6–9) in 0.1 M BB. Two 1-mL portions were withdrawn 0.5 min after mixing. The first one was filtered directly and washed with 0.5 mL of 0.1 M WB. The second one was incubated with 0.1 mM ATP, GTP, and CTP, filtered, and then washed with 2 mL of 0.6 M WB as described under Materials and Methods. Fifteen minutes after mixing two more aliquots were withdrawn. Again, the first was filtered directly and the second was incubated with NTPs. After a 1-min incubation at 37 °C, the samples were filtered and washed with 2 mL of 0.6 M WB. (B) RNAP and *Hae*III-digested T7D111 DNA were incubated at 37 °C in 0.1 M BB. After a 20-min incubation, a 1-mL portion was transferred to a tube containing enough rifampicin for a final concentration of 50 μg/mL (lane 12); after 1 min a mixture of ATP, GTP, CTP, and UTP was added to a final concentration of 0.1 mM, and the sample was filtered after 30 s. A second 1-mL portion was treated the same way except that rifampicin was not added (lane 11). Both filters were washed with 2 mL of 0.6 M WB. The temperature, length of incubation, presence of triphosphates (NTPs), and salt concentration in the wash buffer are all indicated above the gel for the appropriate lanes.

control experiments show that (i) binary complexes are formed after a short (0.5-min) incubation at either temperature, since DNA is retained on the filter after a low salt wash (lanes 2 and 7), and (ii) few of these binary complexes survive a high salt (0.6 M) wash, even after a 15-min incubation at either temperature (lanes 4 and 8). (Two fragments show a higher background level of retention after incubation at 37 °C than at 0 °C.)

Lanes 3, 5, 6, and 9 of Figure 7 show the effect of addition of NTPs to complexes that were formed either at 0 °C (lanes 3 and 5) or at 37 °C (lanes 6 and 9). As observed in lane 5, no detectable amounts of initiation-competent nonpromoter complexes are formed at 0 °C, even after a 15-min incubation in 0.1 M BB. (The 1550-bp promoter fragment is observed on the gel, but since heparin was not present during the short incubation with NTP, these initiation-competent complexes could have formed rapidly after the shift from 0 to 37 °C.) When the incubation in 0.1 M BB is done at 37 °C for 15 min, more fragments remain on the filter following the high salt wash (lane 9). This is not observed after a 0.5-min incubation in 0.1 M BB (lane 6). Initiation-competent complexes are thus formed on many nonpromoter fragments and display a temperature dependence and a time dependence similar to that observed for the formation of TB complexes. Furthermore, the filter-retention patterns for the two types of complexes are similar (compare lane 9 of Figure 7 and lane 9 of Figure 1); in particular those fragments that are absent in one are absent in the other.

In order to eliminate the possibility that our observation is the result of some nonspecific binding of NTPs, we have looked

[4] A few fragments, however, were efficiently protected. The protection was observed even if the incubation with RNAP was brief and even if heparin was present. This cannot be due to the inability of the phosphatase to digest those ends since we verified that in the absence of RNAP all fragment ends are hydrolyzed. This result is evidence of formation of rapidly forming, promoter-like complexes at the ends of a few fragments. One of these, the 350-bp fragment, has been identified as the fragment containing the C promoter characterized by McConnell (1979). The nucleotide sequence shows that position +1 is 18 bases away from the right end of the fragment. End protection caused by binding at that promoter is therefore not surprising. The other two fragments containing protected ends presumably do not contain promoters. It is possible that strong binding sites randomly occur near the restriction site. These results could also mean that such artifactual strong binding sites can be created by cutting with a restriction endonuclease. Although we have not tested the generality of our observation with other nucleases, this information should be kept in mind when promoters on short DNA fragments are studied in vitro.

