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

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

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

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nonmelted "closed" promoter complex proposed as an intermediate in mechanisms of formation of "open" transcriptionally active promoter complexes.

Using a quantitative statistical treatment of the filter binding results, we are able to obtain binding constants for formation of low (0–17 °C) and high (21–37 °C) temperature complexes between holoenzyme and this promoter fragment as a function of ion concentrations and pH. We have analyzed these thermodynamic data using the binding theory of Record et al. (1976, 1978), which was applied previously by deHaseth et al. (1977) and Record et al. (1977) to obtain quantitative information about the sources of stabilization of the specific and nonspecific complexes of *lac* repressor and DNA as well as insight into the molecular details of each type of complex. Although the interactions of RNA polymerase and DNA are more varied than those of *lac* repressor and DNA, making the experimental system and the analysis more complex in the present case, it is nevertheless possible to obtain a partial thermodynamic and molecular characterization of the low- and high-temperature promoter complexes and to compare them with the nonspecific polymerase–DNA complexes investigated by deHaseth et al. (1978) and Lohman et al. (1980a). Characterization of the low-temperature complex is important because it may resemble a closed intermediate in the mechanism of formation of an open promoter complex (Chamberlin, 1974, 1976; Williams & Chamberlin, 1977).

The statistical analysis of the filter binding data used to obtain binding constants, as well as the general thermodynamic analysis of these binding constants, should be applicable to any protein–nucleic acid interaction. It is important to note that the details of the present analysis depend on an independent determination of the nonpromoter binding constants of holoenzyme. A key assumption of the present work is that the nonspecific binding constants determined by deHaseth et al. (1978) and Lohman et al. (1980a) are applicable to describe the interactions of holoenzyme with nonpromoter sites on the fragments investigated. Very recently, some evidence has been obtained to suggest that nonpromoter binding of RNA polymerase is more complex than originally thought (Williams & Chamberlin, 1977; Kadesch et al., 1980a,b; P. Melancon, R. R. Burgess, and M. T. Record, Jr., unpublished work). Therefore, the magnitudes of promoter binding constants presented here may require some revision when more detailed information about modes of nonpromoter binding becomes available. We do not anticipate that such revisions will have any significant effect on the major qualitative conclusions of the present work. Also, in view of the structural, kinetic, and thermodynamic diversity of promoters, these results should not be generalized to other polymerase–promoter interactions until comparative studies have been carried out.

Materials and Methods

The materials and experimental methods used to investigate the selective binding of *E. coli* RNA polymerase holoenzyme to a 1550 base pair HaeIII restriction fragment of T7 D111 DNA containing the A1 and D promoters have been described in the preceding paper (Strauss et al., 1980). Binding constants were calculated from this data according to a statistical model in which the two promoters are assumed to have equal binding affinities for the enzyme (see Theory). Calculations were carried out on a Hewlett-Packard 9820A calculator. Points plotted in the figures are averages of triplicate assays; error bars on these points represent one standard deviation from the mean.

Theory

DNA that contains promoter sites may be retained on nitrocellulose filters by complex formation with RNA polymerase at both promoter and nonspecific sites. The contribution of each of these types of complexes to the retention of DNA on filters (overall efficiency) depends upon the binding constant, the number of sites, and the efficiency of retention of individual complexes of each type (elementary efficiency) [cf. H. S. Strauss, R. S. Boston, M. T. Record, Jr., and R. R. Burgess (unpublished experiments)]. In order to obtain equilibrium binding constants for individual complexes, contributions to DNA retention from promoter and nonspecific binding must be distinguished, and the number of binding sites and elementary efficiencies of these types of complexes must be taken into account. In this section we develop the formalism for calculating binding constants for RNA polymerase–promoter complexes from data generated in the nitrocellulose filter binding assay described in the preceding paper (Strauss et al., 1980). We use probability theory to allow for two classes of binding sites, promoter and nonspecific, and for multiple sites within each class. Giacomoni (1976) used a related approach to account for multiple promoter sites in interpreting filter binding data on intact T7 DNA. We assume that all sites are independent, that all nonspecific sites are identical, and that the average binding densities in the experiments (generally less than one RNA polymerase molecule per DNA fragment) are low enough so that the effects of overlap of nonspecific sites can be neglected [cf. McGhee & von Hippel (1974)]. For nonspecific binding we define potential binding sites and binding constants in terms of the concentration of DNA phosphates and use the nonspecific binding constants determined by deHaseth et al. (1978) and Lohman et al. (1980a). We further assume that if an RNA polymerase–promoter complex exists in solution immediately before the dilution step, it will remain intact throughout the dilution, filtration, and wash steps and the DNA fragment to which the RNA polymerase is bound will remain on the filter. (Equivalently, we assume that the elementary efficiency of retention of promoter complexes is 1.) Evidence concerning the validity of these assumptions will be discussed below.

(*1*) *Separating the Contributions from Specific and Nonspecific Binding.* Each DNA molecule retained on a nitrocellulose filter has one or more RNA polymerase molecules bound either nonspecifically or at the promoter sites. Because of these multiple possibilities, it is easier to consider the fraction of DNA molecules *not retained* on the filter, since in this case exactly zero molecules of polymerase are bound to promoter and nonspecific sites after the filtration and wash steps. The experimental quantity is $1 - \theta_P$, the fraction of promoter-containing DNA molecules not retained on the filter. Then

$$P(N_T = 0) = 1 - \theta_P \quad (1)$$

where $P(N_T = 0)$ is the probability that the total number (N_T) of polymerase molecules bound to a promoter fragment after filtration and wash is zero. This probability can be decomposed into the product of two independent probabilities:

$$P(N_T = 0) = [P(N_P = 0)][P(N_D = 0)] \quad (2)$$

where $P(N_P = 0)$ and $P(N_D = 0)$ are the probabilities that the numbers N_P and N_D of polymerase bound to promoter and nonspecific DNA sites are zero.

We can evaluate $P(N_D = 0)$ from the fractional retention of the nonpromoter DNA fragment, here designated θ_D. For this fragment, nonspecific binding is the only means of retention of DNA on the filter and

$$P(N_D = 0) = 1 - \theta_D \quad (3)$$

Combining eq 1 and 3 we obtain

$$P(N_P = 0) = \frac{1 - \theta_P}{1 - \theta_D} \quad (4)$$

In this expression, the $1 - \theta_D$ term acts as a correction factor. In effect, it factors out the fraction of promoter fragments retained on the filter which only have nonspecific complexes. $1 - \theta_D$ need not be a direct reflection of the number of nonspecific complexes in solution but need only reflect the final retention of nonspecific complexes. If the efficiency of retention of preexisting nonspecific complexes were known, then nonspecific binding constants could be obtained from values of $1 - \theta_D$ (Strauss, 1979).

(2) Probability of Formation of Individual RNA Polymerase–Promoter Complexes. Equation 4 is a measure of the overall contribution of promoter binding to the retention of the promoter fragment on the filter. To proceed in the binding constant calculation, we must find the probability of formation of individual RNA polymerase–promoter complexes. Mathematically, this requires an explicit functional form for $P(N_P = k)$ where k is the number of RNA polymerase–promoter complexes per fragment and $P(N_P = 0)$ is the point at which we know the value of the function. In terms of our filter binding experiment, two limiting functional forms are possible. This is because the promoter fragment contains both the T7 A1 and D promoters which may contribute equally or unequally to the overall promoter binding.

The relative contributions from the two promoters (designated 1 and 2) may be expressed in terms of the ratio of the individual binding constants K_1/K_2. If they contribute equally, $K_1/K_2 = 1$. If they contribute unequally, $K_1/K_2 \neq 1$. The extreme case of unequal contributions is effectively a single promoter. This is a reasonable approximation if $K_1/K_2 > 10$.

Case a: $K_1/K_2 = 1$. If the promoters contribute equally, we can consider $P(N_P = k)$ to be a binomial distribution with two sites:

$$P(N_P = k) = \binom{2}{k} P_b^k (1 - P_b)^{2-k} \quad (5)$$

where P_b is the probability of binding to an individual site, the desired quantity. The subscript b indicates that p arises from the *b*inomial formulation. We can relate P_b to experimental quantities by setting $k = 0$ and combining eq 4 and 5:

$$P_b = 1 - \left(\frac{1 - \theta_P}{1 - \theta_D}\right)^{1/2} \quad (6)$$

Case b: $K_1/K_2 > 10$. The limiting case of two promoters contributing unequally to promoter binding is obtained by assuming that the fragment contains a single promoter. Then

$$P(N_P = 0) + P(N_P = 1) = 1 \quad (7)$$

and, combining eq 3 and 6

$$P(N_P = 1) = p_s = 1 - \frac{1 - \theta_P}{1 - \theta_D} \quad (8)$$

Here p_s is the probability of formation of an individual RNA polymerase–promoter complex. The subscript s denotes that the probability arises from the single promoter limit.

(3) Calculation of K_{obsd}^{RP}. The observed binding constant K_{obsd}^{RP} is defined as

$$K_{obsd}^{RP} = \frac{[RP]}{[R][P]} \quad (9)$$

The concentration of RNA polymerase–promoter complexes in the incubation solution [RP], assuming an efficiency of 100% in both the filter retention and the detection of these complexes, is

$$[RP] = p[P_T] \quad (10)$$

where p is either p_s or p_b, according to the model chosen to interpret the data (cf. eq 6 and 8 above) and $[P_T]$ is the total concentration of promoters.

The free promoter concentration [P] is obtained from the conservation equation

$$[P] = [P_T] - [RP] = [P_T](1 - p) \quad (11)$$

Finally, the concentration of free RNA polymerase [R] is given by

$$[R] = [R_T] - [RP] - [RD] \quad (12)$$

where $[R_T]$ is the total concentration of RNA polymerase and [RD], the concentration of nonspecific polymerase–DNA complexes in the incubation solution, is calculated from the polymerase nonspecific binding constant K_{obsd}^{RD} appropriate to the solution conditions of the experiment (deHaseth et al., 1978):

$$[RD] = K_{obsd}^{RD}[D_T][R] \quad (13)$$

[If elementary efficiencies of retention of nonspecific complexes were known, values of K_{obsd}^{RD} could be calculated from the data of Strauss et al. (1980).] In eq 13, $[D_T]$ is the total concentration of nonspecific DNA sites (the total nucleotide concentration, including both the promoter fragment and the nonspecific fragment). $[D_T]$ is used as an approximation to [D], the free nucleotide concentration. At the low binding densities of the experiments analyzed here, this approximation is accurate to within 1%.

Combining eq 9–13, we obtain

$$K_{obsd}^{RP} = \frac{p}{1 - p} \frac{1 + K_{obsd}^{RD}[D_T]}{[R_T] - p[P_T]} \quad (14)$$

Consequently, K_{obsd}^{RP} can be determined from the experimental quantity p (determined from θ_P and θ_D), the known concentrations $[R_T]$, $[P_T]$, and $[D_T]$, and the nonspecific binding constant K_{obsd}^{RD}.

Results

(1) Calculation of K_{obsd}^{RP} for Different Concentrations of RNA Polymerase and of Promoter DNA. Fractional extents of retention of promoter (θ_P) and nonpromoter (θ_D) fragments were determined as a function of the total concentrations of σ-saturated RNA polymerase and DNA fragments, using the double-label filter assay of Strauss et al. (1980). Experiments were performed at 37 °C with 0.20 M NaCl and 0.01 M Hepes[1] buffer at a pH of 7.50 or 7.35 (as indicated) using both the dilution (A) and no-dilution (B) protocols of Strauss et al. (1980). Accurate data were obtained over a 50-fold range of RNA polymerase concentrations and a 20-fold range of DNA fragment concentrations. Results of these experiments are shown in Table I, which lists mean values and standard deviations of θ_P and θ_D (determined from triplicate assays) as functions of $[R_T]$ and $[P_T]$, the total concentrations of RNA polymerase and of promoters (assuming two promoters per fragment). In individual assays, deviations in θ_P and θ_D from the average were often correlated, and therefore values of K_{obsd}^{RP} were calculated for each pair of θ_P and θ_D values, by using the two-promoter model (eq 6 and 14). Mean values and standard deviations of K_{obsd}^{RP} are listed in Table I. Triplicate determinations of K_{obsd}^{RP} agree to within ±10–50%. Values of K_{obsd}^{RP} vary by no more than a factor of 4 over the

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

Table I: Promoter Binding Constants at Different Concentrations of RNA Polymerase and DNA

protocol[a]	$[R_T]$ (nm)	$[P_T]$[b] (nm)	θ_P	θ_D	$K_{obsd}^{RP} \times 10^{-8}$ (M^{-1})
A	0.4	1.6	0.09 ± 0.01	0.06 ± 0.01	*c, e*
A	1.6	1.6	0.43 ± 0.02	0.16 ± 0.08	2.7 ± 1.1[c]
A	8.0	1.6	0.94 ± 0.05	0.32 ± 0.05	4.6 ± 1.1[c]
A	2.2	3.6	0.53 ± 0.02	0.08 ± 0.01	4.4 ± 0.6[d]
B	0.04	0.16	0.07 ± 0.04	0.07 ± 0.05	*c, e*
B	0.16	0.16	0.16 ± 0.01	0.05 ± 0.01	4.0 ± 0.4[c]
B	0.80	0.16	0.70 ± 0.03	0.11 ± 0.01	10 ± 2[c]
B	0.72	0.19	0.54 ± 0.05	0.05 ± 0.02	7.6 ± 2.4[c]
B	0.73	0.21	0.46 ± 0.12	0.08 ± 0.01	5.4 ± 2.7[c]
				mean (±SD):	5.5 ± 2.7

[a] Protocol A involves a 10-fold dilution before filtration; protocol B has no dilution step. [b] $[P_T]$ is the total promoter concentration, assuming two promoters per promoter fragment (binomial model). [c] Conditions: 37 °C; pH 7.50; 0.20 M NaCl; 0.01 M Hepes buffer. The concentration of the nonpromoter DNA fragment was equal to the concentration of the promoter fragment ($[D_T] = (3.1 \times 10^3)[P_T]$ on a site basis); $K_{obsd}^{RD} = 1.5 \times 10^5$ M^{-1} (deHaseth et al., 1978). [d] Conditions: 37 °C; pH 7.35; 0.20 M NaCl; 0.01 M Hepes buffer. The concentration of the nonpromoter DNA fragment was 1×10^{-9} M ($[D_T] = 8.7 \times 10^{-6}$ M); $K_{obsd}^{RD} = 1.7 \times 10^5$ M^{-1} (deHaseth et al., 1978). The tabulated value of K_{obsd}^{RP} has been corrected to pH 7.5 by using the pH dependence of K_{obsd}^{RP} from Figure 4. (The correction decreases the calculated value of K_{obsd}^{RP} by a factor of 2.) [e] In these experiments, the difference between θ_P and θ_D is not sufficient to allow an accurate estimation of K_{obsd}^{RP}.

concentration ranges examined and show no significant variation with either the reactant concentrations or the protocol employed. The mean value of K_{obsd}^{RP}, based on 21 individual determinations, is 5.5 (±2.7) × 10^8 M^{-1}. The standard deviation (±50% of the mean) is comparable to the error observed by Riggs et al. (1970a,b) in a previous quantitative application of filter binding. Comparable experimental errors are associated with most other methods of determining binding constants of protein–nucleic acid interactions [cf. deHaseth et al. (1977, 1978), Lohman et al. (1980a), and Draper & von Hippel (1979)].

Use of the single-promoter model (eq 8) to interpret the filter retention data yields values of K_{obsd}^{RP} which are larger by two- to threefold, depending on the reactant concentrations. There is no significant effect of the choice of model (eq 6 or 8) on either the invariance of K_{obsd}^{RP} to macromolecular concentrations or the dependences of K_{obsd}^{RP} on solution conditions, discussed below. In what follows, we have chosen to use the two-promoter model in the calculation of K_{obsd}^{RP}.

We conclude from the lack of dependence of K_{obsd}^{RP} on the concentrations of promoters or of RNA polymerase that the binding reaction is at or near equilibrium in the standard 20-min incubation period. Further evidence supporting this assertion is discussed in relation to Figure 4 below.

(2) K_{obsd}^{RP} Is a Sensitive Function of [NaCl]. (a) Dependence of K_{obsd}^{RP} on [NaCl] Is Larger at 37 °C Than at 0 °C. Figure 1 shows the dependence of the logarithm of the observed RNA polymerase–promoter binding constant on the logarithm of the sodium ion concentration. Experiments were performed in the absence of divalent cations at two temperatures, 0 and 37 °C. The binding constants of the complexes formed at both temperatures decrease markedly with increasing $[Na^+]$, although the dependence on $[Na^+]$ of the 0 °C complex is significantly less than that of the 37 °C complex. The 0 °C complex also has a lower binding constant throughout the range of salt concentrations tested, although the magnitude of the difference depends upon the electrolyte concentration.