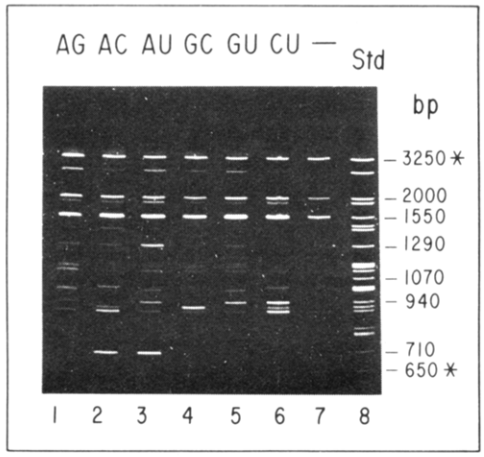

FIGURE 8: Nucleotide specificity of the complex stabilization by NTPs. RNAP and *Hae*III-digested T7D111 DNA were incubated at 37 °C as described in Figure 1A. (Lanes 1–6) After a 15-min incubation, 1-mL aliquots were successively transferred to tubes containing different pairs of NTPs, incubated, filtered, and washed with 0.6 M WB as described for Figure 7. The NTP combinations are indicated above each lane (A stands for ATP etc.). (Lane 7) Control experiment in which the incubation and filtration were done without the addition of NTPs. The filter was washed with 0.6 M WB.

at the effect of rifampicin, which has been shown to block RNA elongation beyond the dinucleotide step (McClure & Cech, 1978) and is therefore expected to prevent the formation of the high salt resistant form. As shown in Figure 7B, addition of rifampicin before NTPs reduces retention to the background level. We therefore conclude that tight nonpromoter complexes can initiate transcription (see Discussion).

(*d*) *Most Tight Nonpromoter Complexes Require Specific Combinations of Nucleotide Triphosphates to Initiate Transcription.* If these initiation-competent nonpromoter sites are specific sequences on the DNA molecule, then in an initiation assay in which only two kinds of NTPs are present, one might expect the formation of high salt resistant complexes to depend on the combination of NTPs chosen. Such an experiment in which the six possible pairs of NTPs were tested is shown in Figure 8. The gel shows that some of the high molecular weight fragments form ternary complexes with any combination of NTPs. The retention of most of the fragments, however, depends on the nature of the NTPs present. For example, in the ~940-bp triplet, the upper band is not well retained if ATP and CTP or if GTP and CTP are used but is retained very well if CTP and UTP are added. The middle band on the other hand seems best retained when the combination GTP and CTP is used. A more dramatic example is the 710-bp fragment which is retained only if either ATP and CTP or ATP and UTP are used. These results imply that the transcription–initiation event is sequence specific.

(*e*) *Measurement of Tight Complexes with Isolated DNA Fragments.* We have followed the formation of heparin-resistant complexes between RNAP and isolated ^{3}H-labeled *Hae*III DNA fragments over periods of up to 40 min. This more quantitative approach has allowed us to confirm the interpretation of the gel assays. Fragments of various sizes were used, and the concentrations of protein and DNA were close to 1 nM. The following results were obtained (data not shown). (i) We have observed that the extent of tight complex formation is fragment specific, as predicted from Figures 1 and 6. (ii) Using fragments of 2000 and 710 bp, we have verified that the formation of tight complexes is virtually abolished by increasing the salt concentration above 0.2 M (with 10 mM $MgCl_2$) or by incubating at 0 °C. (iii) The extent of the reaction also depends on the molar ratio of enzyme to DNA. In 0.1 M BB, at an E:D of 4:1, up to 70% of the DNA molecules are retained on the filter. (Since the level of single-stranded nicks is undetectably low, nicks can be eliminated as the general sites where tight binding occurs.) (iv) The half-time of formation of TB complexes in 0.1 M BB is approximately 2–3 min, much longer than that observed with the A1/D promoter containing fragment under similar conditions (P. Melançon, unpublished results). (v) The half-time of formation of TB complexes is relatively insensitive to the concentrations of RNAP and DNA. The analysis of these data has not yet yielded a unique reaction order or mechanism. (Further work is in progress in this area.)

Discussion

(*1*) *Summary of the Results and Description of a Model.* When *E. coli* RNA polymerase is mixed with a *Hae*III digest of T7D111 DNA in our standard incubation buffer (100 mM NaCl/10 mM $MgCl_2$, pH 7.2), at least three distinct classes of interactions are observed by the filter binding assay. Open-promoter complexes are formed rapidly ($t_{1/2} < 30$ s) at 37 °C and are resistant to a short (10 s) challenge with a low concentration (10 μg/mL) of heparin. A second type of heparin-resistant interaction, called tight binding (TB), is detected on a subset of restriction fragments. This mode of binding (i) has a much slower rate of formation ($t_{1/2}$ = 2–3 min), (ii) is resistant to a 10-s heparin challenge, (iii) is abolished by lowering the temperature to 0 °C or by increasing [NaCl] to 0.2 M, and (iv) allows selective rifampicin-sensitive stabilization by ribonucleoside triphosphates. Heparin-resistant complexes are not observed at 0 °C. In addition, weak complexes, detected only in the absence of a heparin challenge, are formed rapidly on all the DNA fragments we examined. This heparin-sensitive interaction, which is well described by an end-binding model, (i) is observed at either 0 or 37 °C, (ii) is quite dependent on salt concentration [$-(\mathrm{d} \log K_{obsd}/\mathrm{d} \log [Na^+])_{[Mg^{2+}]=0} = 6 \pm 1$ in the absence of Mg^{2+}], and (iii) is not stabilized by NTPs.