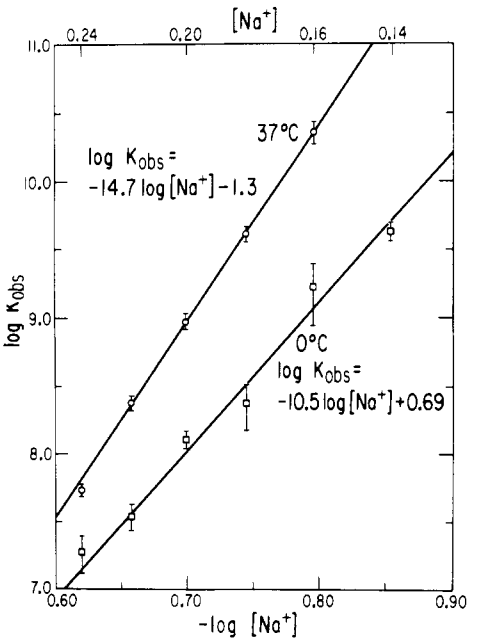

FIGURE 1: Dependence of the observed binding constant of the RNA polymerase–promoter complex on the concentration of added NaCl, at two temperatures, 0 and 37 °C, in the absence of divalent cations. Binding constants were calculated from the binomial promoter model by using data from Strauss et al. (1980) obtained by protocol A. Solid lines represent the linear least-squares fit through the data points. Concentrations based on 50 μL: RNA polymerase, 2.2×10^{-9} M; ^{32}P-labeled promoter fragment, 1.80×10^{-9} M; ^3H-labeled nonpromoter fragments, 1.01×10^{-9} M. Binding buffer: 0.01 M Hepes, pH 7.35 at 37 °C and pH 7.51 at 0 °C; 10^{-3} M DTT; 10^{-3} M Na_3EDTA; 50 μg/mL BSA; plus the indicated concentrations of NaCl. Symbols: (○) incubation at 37 °C; (□) incubation at 0 °C.

Equations for the solid lines in Figure 1 were calculated by using a linear least-squares analysis of the data points at each temperature. They are, for 37 °C

$$\log K_{obsd}^{RP} = -14.7 \log [Na^+] - 1.3 \qquad (15)$$
$$0.14 \leq [Na^+] \leq 0.24$$

and for 0 °C

$$\log K_{obsd}^{RP} = -10.5 \log [Na^+] + 0.69 \qquad (16)$$
$$0.14 \leq [Na^+] \leq 0.24$$

The slope of each line is a direct measure of the number of low molecular weight ions released upon complex formation at that temperature (Record et al., 1976, 1978). Thus, Figure 1 shows that more ions are released upon formation of the 37 °C complex than upon formation of the 0 °C complex.

For comparison, deHaseth et al. (1978) found that the binding constants for RNA polymerase holoenzyme–nonspecific DNA complexes at both 0 and 37 °C can be represented by

$$\log K_{obsd}^{RD} = -10.8 \log [Na^+] - 2.3 \qquad (17)$$
$$0.20 \leq [Na^+] \leq 0.26$$

The number of ions released upon formation of RNA polymerase–nonspecific DNA complexes is similar to that observed for the 0 °C RNA polymerase–promoter complex and less than that observed for the 37 °C RNA polymerase–promoter complex. At 0.2 M Na^+, the nonspecific binding constant is 1.8×10^5 M^{-1}, making it 600-fold and 5000-fold less than the binding constants obtained for the 0 °C and 37 °C complexes, respectively.

From the dependences of log K_{obsd} on log $[Na^+]$ given in eq 15–17, information can be obtained concerning the relative

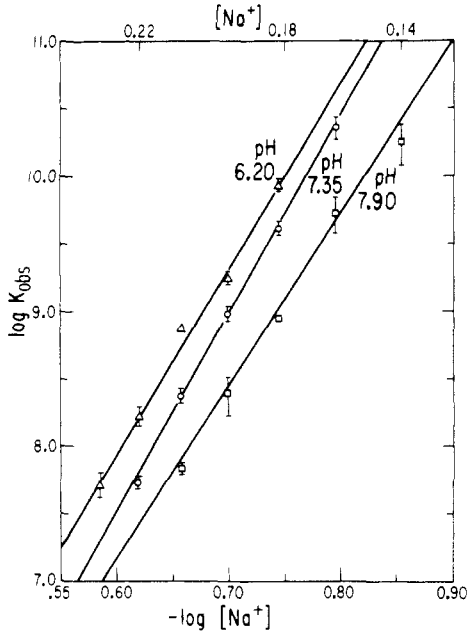

FIGURE 2: Dependence of the observed binding constant of the RNA polymerase–promoter complex on the concentration of added NaCl, at three pH values, 6.20, 7.35, and 7.90. Log–log plots. Incubation temperature was 37 °C; no divalent cations were present in the solution. Binding constants were calculated from the binomial promoter model from experiments performed according to protocol A. Solid lines represent the linear least-squares fit through the data points. The concentrations of RNA polymerase and DNA and the binding buffer were the same as in Figure 1 except 0.01 M Hepes, pH 7.35 at 37 °C, was replaced with either 0.01 M sodium cacodylate, pH 6.20 at 37 °C, or 0.01 M Hepes, pH 7.90 at 37 °C. Symbols: (△) pH 6.20; (○) pH 7.35; (□) pH 7.90.

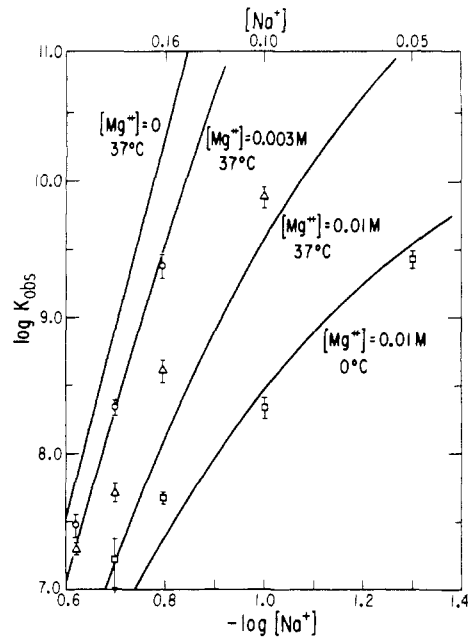

FIGURE 3: Dependence of the observed binding constant of the RNA polymerase–promoter complex on the concentration of added NaCl at three concentrations of Mg^{2+}. Log–log plots. Binding constants were calculated from the binomial promoter model by using data from Strauss et al. (1980) obtained by protocol A. The solid curves were calculated by using the binding theory described in Record et al. (1978). The concentrations of RNA polymerase and DNA and the binding buffer were the same as in Figure 1 except the indicated concentration of $MgCl_2$ was added and the concentration of Na_3EDTA was decreased to 10^{-4} M. Incubations were at the temperatures indicated. Symbols: (○) $[Mg^{2+}]$ = 0.003 M, 37 °C incubation; (△) $[Mg^{2+}]$ = 0.01 M, 37 °C incubation; (□) $[Mg^{2+}]$ = 0.01 M, 0 °C incubation.

contributions of electrostatic and nonelectrostatic interactions to the stability of the various polymerase DNA complexes and about the maximum number of charged groups involved in the binding process. This interpretation is given under Discussion.

(b) Dependence of K_{obsd}^{RP} on [NaCl] at 37 °C Is Independent of pH. Figure 2 shows the dependence of K_{obsd}^{RP} on the [NaCl] at 37 °C at three pH values, 6.20, 7.35, and 7.90. The data can be adequately described by the equations (linear least-squares lines): for pH 6.20

$$\log K_{obsd} = -13.7 \log [Na^+] - 0.29 \qquad (18)$$

$$0.18 \leq [Na^+] \leq 0.24$$

and for pH 7.90

$$\log K_{obsd} = -12.6 \log [Na^+] - 0.41 \qquad (19)$$

$$0.14 \leq [Na^+] \leq 0.22$$

The data in Figure 2 at pH 7.35 are taken from Figure 1 (cf. eq 15).

Again, the slopes of the lines are a direct measure of the number of low molecular weight ions released upon complex formation. The slopes at all three pH values are within experimental error of each other and give a mean value of 13.7 ± 1. Throughout the salt range studied, the observed binding constants are higher at lower pH values. This effect is further demonstrated in Figure 4 and discussed below.

(c) Addition of $MgCl_2$ Reduces Both K_{obsd}^{RP} and the Magnitude of Its Dependence on [NaCl]. Figure 3 summarizes the effects of Mg^{2+} on K_{obsd}^{RP} at 0 and 37 °C, as a function of the Na^+ concentration. (i) The presence of Mg^{2+} (0.01 M) does not affect either the relative stabilities or relative salt sensitivities of the 0 and 37 °C complexes. Both K_{obsd}^{RP} and d log K_{obsd}^{RP}/d log $[Na^+]$ are greater for the 37 °C complex than for the 0 °C complex, as is observed in the absence of Mg^{2+} (cf. Figure 1). (ii) For both types of complex, K_{obsd}^{RP} and d log K_{obsd}^{RP}/d log $[Na^+]$ decrease with increasing Mg^{2+} concentration. These effects become more pronounced as the Na^+ concentration is reduced.