We doubt that our observations are binding artifacts that result from damage to the DNA or the RNAP. We have verified that (i) at least 70% of the DNA can be retained by either weak or tight nonpromoter binding, (ii) variations in RNAP and DNA preparations do not affect our observations, (iii) slow enzyme denaturation cannot account for the slow formation of TB complexes, (iv) core polymerase does not cause time-dependent retention of DNA fragments on the filter, and (v) the addition of an equimolar amount of σ subunit to the core–DNA mixture restores the binding behavior observed with the holoenzyme.

On the basis of our observations and other studies of RNAP–DNA interactions, we propose a description of the temporal sequence of events upon mixing a large excess of RNAP with DNA molecules containing various classes of binding sites (such as the restriction digest we have used):

(i) On the time scale of mixing for the range of concentrations investigated, an equilibrium is established between free enzyme, DNA fragment ends (at which filter-retainable complexes are formed), and the large number of weaker binding sites (complexes which are not filter retainable). The free enzyme concentration is therefore dependent on the nature and number of the weak sites. At this time, no heparin-resistant complexes have formed.

(ii) Within a short time thereafter, either through one-dimensional diffusion of weakly bound enzyme along the DNA chain or after multiple, rapid association and dissociation events, a promoter site is located. An open complex is then

rapidly formed. A short heparin challenge does not affect such promoter complexes.[5]

(iii) At a later time, limited by some still unknown process, open complexes are also formed at TB sites; these too are resistant to the heparin challenge.

(*2*) *Comparison with Previous Work on Nonpromoter Interactions of RNAP.* deHaseth et al. (1978), using calibrated DNA–cellulose chromatography, measured the affinity of RNAP for calf thymus DNA. The data were interpreted in terms of a nonspecific random model, and values of K_{obsd}^{R} were obtained as a function of NaCl, both in the presence and in the absence of Mg^{2+}. They observed that K_{obsd}^{R} decreased strongly with increasing salt concentration, $-(\text{d log } K_{obsd}^{R}/\text{d log } [Na^+])_{[Mg^{2+}]=0} = 11 \pm 1$, with a value of K_{obsd}^{R} of 1.2×10^5 M^{-1} characterizing the interaction at 0.1 M NaCl/10 mM $MgCl_2$. Lohman et al. (1980) used difference sedimentation to investigate the interaction of RNAP with T7 DNA at higher [NaCl]. The combined results of these two studies gave a linear plot of log K_{obsd} vs. log $[Na^+]$ (no Mg^{2+}) over the range 0.20–0.32 M NaCl with $-(\text{d log } K_{obsd}/\text{d log } [Na^+])_{[Mg^{2+}]=0} = 8 \pm 1$. Revzin & Woychik (1981) have recently measured binding isotherms with a thermodynamically rigorous centrifugation technique and obtained values of K_{obsd}^{R} in general agreement with those described above. However, their salt dependence (based on only two points) is somewhat smaller $[-(\text{d log } K_{obsd}^{R}/\text{d log } [Na^+])_{[Mg^{2+}]=0} = 6.2]$ than that of Lohman et al. (1980). A unique nonpromoter binding constant provided good fits to the binding isotherms. This justified the assumption that the contribution of specific binding to promoter sites on the DNA could be ignored under the conditions (20 °C) of the experiment [as assumed previously by Lohman et al. (1980)].

Kadesch et al. (1980a,b) have challenged the assumption that there is a single class of nonpromoter interactions of RNAP. By electron microscopy, they obtained evidence for two modes of nonpromoter interactions: (i) a random mode (independent of DNA sequence) that occurred at both 0 and 37 °C, with a value of K_{obsd}^{R} (10^4 M^{-1} in 0.1 M NaCl/10 mM $MgCl_2$) and a salt dependence $[-(\text{d log } K_{obsd}^{R}/\text{d log } [Na^+])_{[Mg^{2+}]=0} = 2.8$; see Figure 6A] which were significantly lower than those obtained by deHaseth et al. (1978) and Revzin & Woychik (1981), and (ii) a TB mode in which RNAP formed long-lived complexes at a slow rate ($k_a = 2 \times 10^5$ M^{-1} s^{-1}) at many sites (about 100 on whole T7 DNA) with some preference for three or four regions of T7 DNA; TB complexes were not observed at 0 °C and their lifetime (>5 min at 0.1 M NaCl/10 mM $MgCl_2$) was quite dependent on NaCl concentration.

Evidence for a mode of binding with properties similar to the TB mode had been reported previously. Zarudnaya et al. (1976) observed that RNAP can bind to DNA and locally destabilize the DNA helix, as shown by its increased reactivity with formaldehyde. This type of interaction was very sensitive to temperature and was not observed at 0 °C. Further investigation with both the kinetic formaldehyde method and electron microscopy (Cherny et al., 1977) showed that the helix-destabilizing complexes occurred at a limited number of sites on T7 and T2 DNA and that the number of such complexes was dramatically reduced by an increase in the NaCl concentration from 0.10 to 0.20 M (with 10 mM $MgCl_2$). Finally, DNA sites have been recently described where RNAP can bind firmly but from which no detectable amount of RNA is synthesized in vitro (West & Rodriguez, 1980; Pirotta et al., 1980).

Since we have detected the tight-binding mode of nonpromoter interaction by filter binding, one may ask why tight complexes were not observed by chromatography and centrifugation. In the chromatographic technique only the eluted protein is monitored, and therefore any tightly DNA-bound protein that remained on the column would not significantly affect the measurements. [Actually, deHaseth et al. (1978) observed some dependence of K_{obsd}^{R} for binding of RNAP to calf thymus DNA on the flow rate and initial binding density that could be explained by heterogeneous binding; this was not seen in their work on *lac* repressor]. The centrifugation studies (Lohman et al., 1980; Revzin & Woychik, 1981), in principle, should have been able to detect tight binding. However, most of the information was obtained near 20 °C; in addition, the measurements of Lohman et al. (1980) were all made above 0.24 M NaCl. We would conclude from our data that little tight nonpromoter binding should have occurred at the lower incubation temperature and/or higher NaCl concentrations of their experiments.

The above nonpromoter interactions will affect all in vitro studies of RNAP–promoter interactions by lowering the free protein concentration and, in particular, will affect the filter assay by causing filter retention. Strauss et al. (1980a) developed a double-label filter binding assay in which competitors were not used. Instead, the filter retention of a promoter-containing fragment due to nonpromoter complexes was internally determined by comparison with a promoter-free DNA fragment of similar size. In their work, Strauss et al. assumed that the extent of formation of nonpromoter complexes was the same on both fragments. Since in these experiments RNAP was not in excess and since TB complexes form slowly, we conclude that the presence of TB sites should have had little effect on their results. These authors in fact found that, under their experimental conditions, the retention of the promoter-free DNA (now known to contain TB sites) was temperature independent and sensitive to dilution, thereby proving that no significant amount of binding was occurring at TB sites. However, the use by Strauss et al. (1980b) of the nonpromoter binding data of deHaseth et al. (1978) to estimate the free protein concentration was probably inappropriate, as will be discussed below.

(*3*) *Interpretation of the Heparin-Sensitive Retention.* (*a*) *Calculation of Binding Constants.* In the analysis of our data we have assumed (i) that all enzyme molecules are capable of forming filter-retainable complexes, (ii) that the efficiency of filter retention is 100% and independent of solution conditions, and (iii) that a single class of sites leads to the heparin-sensitive retention. We can justify the first assumption on the basis of the results of Revzin & Woychik (1981), who observed that the value of K_{obsd}^{R} was not sensitive to the fraction of their enzyme preparation that was competent in a transcription assay. The second assumption [also made by Strauss et al. (1980a,b)] cannot be formally verified, although we have obtained supportive evidence for it: (i) We have shown that the retention of TB complexes is not affected by an adjustment of the [NaCl], immediately prior to filtration, from 0.1 to 0.2 M NaCl. It is thus unlikely that the process of trapping preformed complexes on the filter is very salt sensitive. (ii) We have observed that filter-bound complexes are stabilized relative to those in solution, since the level of