Qualitatively, the effects of Mg^{2+} on K_{obsd}^{RP} and d log K_{obsd}^{RP}/d log $[Na^+]$ can be explained as follows. Mg^{2+} binds to DNA (like any other polyvalent cationic ligand), competes with RNA polymerase for phosphate binding sites, and thereby lowers the observed binding constant for the polymerase–promoter interaction. Since the binding of Mg^{2+} releases Na^+ ions from the DNA, the extent of binding of Mg^{2+} increases as the Na^+ concentration decreases. Consequently, the competitive effect of a given concentration of Mg^{2+} is greater at low Na^+ concentration. Finally, the derivative d log K_{obsd}^{RP}/d log $[Na^+]$ is a measure of the amount of Na^+ release accompanying formation of the polymerase–promoter complex. At low Na^+ concentration in the presence of Mg^{2+}, little Na^+ remains associated with the DNA (having been replaced by Mg^{2+}); consequently, little Na^+ is released when polymerase binds. Instead, Mg^{2+} is released, but this release does not contribute to d log K_{obsd}^{RP}/d log $[Na^+]$.

In Figure 3, the solid curves are theoretical curves based on a quantitative treatment of the roles of Mg^{2+} as a competitive ligand. This approach was successful in analyzing the effect of Mg^{2+} on the specific and nonspecific interactions of *lac* repressor with DNA (Record et al., 1977, 1978) and on the interaction of pentalysine with DNA (Lohman et al., 1980b). Further discussion of this analysis, and of possible origins of the discrepancies between theory and experiment in Figure 3, is given below.

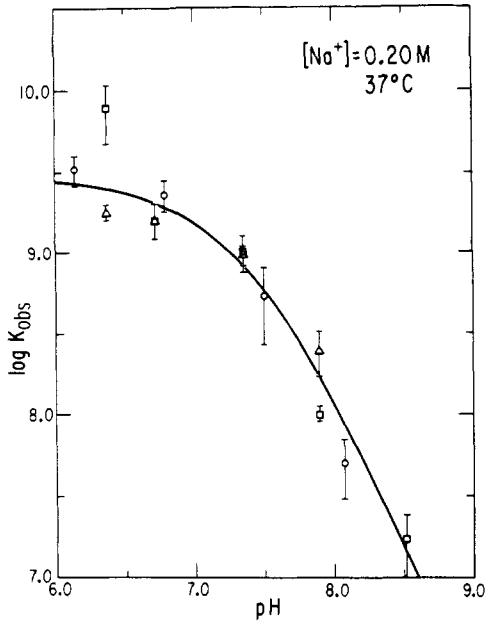

FIGURE 4: Dependence of the observed binding constant of the RNA polymerase–promoter complex on the pH of the incubation solution. Binding constants were calculated from the binomial promoter model by using data obtained by protocols A and B. The solid curve was calculated by using the binding theory described in Record et al. (1978), assuming a requirement for protonation of two groups with pK values of 7.4 and a value of log K_{obsd}^{RP} (1 M) = −0.8. Binding buffer: 10^{-3} M Na_3EDTA, 10^{-3} M DTT, 50 μg/mL BSA, and 0.20 M NaCl at 37 °C; also, as appropriate, 0.01 M sodium cacodylate, 0.01 M Hepes, and 0.01 M Bicine at the indicated pH values. Symbols: (□) protocol A, [RNA polymerase] = 2.2×10^{-9} M, [^{3}H-labeled promoter fragment] = 6.3×10^{-10} M, [^{32}P-labeled nonpromoter fragments] = 1.05×10^{-9} M; (△) protocol A, data taken from Figure 2; (○) protocol B, [RNA polymerase] = 7.35×10^{-10} M, [^{32}P-labeled promoter fragment] = 1.07×10^{-10} M, [^{3}H-labeled nonpromoter fragments] = 1.5×10^{-10} M.

(3) K_{obsd}^{RP} Decreases with Increasing pH. Figure 4 shows the dependence of the logarithm of the observed binding constant of the polymerase–promoter complex formed at 37 °C on the pH of the solution. At low pH, K_{obsd}^{RP} is only weakly pH dependent; above neutral pH, K_{obsd}^{RP} decreases with increasing pH. The derivative −d log K_{obsd}^{RP}/d pH approaches the value 2 at alkaline pH. No tendency for K_{obsd}^{RP} to level off at high pH is apparent over the range investigated. The solid curve in Figure 4 was calculated by using the binding theory of deHaseth et al. (1977) and Record et al. (1978) by assuming that the protonation of two groups on polymerase, each with a pK of 7.4, is *required* for formation of the 37 °C complex. Alternative models involving different numbers of protons or in which it is assumed that protonation of polymerase merely increases the binding affinity but is not required for binding [cf. Record et al. (1978)] do not fit the data of Figure 4.

The observed binding constant of the polymerase–promoter complex formed at 0 °C also shows a large pH dependence, although the data in this case are more limited [cf. Figure 6 of Strauss et al. (1980)]. We infer from this behavior that the uptake of at least one and possibly two protons is required for formation of the 0 °C complex. Both the 0 and 37 °C complexes show much greater dependences of K_{obsd}^{RP} on pH than those seen by deHaseth et al. (1978) for the nonspecific complexes of core and holoenzyme forms of RNA polymerase with DNA. (For these nonspecific complexes, −d log K_{obsd}^{RD}/d pH did not exceed 0.5 from pH 6 to pH 9.)

The data in Figure 4 were obtained by using two different protocols (Strauss et al., 1980) and ranges of three- and sixfold

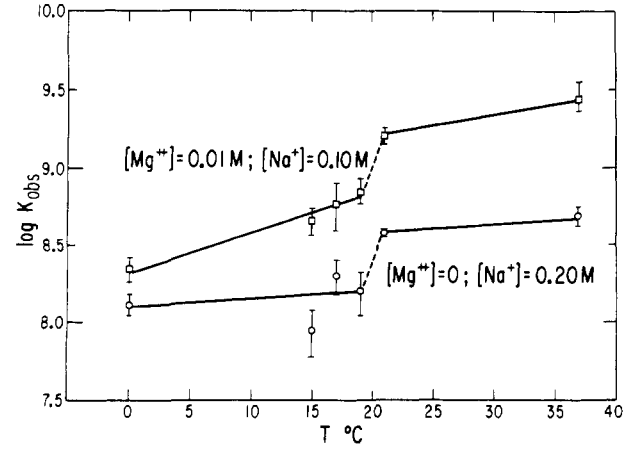

FIGURE 5: Dependence of the observed binding constant of the RNA polymerase–promoter complex on the incubation temperature at two ionic conditions. Binding constants were calculated from the binomial promoter model by using data from Strauss et al. (1980). The binding constants were not corrected for the temperature dependence of the pH of the buffer which ranged between 7.51 at 0 °C and 7.35 at 37 °C. The lines drawn through the data are interpretative and do not correspond to any theory. Binding buffer: 0.01 M Hepes, 10^{-3} M DTT, 50 μg/mL BSA, and either 0.01 M $MgCl_2$, 0.10 M NaCl, and 10^{-4} M Na_3EDTA (□) or 0.20 M NaCl and 10^{-3} M Na_3EDTA (○).

in the RNA polymerase and DNA concentrations in the incubation solution, respectively. That the binding constants calculated from these different concentrations agree within experimental error demonstrates again the likelihood that the reaction is at equilibrium (see above).

(4) K_{obsd}^{RP} Increases with Increasing Temperature (0–37 °C). Figure 5 shows the effect of temperature on the observed RNA polymerase–promoter binding constants at two ionic conditions. The lines drawn through the points at both ionic conditions are interpretative and are not based on any theoretical model. The observed binding constant increases gradually in the ranges 0–17 and 21–37 °C and increases more abruptly between 17 and 21 °C at both ionic conditions. There is approximately a 10-fold increase in the binding constant, the exact magnitude depending on ionic conditions, between 0 and 37 °C (see also Figures 1 and 3). For comparison, deHaseth et al. (1978) found that the interaction of RNA polymerase with nonspecific DNA sites exhibited little temperature dependence and gave a binding constant of 10^5 M^{-1} at both ionic conditions shown here.