[5] It has been shown that RNAP can be displaced from open-promoter complexes by exposure to heparin at higher concentrations and/or for longer times than those used here (Pfeffer et al., 1977; Giacomoni et al., 1977). In addition, Pfeffer et al. (1977) have found that this sensitivity is promoter dependent. Our operational definition of open complexes is therefore not completely general.

retention of weak complexes is not much affected by a change in the volume of the wash from 0.5 to 5 mL of 0.1 M WB (P. Melançon, unpublished results). However, if the retention efficiency does decrease with increasing NaCl concentration due to dissociation of complexes on the filter, one expects a systematic underestimation of K_{obsd} that will lead to an overestimation of the salt dependence. As was shown in Table IIA the values of K_{obsd} depend on both the protein and DNA concentrations in the assay; we note a systematic decrease in the value of K_{obsd} when either concentration is increased. The variation is sufficiently small to justify our equilibrium analysis. In order to reduce the effect of such small variations, we have made all other measurements at a fragment concentration of 1 nM and at a protein:DNA molar ratio of 2:1 or 1:1.

(*b*) *Nature of the Sites.* We favor a model in which the heparin-sensitive interactions detected by the filter assay occur at DNA ends for the following reasons: (i) The inconsistency between the values of K_{obsd}^{R} obtained by filter binding for the two fragment sets is reduced or eliminated. The small residual difference in K_{obsd}^{E} (if indeed significant) could imply that the binding properties of the ends are slightly different on average in the two fragment sets. (ii) Interpretation of the filter-binding results by the end-binding model does not require us to postulate that complexes with lifetimes in the millisecond range are filter retainable, since the values of K_{obsd}^{E} are much larger (3×10^7–5×10^8 M^{-1}) than those of K_{obsd}^{R} (3×10^4–5×10^6 M^{-1}) and correspond to lifetimes of seconds (assuming a diffusion-controlled k_a of 10^7–10^8 M^{-1} s^{-1}). (iii) If the end-binding model is correct, then the filter binding results are not comparable with those obtained by using high molecular weight DNA (deHaseth et al., 1978; Lohman et al., 1980; Revzin & Woychik, 1981) since these contain far fewer ends. Therefore there need not be a discrepancy. End binding has been observed by electron microscopy (Hirsch & Schleif, 1976; Williams, 1977; Kingston et al., 1981), but it has not yet been systematically investigated.

For the calculation of K_{obsd}^{E} we have assumed that other weak (e.g., random) RNAP–DNA complexes, even though present, do not contribute to filter retention. Furthermore, we have neglected the decrease in free protein concentration caused by such complexes. The effect of this omission will depend on whether one uses the data of deHaseth et al. (1978) or Kadesch et al. (1980a) to correct for the presence of non-filter-retainable complexes. If the results of deHaseth et al. (1978) were used, an increase in the value of $-(\mathrm{d} \log K_{obsd}/\mathrm{d} \log [Na^+])_{[Mg^{2+}]=0}$ would result, since the values of K_{obsd}^{E} on a per molecule basis are the same order of magnitude and the salt dependence of K_{obsd}^{R} of deHaseth et al. (1978) is higher, such that the value of K_{obsd}^{E} is increasingly underestimated as the NaCl concentration is reduced. The values of K_{obsd}^{R} from Kadesch et al. (1980a), on the other hand, are so small that the correction does not change the values of K_{obsd}^{E} significantly. Further interpretation of our results must await an investigation of the binding properties of DNA fragments over a wide range of sizes and types of ends.

Whatever the nature of the sites, it remains clear that this class of heparin-sensitive nonpromoter interactions can be detected by a filter binding assay and can affect the level of free enzyme as well as the extent of DNA retention on the filter.

(*4*) *Interpretation of the Heparin-Resistant Interactions.* (*a*) *Kinetic Discrimination between TB and Promoter Complexes.* As we qualitatively showed in Figure 1A, even though RNAP can form nonpromoter complexes with properties similar to those of promoter complexes, the rate of formation is such that under competitive conditions, at short incubation time, promoter complexes are preferentially formed, and the two modes of binding can thus be resolved kinetically. We have been unable to obtain an unambiguous analysis of kinetic studies with the labeled *Hae*III fragments and so have not estimated a rate constant for complex formation at TB sites. The half-time of formation measured at nanomolar concentrations in 0.1 M BB (2–3 min) is, however, clearly much longer than that observed in studies with the fragment containing the A1/D promoters. On the other hand, this ability to resolve the binding of RNAP at TB and promoter sites on the basis of their rates of formation would not exist for promoters that have a slow rate of formation of open complexes.

(*b*) *Tight Nonpromoter Binding Occurs at Unique Sites.* There are two major observations to support our conclusion that tight nonpromoter binding occurs at specific sites: (i) many fragments in the *Hae*III digests of SV40, λCI47, P22, and T7D111 DNAs do not form heparin-resistant complexes to a significant extent, and (ii) for most fragments only a limited number of combinations of NTPs are effective in stabilizing TB complexes, and these combinations are different for different fragments. Unless the tight binding occurred at particular sites on the DNA, one could not explain the specificity of the nucleotide stabilization or the fact that some fragments form very few TB complexes.

(*c*) *RNAP Can Form Open Complexes at TB Sites.* As initially reported by Anthony et al. (1966), RNAP–promoter complexes, if allowed to initiate transcription and form ternary RNAP–DNA–RNA complexes, are retained on nitrocellulose filters even after the filters are washed with a high salt solution. Taylor & Burgess (1979) have further characterized this observation by showing that (i) initiated complexes are relatively unstable in a 0.6 M NaCl solution until mRNA elongation has proceeded beyond two to four bases and (ii) addition of 50 μM rifampicin before the NTPs prevents stabilization to the 0.6 M wash, in agreement with the previous observation that rifampicin allows synthesis of only dinucleotides (McClure & Cech, 1978).

We have shown that the tight nonpromoter complexes can be protected against a 0.6 M wash by the addition of NTPs. It is unlikely that simple binding of NTPs to the enzyme is responsible for our observation since we have demonstrated that initiation at many TB complexes shows stringent requirements for certain NTPs and that the effect is sensitive to the addition of rifampicin. By analogy to the work on promoters described above, we conclude that multiple nonpromoter sites where RNAP can bind and initiate transcription exist on each of the four genomes we have studied.

If the tight nonpromoter complexes are able to initiate transcription, they must resemble open complexes since it seems unlikely that initiation could occur without prior disruption of the DNA helix. The results of Zarudnaya et al. (1976) and Cherny et al. (1977) provide more support for this conclusion. It is quite plausible that these authors were studying TB complexes since the helix-destabilizing complexes described by them have properties similar to those we observed for TB complexes, in which case the helix destabilization can be interpreted as resulting from the opening of the helix required for transcription initiation.

The initiation of transcription at sites other than promoters has also been observed with the *B. subtilis* RNA polymerase. Achberger & Whiteley (1981) have shown that at low salt (0.1 M NaCl) or in the absence of the δ subunit, the RNA polymerase (core σ) bound to and transcribed regions of the genome of phage SP82 other than those containing early phage

genes. An increase in [NaCl] to 0.3 M or the addition of δ restored a specific pattern of transcription.

(*d*) *Are TB Complexes Formed in Vivo*? Kadesch et al. (1980b) have discussed the possibility that TB sites are regions of DNA that fortuitously have partial homology with the canonical promoter sequence, most likely the AT-rich Pribnow box thought to be involved in the DNA melting step. Our demonstration that TB sites are discrete sequences where transcription initiation can occur is certainly consistent with that model. This suggests that TB sites may be considered as mutant promoters with a very small rate of formation of open complexes.

TB sites are most likely also present on the *E. coli* chromosome. Even though the rate constant for the formation of TB complexes is small, the presence of excess enzyme and the high intracellular concentrations of RNAP and DNA will probably allow some of these complexes to be formed. There is no function yet known for these complexes. Cherny et al. (1977) had suggested that these complexes may be involved in the synthesis of RNA primers during DNA replication. It has been shown, however, that the synthesis of primers is accomplished by primase, the product of gene dnaG [for a review, see Wickner (1978)]. Kadesch et al. (1980b) could not detect efficient initiation of long RNA transcripts at TB sites. The lack of RNA synthesis could result from the fact that TB complexes cannot go beyond the initiation step. (We have not looked at possible abortive initiation occurring at TB sites.) This apparent inability to elongate, as well as the slow rate of formation, may be due to the lack of the proper DNA sequences that make up an effective in vivo promoter. Only in vivo work with TB sites artifically transferred upstream from genes with readily assayable products will allow one to fully answer the above questions.