Equilibrium Selectivity Ratio (ESR). In the preceding paper, Strauss et al. (1980) used a double-label filter binding assay to demonstrate in a qualitative manner the *existence* of selectivity of RNA polymerase holoenzyme for the T7 A1/D promoter fragment. To discuss the *extent* of selectivity under various solution conditions, the ratio of specific and nonspecific binding constants is required. We call this ratio the equilibrium selectivity ratio (ESR):

$$\mathrm{ESR} = K_{obsd}^{RP}/K_{obsd}^{RD}$$

The ESR is a measure of the selective affinity of polymerase holoenzyme for a promoter site as compared to a nonspecific site (where the number of nonspecific sites equals the number of nucleotides). The logarithm of the ESR is proportional to the standard free energy difference between promoter and nonspecific complexes under the given solution conditions. If the ratio of promoter sites to nonspecific sites on a DNA molecule is known, the product of this site ratio and the ESR specificies the equilibrium distribution of polymerase between promoter and nonpromoter sites, in the limit of low polymerase binding density. For example, the site ratio for the HaeIII

Table II: Equilibrium Selectivity Ratios: ESR = $K_{obsd}^{RP}/K_{obsd}^{RD}$

ESR as a Function of Na^+ and Mg^{2+} Concentrations

	ESR at Mg^{2+} concentrations of			
[Na^+]	none (37 °C)[b]	none (0 °C)[c]	0.01 M (37 °C)[b]	0.01 M (0 °C)[c]
0.05		8×10^2		9×10^2
0.10	8×10^4[a]	8×10^2	2.5×10^4[a]	8×10^2
0.16	1×10^4	8×10^2	1.5×10^4	2×10^3
0.18	7×10^3			
0.20	5×10^3	8×10^2	1×10^4	4×10^3
0.22	4×10^3			
0.24	3×10^3		5×10^3	

ESR Values for Complexes as a Function of pH (0.2 M NaCl)

pH	ESR (37 °C)	pH	ESR (0 °C)
6.14	5×10^3	5.87	9×10^2
6.78	6×10^3	6.98	6×10^2
7.35	6×10^3	7.51	8×10^2
7.49	5×10^3	7.69	$<10^2$
7.90	1×10^3	8.27	$<10^2$
8.07	8×10^2	8.92	$<10^2$
8.52	3×10^2		
8.59	$<10^2$		

[a] Extrapolated value. [b] pH 7.35. [c] pH 7.51.

fragment used in this work is 1550. Consequently, at low binding density, and an ESR of 1550, half of the bound polymerase will be at promoter sites and half will be at nonspecific sites in the population. For this fragment, an ESR of 10^2 corresponds to a distribution of polymerase (at low binding densities) in which there is 10^2 times as much polymerase at promoter sites than at any *individual* nonspecific site, but in which 94% of the bound polymerase is at nonspecific sites, and only 6% is at promoter sites. This distribution represents the lower limit of detection of specificity of binding in our assay. Use of a shorter fragment would extend the range of accessible ESR values.

Values of the ESR as a function of solution conditions are listed in Table II. Experimentally accessible values range from 10^2 to 10^4. (Under solution conditions giving an ESR above 10^4, K_{obsd}^{RP} is too large to determine in our assay.) At 0 °C, the ESR appears to be independent of [NaCl] and [$MgCl_2$], but decreases with increasing pH from a value of $\sim 10^3$ near pH 6 to a value of $<10^2$ above pH 7.7. (Above pH 7.7, selectivity of binding at 0 °C still presumably exists but is not detectable above the base-line level of nonspecific binding to this fragment. Under these conditions, the difference in standard binding free energies between specific and nonspecific complexes has decreased to less than 3 kcal/mol.) At 37 °C, the ESR decreases with increasing [NaCl] and increasing pH, from values in excess of 10^4 at low salt and/or low pH to values of 10^2 or less above pH 8.6 (at 0.2 M NaCl). (At the low-salt conditions of some binding and transcription assays (0.05 M NaCl and 0.01 M $MgCl_2$), we estimate that the ESR is $\sim 10^5$, which would give relatively selective binding to promoters even on intact T7 DNA.) The apparent variations in ESR of the 37 °C complex upon addition of $MgCl_2$ at constant [NaCl] are within the experimental uncertainty (approximately ±50%) in these ratios.

Discussion

Accuracy of K_{obsd}^{RP}. Random experimental errors contribute an uncertainty of approximately ±50% in K_{obsd}^{RP}, as judged from standard deviations of the mean in triplicate assays or from the reproducibility of independent determinations of K_{obsd}^{RP} (cf. Table I). This uncertainty includes random errors in concentrations of macromolecular and low molecular weight components of the reaction mixture as well as random errors associated with the binding assay (variations in the dilution and wash procedures, temperature variations, and/or uncertainties introduced by the crossover correction and background corrections applied to the data from liquid scintillation counting). Of these sources of error, the uncertainty in the background correction for DNA retained on the filter in the absence of RNA polymerase is probably the most important under our experimental conditions. The background fractional retention of DNA was generally in the range 0.01–0.04, although values as high as 0.06 were encountered (Strauss et al., 1980). Since the source of variability in the background retention is unknown, all background corrections are subject to a substantial (~50%) uncertainty. Consequently, values of $\theta_P \geq 0.1$ are generally required to obtain binding constants of more than order-of-magnitude accuracy. Because values of $\theta_D < 0.1$ were often obtained, they are subject to a large relative error from the uncertainty in the background correction. This does not significantly affect the calculation of K_{obsd}^{RP} as long as $\theta_P \geq 0.1$.

Since an error of ±50% in K_{obsd}^{RP} results in an error of less than ±5% in log K_{obsd}^{RP} (the significant quantity in the thermodynamic calculations performed with the data), our major concern is not with random error but rather with systematic errors, particularly those which might affect the determination of K_{obsd}^{RP} to different extents as a function of solution conditions. The most likely sources of systematic error appear to be the following assumptions: (a) the binomial-promoter model is applicable under all solution conditions; (b) values of K_{obsd}^{RD} obtained by deHaseth et al. (1978) are appropriate for determination of the competitive role of nonpromoter binding of polymerase in our experiments; (c) all RNA polymerase molecules are active in promoter binding under all solution conditions; (d) the elementary efficiency of retention of polymerase–promoter complexes is 100% under all solution conditions.

We cannot rule out the possibility that these assumptions are incorrect. However, some arguments in favor of their validity can be made.

(a) The single-promoter and binomial-promoter models represent the two extremes of behavior. Values of K_{obsd}^{RP} calculated from the filter retention data are based on the binomial model. Consistent use of the single-promoter model would increase all values of K_{obsd}^{RP} by a factor which varies from 1.5 to 3 or more, depending on the reactant concentrations and the binding density. However, use of the single promoter model does not affect the values of the derivatives of log K_{obsd}^{RP} with respect to log [Na^+] or pH, within experimental uncertainty (Strauss, 1979). There is no guarantee, however, that either model is applicable to *all* solution conditions examined. If the two promoters (A1 and D) differ in their sensitivity to ion concentrations, pH, or temperature, then the analysis used in this paper is oversimplified. Even so, the maximum error in the derivatives of log K_{obsd}^{RP} with respect to log [Na^+] or pH introduced by such an effect should be no more than 10–15% (a systematic shift in values of K_{obsd}^{RP} by a factor of 1.5–3 occurring over a range in K_{obsd}^{RP} of 2–3 orders of magnitude).

(b) Values of K_{obsd}^{RD} were determined by deHaseth et al. (1978) by analysis of the elution of polymerase from columns of immobilized nonpromoter DNA. This method is subject to the criticisms that the physical state of the adsorbed DNA may differ from that of DNA in solution and that the analysis of the elution profile is based on discrete extraction theory rather than a more rigorous chromatography theory. However,

values of K_{obsd}^{RD} determined by deHaseth et al. (1978) have recently been confirmed by Lohman et al. (1980a) using a difference sedimentation technique and intact T7 DNA (under ionic conditions where we estimate that ~90% of the polymerase is nonspecifically bound). Lohman et al. (1980a) conclude that the error in K_{obsd}^{RD} is less than a factor of 2; standard methods of error analysis show that this introduces an uncertainty of less than a factor of 2 into the determination of K_{obsd}^{RP}.

In principle, it is also possible that the binding of RNA polymerase to the nonpromoter fragment is not the same as the nonspecific binding studied by deHaseth et al. (1978) and Lohman et al. (1980a) but rather is binding to ends (or other tight-binding sites; Kadesch et al., 1980b) to form more stable complexes. Under conditions where these sites are present in excess, eq 14 remains applicable for the calculation of K_{obsd}^{RP}, but the term $K_{obsd}^{RD}[D_T]$ should be replaced by the product of the appropriate site binding constant and site concentration. However, since both experimental observation (Strauss et al., 1980) and calculations based on the data of deHaseth et al. (1978) and Lohman et al. (1980a) agree that nonspecific binding and promoter binding are of comparable importance in the population under the conditions of our experiments, it appears that the term $K_{obsd}^{RD}[D_T]$ [which is of comparable magnitude to K_{obsd}^{RP}; see Equilibrium Selectivity Ratio (ESR)] is at least a reasonable parameterization of the contributions of nonpromoter binding by RNA polymerase to the distribution of polymerase in the solution. Further work is in progress to define the modes of nonpromoter binding of RNA polymerase.