(*5*) *Predictions for in Vitro Measurements.* Because RNAP can bind at so many sites with promoter-like properties, one might expect some difficulty with the identification of new promoters and the in vitro measurements of their properties. Interference can also be expected from the other nonpromoter interactions. These are problems particularly with techniques that do not distinguish promoters on the basis of some template activity such as the synthesis of fixed length (runoff) transcripts. However, a less discriminatory technique like filter binding can be preferable, especially when one wishes to study the DNA binding step independently of subsequent events; studies on p^- L157, a mutant of the lactose operon promoter, have already shown that changes in DNA sequence do not necessarily affect the binding and transcription–initiation steps in the same way (Maquat & Reznikoff, 1978). In addition, enzymatic assays usually place more stringent requirements on the reaction conditions (temperature, pH, and presence of Mg^{2+}) than those necessary for the retention of protein–DNA complexes on nitrocellulose filters (Strauss et al., 1981) so that the binding reaction cannot be studied over as wide a range of solution conditions.

Binding at TB sites may not always have to be considered as a competitive reaction to promoter binding. Many DNA fragments do not contain TB sites, so that performing in vitro experiments with selected fragments may eliminate the problem altogether. Also, because the formation of tight nonpromoter complexes is so slow, kinetic measurements of fast RNAP–promoter interactions can be done with minimal interference from TB sites. For promoters which form specific complexes with RNAP slowly, the ability to discriminate kinetically is not present, and a different approach must be chosen.

Our measurements indicate that the interaction giving rise to the heparin-sensitive filter retention (be it end binding or other) can be quantified, and therefore its contribution can be estimated. A double-label approach like that of Strauss et al. (1980a), in which a promoter-free DNA fragment is present in the incubation mixture, can also be used. More needs to be known, though, about the relative binding properties of the ends of various fragments. We have also shown that under conditions where promoter complexes are sufficiently stable, a challenge with competitor can be used to eliminate nonpromoter retention. However, except for the first-order analysis of dissociation kinetic experiments, one still needs to know the concentration of complexes in order to estimate the correct free protein concentration. The weaker nonfilter-retainable complexes must also be taken into consideration. Our work does not provide clues as to which value to choose among the various conflicting estimates of the random nonspecific interactions.

In conclusion, since we have identified DNA fragments containing only weak sites (without TB or promoter sites) or having TB sites but no promoter sites, we are able to define experimental conditions (temperature, time, and heparin challenge) under which one can study both promoter and nonpromoter interactions (weak and tight). On the basis of our knowledge of these various interactions, we can now address questions about the nature of filter retention and some properties of filter-bound complexes more systematically (P. Melançon et al., unpublished results) and thus can verify some of the assumptions that must be made when the quantitative filter binding assay is used.

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References

Achberger, E. C., & Whiteley, H. R. (1981) *J. Biol. Chem. 256*, 7424–7432.

Anthony, D. D., Zeszotek, E., & Goldthwait, D. A. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 1026–1033.

Belintsev, B. N., Zavriev, S. K., & Shemyakin, M. F. (1980) *Nucleic Acids Res. 8*, 1391–1404.

Berg, O. G., Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry 20*, 6929–6948.

Blakesley, R. W., & Wells, R. D. (1975) *Nature (London) 257*, 421–422.

Burgess, R. R. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 69–100, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.

Chaconas, G., & van de Sande, J. H. (1980) *Methods Enzymol. 65*, 75–88.

Chamberlin, M. J., Nierman, W. C., Wiggs, J., & Neff, N. (1979) *J. Biol. Chem. 254*, 10061.

Cherny, D. I., Alexsandrov, A. A., Zarudnaya, M. I., Kosaganov, Y. N., Lazurkin, Y. S., Beabealashvilli, R. S., & Savochkina, L. P. (1977) *Eur. J. Biochem. 79*, 309–317.

Cohen, A., Fisher, W. D., Curtiss, R., III, & Adler, H. I. (1968) *Cold Spring Harbor Symp. Quant. Biol. 33*, 635–639.

deHaseth, P. L., Lohman, T. M., Burgess, R. R., & Record, M. T., Jr. (1978) *Biochemistry 17*, 1612–1622.

Giacomoni, P. U., Delain, E., & LePecq, J. B. (1977) *Eur. J. Biochem. 78*, 215–220.
Hansen, J. N. (1976) *Anal. Biochem. 76*, 37–44.
Hinkle, D. C., & Chamberlin, M. J. (1972a) *J. Mol. Biol. 70*, 157–185.
Hinkle, D. C., & Chamberlin, M. J. (1972b) *J. Mol. Biol. 70*, 187–195.
Hirsh, J., & Schleif, R. (1976) *J. Mol. Biol. 108*, 471–490.
Jones, B. B., Chan, H., Rothstein, S., Wells, R. D., & Reznikoff, W. S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4914–4918.
Jones, O. W., & Berg, P. (1966) *J. Mol. Biol. 22*, 199–209.
Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980a) *J. Mol. Biol. 136*, 65–78.
Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980b) *J. Mol. Biol. 136*, 79–93.
Kaiser, A. D. (1957) *Virology 3*, 12–64.
Kingston, R. E., Gutell, R. R., Taylor, A. R., & Chamberlin, M. J. (1981) *J. Mol. Biol. 146*, 433–449.
Koller, T., Kubler, O., Portmann, R., & Sogo, J. (1978) *J. Mol. Biol. 120*, 121–131.
Lescure, B., Oudet, P., Chambon, P., & Yaniv, M. (1976) *J. Mol. Biol. 108*, 83–97.
Lin, S. Y., & Riggs, A. D. (1972) *J. Mol. Biol. 72*, 671–690.
Lohman, T. M., Wensley, C. G., Cina, J., Burgess, R. R., & Record, M. T., Jr. (1980) *Biochemistry 19*, 3516–3522.
Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.
Mangel, W. F., & Chamberlin, M. J. (1974) *J. Biol. Chem. 249*, 3007–3013.
Maquat, L. E., & Reznikoff, W. S. (1978) *J. Mol. Biol. 125*, 467–490.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
McClure, W. R., & Cech, C. L. (1978) *J. Biol. Chem. 253*, 8949–8956.
McConnell, D. J. (1979) *Nucleic Acids Res. 6*, 525–544.
McMaster, G. K., & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835–4838.
Miller, J. S., & Burgess, R. R. (1978) *Biochemistry 17*, 2054–2059.
Oeschger, M. P., & Berlyn, M. K. B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 911–915.
Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.
Pfeffer, S. R., Stahl, S. J., & Chamberlin, M. J. (1977) *J. Biol. Chem. 252*, 5403–5407.
Pirotta, V., Ineichen, K., & Walz, A. (1980) *Mol. Gen. Genet. 180*, 369–376.
Record, M. T., deHaseth, P. L., & Lohman, T. M. (1977) *Biochemistry 16*, 4791–4796.
Revzin, H., & Woychik, R. P. (1981) *Biochemistry 20*, 250–256.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67–83.
Rünzi, W., & Matzura, H. (1976) *J. Bacteriol. 125*, 1237–1239.
Saragosti, S., Croissant, O., & Yaniv, M. (1980) *Eur. J. Biochem. 106*, 25–31.
Stahl, S. J., & Chamberlin, M. J. (1977) *J. Mol. Biol. 112*, 577–601.
Strauss, H. S., Burgess, R. R., & Record, M. T., Jr. (1980a) *Biochemistry 19*, 3496–3504.
Strauss, H. S., Burgess, R. R., & Record, M. T., Jr. (1980b) *Biochemistry 19*, 3504–3515.
Strauss, H. S., Boston, R. S., Record, M. T., Jr., & Burgess, R. R. (1981) *Gene 13*, 75–87.
Studier, F. W. (1975) *J. Mol. Biol. 94*, 283–295.
Studier, F. W., Rosenberg, A. H., Simon, M. N., & Dunn, J. J. (1979) *J. Mol. Biol. 135*, 917–937.
Taylor, W. E., & Burgess, R. R. (1979) *Gene 6*, 331–365.
von Hippel, P. H. (1979) in *Biological Regulation and Development* (Goldberger, R. F., Ed.) pp 279–347, Plenum Press, New York.
West, R. W., & Rodriguez, R. L. (1980) *Gene 9*, 175–193.
Wickner, S. H. (1978) *Annu. Rev. Biochem. 47*, 1163–1191.
Williams, R. C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2311–2315.
Williams, R. C., & Chamberlin, M. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3740–3744.
Zain, B. S., Weisman, S. M., Dhar, R., & Pan, J. (1974) *Nucleic Acids Res. 1*, 577.
Zarudnaya, M. I., Kosaganov, Y. N., Lazurkin, Y. S., Frank-Kamnetskii, M. D., Beabealashvilli, R. S., & Savochkina, L. P. (1976) *Eur. J. Biochem. 63*, 607–615.