(c) A minimum estimate of the fraction of molecules of σ-saturated RNA polymerase that is able to bind specifically to promoter sites can be obtained by analysis of those double-label binding experiments performed with limiting concentrations of polymerase ($[R_T] < [P_T]$) at low salt concentration (e.g., 0.16 M NaCl or 0.01 M $MgCl_2$ plus 0.10 M NaCl) at 37 °C, where more than 90% of the polymerase is bound to the DNA [see Figures 3 and 4 of Strauss et al. (1980)]. Under these conditions, assuming that both promoters bind polymerase with equal affinity, we calculate that between 66 and 78% of the polymerase is bound to promoter sites. Most of the remainder is nonspecifically bound. It is consistent with all the available data to conclude that nonspecifically bound polymerase can bind specifically as well and that the observed distribution of polymerase between promoter and nonspecific sites is a consequence of the relatively low ESR values and the large excess of nonspecific sites under the conditions investigated. Therefore, we conclude that more than 90% of the RNA polymerase molecules are capable of binding to DNA. (Using the alternative single promoter model to analyze these same binding data, we find that between 53 and 60% of polymerase molecules are bound to promoter sites, and more than 88% are bound to either a promoter or nonpromoter site on DNA.) In the calculations reported here, we assume that all the RNA polymerase is active in promoter and nonspecific binding.

(d) The assumption that the elementary efficiency of retention of polymerase–promoter complexes is 100% under all solution conditions examined cannot be simply justified by theoretical arguments. Binding constants as low as 2×10^7 M^{-1} were determined by the filter assay. Published values of the bimolecular association rate constant for various polymerase–promoter complexes are in the range 10^7–10^8 M^{-1} s^{-1} or larger (Hinkle & Chamberlin, 1972b; Seeburg et al., 1977; Giacomoni, 1979). Consequently, the lifetimes of complexes with binding constants below 10^9 M^{-1} may be comparable to or less than the time of the dilution, filtration, and wash steps. Therefore, the elementary efficiency of retention of these complexes may be less than 100%. The effect of this would be to introduce a systematic error into both the absolute and relative values of K_{obsd}^{RP} determined as a function of solution conditions (ion concentrations, pH, or temperature) where the elementary efficiency is not constant at 100%. Consequently, the magnitudes of the derivatives of log K_{obsd}^{RP} with respect to these solution variables may be overestimated. This effect, if present, may not be too serious because the binding constants estimated by extrapolation of our data to the lower ionic conditions used by others to study RNA polymerase–promoter interactions are generally in order-of-magnitude agreement with published results (see below). Moreover, from comparison of values of θ_D (in the region $\theta_D \geq 0.1$ where the uncertainty in the background correction is small) with the calculated distribution of RNA polymerase molecules over nonspecific sites, we find that the elementary efficiency of filter retention of nonspecific complexes is in the range 20–60% under all conditions where K_{obsd}^{RD} is in the range 10^5–10^6 M^{-1}. (These calculated efficiencies do not show any strong correlation with the values of K_{obsd}^{RD}.) We cannot explain why the efficiency of filter retention of these weak complexes is so large. Nevertheless, this result gives us more confidence that the assumption of 100% efficiency of retention of specific complexes with $K_{obsd}^{RD} > 10^7$ M^{-1} may be reasonable.

Comparisons with Previous Work. Equilibrium binding constants for the complexes of RNA polymerase with various promoters have been determined by filter binding (Hinkle & Chamberlin, 1972a,b; Seeburg et al., 1977), electron microscopy (Giacomoni et al., 1977a,b; Williams & Chamberlin, 1977), and a steady-state initiation assay (W. McClure and C. Cech, personal communication). Most of these determinations are for a single set of ion concentrations, typically lower than those investigated by us. Consequently, a comparison of our results with those obtained by others requires extrapolation of our values of K_{obsd}^{RP}. The uncertainty in this extrapolation, combined with the uncertainty in the determination of absolute values of K_{obsd}^{RD}, implies that only order-of-magnitude comparisons should be attempted. In addition, as noted above, there is no reason to expect identical binding constants for different promoters.

Williams & Chamberlin (1977) estimated by electron microscopy that the binding constant for the interaction of RNA polymerase with principally the A1 and A3 promoters on a 1100 base pair fragment of T7 DNA is in the range 10^8–10^9 M^{-1} at 0 °C (0.05 M NaCl, 0.01 M $MgCl_2$, and Tris, pH 8). We obtain $K_{obsd}^{RP} = 3 \times 10^9$ M^{-1} at 0 °C (0.05 M NaCl and 0.01 M $MgCl_2$, pH 7.5). This will be reduced by ~3–10-fold at pH 8 (depending on the exact magnitude of the pH dependence of K_{obsd}^{RP} at 0 °C) and consequently is in agreement with the result of Williams & Chamberlin (1977). However, as noted by deHaseth et al. (1978), the values of K_{obsd}^{RD} used in our calculation do not agree with the nonspecific binding constant determined by Williams & Chamberlin (1977).

Hinkle & Chamberlin (1972a,b) estimated a binding constant of 10^{14} M^{-1} (at 0.05 M NaCl, 0.01 M $MgCl_2$, 37 °C, and Tris, pH 7.9) for the interaction of polymerase with promoters on intact T7 DNA. Giacomoni (1976) used Poisson statistics to obtain an average binding constant for the interaction of polymerase with a single promoter of $\sim 2 \times 10^{12}$ M^{-1} from this result. An attempt to directly measure K_{obsd}^{RP} under these ionic conditions gave a result (3×10^{10} M^{-1} at

pH 7.5) which is probably in error [cf. Figure 4 of Strauss et al. (1980)] because the value of θ_P is anomalously low. Extrapolation of data obtained at higher NaCl concentration yields an estimate in excess of 10^{11} M^{-1} under the conditions used by Hinkle & Chamberlin (1972a,b).

W. McClure and C. Cech (personal communication) have analyzed kinetic results obtained from a steady-state initiation assay according to a model in which an initial rapid equilibrium involving formation of a closed complex is followed by formation of an open complex. For the A2 promoter of T7 DNA, they find an overall binding constant (for formation of an open complex) of 6×10^{10} M^{-1} and a binding constant of 2×10^8 M^{-1} for formation of the intermediate closed complex at 0.08 M KCl and 0.01 M $MgCl_2$, pH 7.7 at 37 °C. Under these conditions, we estimate K_{obsd}^{RP} to be 4×10^{10} M^{-1} for the 37 °C complex and 10^8 M^{-1} for the 0 °C complex. Though this agreement is encouraging, it again involves a comparison of two different promoters.

Giacomoni et al. (1977a,b) obtained binding constants for RNA polymerase–promoter complexes on the replicative form of fd phage (fd RF) from an equilibrium binding curve using electron microscopy. They estimate a lower bound for K_{obsd}^{RP} of 10^9 M^{-1} at 0.12 M NaCl, 0.01 M $MgCl_2$, and Tris, pH 7.9, at 37 °C. Under the same ionic conditions, Seeburg et al. (1977) have measured the rate of association and dissociation of polymerase complexed with individual DNA restriction fragments of fd RF. From the ratio of rate constants, Seeburg et al. (1977) found the binding constants of fd RF promoters ranged between 2×10^8 and 2×10^{11} M^{-1}. (The number of strong sites per restriction fragment was not included in these binding constant calculations, however.) Although comparisons of different promoters may not be meaningful, we note that extrapolation of our data to these ionic conditions yields a binding constant of $\sim 10^9$ M^{-1}.

Thermodynamic and Molecular Interpretation of Polymerase–Promoter Binding Data. Both thermodynamic information and molecular information concerning the interactions stabilizing a noncovalent protein–nucleic acid complex can be obtained by analysis of the dependences of the observed binding constants on solution conditions [cf. Record et al. (1976, 1977, 1978), deHaseth et al. (1977, 1978), and Lohman et al. (1980a,b)]. Here we apply this analysis to the 37 and 0 °C polymerase–promoter complexes and compare their properties and sources of stability to those of nonspecific polymerase–DNA complexes.

(*a*) *General Interpretation of Electrolyte Effects on K_{obsd}.* deHaseth et al. (1978) demonstrated that the nonspecific binding of RNA polymerase holoenzyme to double-helical DNA was extremely sensitive to monovalent and divalent cation concentrations. We find analogous behavior for the specific binding of RNA polymerase holoenzyme to the A1/D promoter fragment at 0 and 37 °C. In the absence of divalent cations, the logarithms of the observed binding constants (K_{obsd}) are linear functions of the logarithm of the NaCl concentration (cf. eq 15–19 above).

Record et al. (1976, 1978) analyzed the binding of cationic ligands to DNA and showed that the slope $-(\mathrm{d} \log K_{obsd}/\mathrm{d} \log [Na^+])$ is a measure of the number of low molecular weight ions (Na^+; Cl^-) released from the DNA and the ligand in forming the complex. From this slope, the number of positively charged groups on the ligand that interact with DNA phosphates can be estimated. In addition, if the majority of ion release occurs from the nucleic acid, as appears to be the case in situations where the charge on the ligand is small (see below), the magnitude of K_{obsd} at any fixed NaCl concentration can be decomposed into contributions from electrostatic and nonelectrostatic interactions. We summarize this analysis here briefly. Two factors contribute to the dependence of K_{obsd} on NaCl concentration in the salt concentration range of usual interest (Record et al., 1976, 1978).

(1) Low molecular weight ions, previously condensed (Manning, 1969) on the nucleic acid or bound to cationic groups on the ligand, are displaced by the binding of the ligand to the nucleic acid. These ions are direct stoichiometric participants in the reaction. Since K_{obsd} is defined in terms of the concentrations of the macromolecular reactants only, it will vary as the concentration of these low molecular weight participants is varied.

(2) Low molecular weight ions form screening atmospheres about the unneutralized phosphates on the DNA (and the cationic groups on the ligand). Neutralization of these charges by complex formation will affect the distribution of electrolyte ions involved in screening. These screening effects are introduced by way of the activity coefficients of the macromolecular components, which are therefore functions of the electrolyte concentration. Since K_{obsd} is written in terms of macromolecular concentrations and not activities, an additional dependence of K_{obsd} on salt concentration results.

Record et al. (1976, 1978) showed, using polyelectrolyte theory (Manning, 1969, 1978) that the condensation (binding) and screening effects of a 1:1 salt like NaCl on helical DNA are *thermodynamically equivalent* to the binding of 0.88 of a cation per DNA phosphate. This extent of thermodynamic binding is independent of the NaCl concentration in the bulk solution. If a Z-valent cationic ligand binds to a region of DNA and interacts electrostatically with Z phosphates, then the thermodynamic binding of Na^+ to the DNA will be reduced by the amount $0.88Z$. If, in addition, k anions are displaced from the ligand (again in the thermodynamic sense), the total ion release detected by a thermodynamic method will be $0.88Z + k$. Examination of the dependence of K_{obsd} on NaCl concentration is such a thermodynamic method, and it is readily shown that (Record et al., 1976, 1978)

$$-\frac{\mathrm{d} \log K_{obsd}}{\mathrm{d} \log [Na^+]} = 0.88Z + k \qquad (20)$$

If the anion coefficient k is known or can be neglected, the number of cationic groups on the ligand interacting with DNA phosphates can be determined from the salt dependence of the binding constant. Even in situations where k may be non-negligible, an upper bound on Z can be determined. The maximum value of Z, Z_{max}, is given by

$$-\frac{\mathrm{d} \log K_{obsd}}{\mathrm{d} \log [Na^+]} = 0.88Z_{max} \qquad (21)$$

For a number of small cationic ligands of known valence (e.g., Mg^{2+}, oligolysines, and dyes), for which the interactions of anions with the ligand can reasonably be neglected ($k = 0$), eq 20 has been shown to be in agreement with the experimental salt dependence of K_{obsd} (Record et al., 1976, 1978; Capelle et al., 1979; Lohman et al., 1980b). It should be emphasized, however, that no structural features of the ligand or the complex are incorporated into the derivative of eq 20 and that the DNA is modeled as a one-dimensional array of charges separated by an average distance of 1.7 Å. End effects and other effects arising from the actual three-dimensional distribution of charges on the ligand and the DNA are neglected. Conformational changes in the DNA that might accompany binding of the ligand are also not considered. In addition, if the Z-valent ligand is itself delocalized (condensed)

on the DNA, instead of being site bound, then Z monovalent cations (rather than $0.88Z$) will be released from the DNA when it binds (Manning, 1978). In spite of these approximations and assumptions, eq 20 and our interpretation of ion effects appear applicable to protein–nucleic acid interactions [cf. Record et al. (1978)]. In particular, the number of interactions between DNA phosphates and cationic groups on *lac* repressor in the specific repressor–operator complex predicted from binding data by using eq 20 (8 ± 1; Record et al., 1977) agrees well with the number of phosphates whose modification weakens the binding of repressor to operator (7; W. Gilbert, personal communication).

Since binding constants K_{obsd} for the interactions of cationic ligands with nucleic acids are so sensitive to the ionic environment, it is of interest to obtain the thermodynamic equilibrium constant for the reaction, which includes the direct and indirect effects of ions and is therefore independent of ion concentrations. While sufficient information is not available to determine exact thermodynamic equilibrium constants [cf. Record et al. (1978) for an attempt along these lines], a first approximation is to use the extrapolated value of K_{obsd} at 1 M NaCl [denoted K_{obsd} (1 M)]. This binding constant refers to pseudo-standard-state conditions, i.e., conversion of reactants at concentrations of 1 M to products (including free electrolyte ions) at 1 M. For oligolysines with chain lengths N in the range three to eight residues, values of K_{obsd} (1 M) are of order of magnitude unity; $-\log K_{obsd}$ (1 M) $\simeq 0.2N$ at neutral pH (Record et al., 1976, 1978; Lohman et al., 1980b). Consequently, the contribution to the binding free energy from a lysine–phosphate interaction at an electrolyte concentration of 1 M is essentially zero; this is reasonable because the process is in reality the replacement of a counterion–phosphate interaction by the lysine–phosphate interaction. As the electrolyte concentration is reduced from 1 M, the binding affinity of the oligolysine (or any cationic ligand, including proteins with cationic binding sites) increases due to the entropic contribution to the free energy of binding that arises from the release of Na^+ ions into a dilute salt solution (a free energy of dilution). Manning (1978) has provided a molecular thermodynamic justification of these results.

In situations where anion effects can be neglected ($k = 0$), extrapolation of the observed binding constant for a protein–nucleic acid interaction to 1 M NaCl provides an estimate not only of the thermodynamic equilibrium constant but also of the relative contributions of electrostatic and nonelectrostatic effects to the interaction. If the extrapolated binding constant is significantly larger than that estimated for Z lysine–phosphate electrostatic interactions [$-\log K_{obsd}$ (1 M) $\simeq 0.2Z$], then the difference reflects the nonelectrostatic (salt-independent) contribution to the binding free energy. If k is not negligible, the above procedure still provides a minimum estimate of the nonelectrostatic term. [If the observed binding constant is also a function of pH, accurate evaluation of the nonelectrostatic component may be more complex (Record et al., 1978; Lohman et al., 1980b)].

(*b*) *Interpretation of Salt Effects on RNA Polymerase–DNA Complexes.* From eq 15–19, the numbers of ions released (in the thermodynamic sense defined above) in the formation of the various complexes between RNA polymerase holoenzyme and double-stranded DNA are as follows: nonspecific complex, 10.8 ± 2; 0 °C promoter complex, 10.5 ± 1.5; 37 °C promoter complex, 13.7 ± 1.0. For the 37 °C complex, the error estimate is the standard deviation of the results of experiments at pH 6.20, 7.35, and 7.90. For the other complexes, the error is estimated graphically from linear fits to the data with maximum and minimum slopes. In our opinion, the difference in the amount of ion release obtained in forming the 0 and 37 °C promoter complexes is significant. The amount of ion release in forming the 0 °C promoter complex is the same as that observed by deHaseth et al. (1978) for the nonspecific complex of holoenzyme with nonpromoter DNA; more ions are released in forming the 37 °C promoter complex. For comparison, we note that 21 ± 3 ions are released in the formation of the complex between core polymerase and double-stranded DNA and 18 ± 5 ions are released in forming the complexes between either holoenzyme or core RNA polymerase and single-stranded DNA (deHaseth et al., 1978).

For the nonspecific complex of holoenzyme with double-stranded DNA, deHaseth et al. (1978) found that $\log K_{obsd}^{RD}$ (1 M) $= -2.5 \pm 1.5$. The binding constant was independent of temperature, and only weakly pH dependent. For this complex, $Z_{max} = 12 \pm 2$. The simplest interpretation of these binding data is to assume that anion release is not a significant factor ($k = 0$), that ~ 12 cationic groups on polymerase interact with DNA phosphates, and that the entropic contribution to the binding free energy resulting from the release of ions from the DNA drives the binding reaction. Within error, the value of $\log K_{obsd}^{RD}$ (1 M) can be accounted for by the formation of 12 ionic interactions between cationic amino acid residues and DNA phosphates. The data thus suggest that there is no major nonelectrostatic or enthalpic contribution to the binding free energy, as would be the case if substantial conformational changes in polymerase or DNA occurred in the reaction. The nonspecific complex is presumably an outside or closed complex, in which the interactions are with phosphates on the outside of the helix and not with functional groups on the bases.

Our data suggest that the 0 °C promoter complex retains the electrostatic component of the nonspecific holoenzyme–DNA complex but has in addition a net favorable nonelectrostatic contribution to the binding free energy which presumably relates to the specificity of its formation. The amount of ion release is the same for both complexes. It is reasonable to assume that the same binding site on polymerase is involved in both interactions. If this is the case, then the same cationic groups (a maximum of 12) are probably involved in both complexes.

deHaseth et al. (1978) proposed that the nonspecific complex was stabilized by electrostatic interactions only. The value of K_{obsd}^{RP} (1 M) for the 0 °C promoter complex at pH 7.5 is probably larger than that obtained for the nonspecific complex (0.7 ± 1.5 as compared to -2.5 ± 1.5). We therefore estimate that the minimum nonelectrostatic contribution to K_{obsd}^{RP} (at all electrolyte concentrations) is a factor of $\sim 10^3$. This is a minimum estimate because of the possible roles of anions and protons (see below) in the promoter binding reaction at 0 °C.

These results are consistent with a picture in which the 0 °C promoter complex is also an outside or closed complex, like the nonspecific complex, but in which additional nonelectrostatic interactions contribute to the binding free energy. Under roughly physiological ionic conditions (0.003 M $MgCl_2$ and 0.2 M NaCl), we estimate K_{obsd}^{RP} to be 4×10^7 M^{-1} for the 0 °C promoter complex, compared to $K_{obsd}^{RD} = 3 \times 10^5$ M^{-1}. Consequently, more than 60% of the standard binding free energy under these conditions is provided by the electrostatic component of the binding interaction.

A total of 3.2 ± 2.5 additional ions are released in forming the 37 °C promoter complex, as compared to the 0 °C promoter complex (13.7 ± 1 vs. 10.5 ± 1.5). This larger amount

of ion release may result either from an increase in Z (Z_{max} would increase from 12 ± 2 to 16 ± 2) or from a conformational change in the DNA affecting its ion binding properties or from a composite of these or other factors. The 37 °C complex is thought to be an open complex, requiring the local unwinding of the double helix. It is of interest to note that the additional ion release involved in forming the 37 °C complex could in principle be accounted for by the denaturation of approximately 3.2/0.34 = 9–10 base pairs, assuming that the extent of ion release per base pair denatured (0.34 ion) is the same as in the denaturation of unliganded DNA [see Record et al. (1978)]. This calculation assumes that the phosphates of these base pairs in both the native and denatured forms interact only with Na^+ (and not with polymerase) and that the denatured region has an axial phosphate spacing similar to that found for denatured DNA in the absence of RNA polymerase. Although it is very approximate, this estimate is in general agreement with the amount of unwinding found by Wang et al. (1977) and Siebenlist (1979) for formation of a 37 °C polymerase–promoter complex.

Finally, the data show that the net nonelectrostatic contribution to the standard binding free energy of the 37 °C polymerase–promoter complex is small. [The maximum value of log K_{obsd}^{RP} (1 M) at the low pH limit (pH 6, see below) is approximately zero.] It is reasonable to conclude that any favorable contribution to the binding free energy from specific contacts between polymerase and functional groups on the bases is compensated to a large extent by the unfavorable free energy of conformational changes in the promoter and/or polymerase. As a result, the driving force for formation of the 37 °C polymerase–promoter complex under physiological ionic conditions is predominantly electrostatic and is a consequence of the release of low molecular weight ions from the DNA (and possibly from polymerase) in the binding reaction.

(*c*) *Interpretation of the Effect of Mg^{2+} on K_{obsd}^{RP} at 0 and 37 °C.* Record et al. (1977) and Lohman et al. (1980b) have demonstrated that the only effect of Mg^{2+} on the binding of *lac* repressor and pentalysine to DNA is as a competitor, with cationic groups on the ligand, for phosphate groups on the DNA. This competitive effect of Mg^{2+} is readily analyzed by using binding theory. The calculation requires knowledge of the parameters describing the dependence of K_{obsd} on monovalent salt concentration in the absence of Mg^{2+} [Z, k, and log K_{obsd} (1 M)]. In addition, the observed binding constant for Mg^{2+} to DNA as a function of the NaCl concentration is needed. This is theoretically expected to be of the form

$$\log K_{obsd}^{Mg\text{-}DNA} = A + B \log [Na^+]$$

where the intercept term A (the value of $K_{obsd}^{Mg\text{-}DNA}$ at 1 M NaCl) is close to zero and the slope B is in the range 1.7–2.0, depending on the theoretical model used (Record et al., 1978; Manning, 1978). Since sufficiently accurate data for $K_{obsd}^{Mg\text{-}DNA}$ are not available, A and B are used as adjustable parameters and are obtained from the best fit of the competition binding theory to the experimental data. The fit to the polymerase binding data, shown in Figure 3, is obtained with $A = 0.32$ and $B = 1.75$, in good agreement with the parameters used by Record et al. (1977) and Lohman et al. (1980b). Appropriate values of Z and log K_{obsd} (1 M) were used for the 0 and 37 °C complexes; it was assumed in the calculations that $Z = Z_{max}$ and $k = 0$. The fit is adequate but not as good as that obtained for other ligands (Record et al., 1977; Lohman et al., 1980b). This might be the result of inadequacies in the model (e.g., the existence of conformational changes, anion effects, etc.), in the implicit assumption that A and B are independent of temperature between 0 and 37 °C, and/or in the experimental data. In any case, the semiquantitative agreement between the experimental results and the behavior predicted from a competition model leads us to conclude that the dominant role of Mg^{2+} is as a competitor with polymerase for DNA phosphates. The large effects of Mg^{2+} on K_{obsd}^{RP} illustrate again the importance of electrostatic interactions in these specific RNA polymerase–promoter complexes.

(*d*) *Interpretation of the Effects of pH on K_{obsd}^{RP} and d Log K_{obsd}^{RP}/d Log [Na^+].* At 37 °C, the dependence of K_{obsd}^{RP} on pH is accurately described by assuming that protonation of two groups on polymerase (average pK = 7.4) is required for formation of the polymerase–promoter complex (cf. Figure 4). Presumably these groups are either histidines and/or α-amino groups. Very similar behavior was found by deHaseth et al. (1977) for the pH dependence of nonspecific binding of *lac* repressor to DNA. The protonated groups may be directly involved in the interactions with DNA phosphates. Alternatively, protonation may be coupled to a conformational change in polymerase that accompanies DNA binding. We favor the latter alternative, since it is not obvious why a strict requirement for protonation would exist in the former case.

The pH dependence of formation of the 0 °C holoenzyme–promoter complex is consistent with a requirement for protonation at one or two sites with similar pK values (~7.4). More accurate data will be required to determine the exact number of sites and the role of these groups (i.e., whether they interact with DNA or are conformationally linked to DNA binding). Since equal numbers of ions are released in forming the nonspecific and 0 °C complexes, but nonelectrostatic interactions contribute to the 0 °C complex, it is reasonable to propose that a conformational change in polymerase accompanies binding to a promoter at 0 °C (relative to the nonspecific binding conformation) to permit formation of these specific interactions and that the protonation event(s) may be involved in this conformational change. However, the experimental error in determining d log K_{obsd}/d log [Na^+] for the nonspecific and 0 °C promoter complexes is too large to permit a definite statement regarding this model.

(*e*) *Interpretation of the Effects of Temperature on K_{obsd}^{RP}.* As a consequence of the sigmoid dependence observed for K_{obsd}^{RP} as a function of temperature (0 to 37 °C) and of the different dependences of K_{obsd}^{RP} on Na^+ concentration observed for the 0 and 37 °C polymerase–promoter complexes (both in the presence and in the absence of Mg^{2+}), we have concluded that the 0 and 37 °C complexes differ at the molecular level. We have tentatively identified these as examples of the closed and open complexes between RNA polymerase and promoters assumed in models of the mechanism of polymerase action. For both complexes, the entropic contribution from counterion release (a free energy of dilution) to the binding free energy under physiological ionic conditions is substantial, as discussed under Interpretation of Salt Effects on RNA Polymerase–DNA Complexes. Estimates of the enthalpies of formation of these complexes are required to complete this description of the thermodynamics of complex formation. In principle, these can be obtained from the temperature dependences of K_{obsd}^{RP} in the low (0–17 °C) and high (21–37 °C) regions. Based on the limited data available (cf. Figure 5), these enthalpies are probably not negative; the binding reactions appear to be entropy driven, in large part by counterion release. In the region of the transition between the two complexes, log K_{obsd}^{RP} increases by ~0.4 over a temperature interval of ~3 °C. This corresponds to a van't Hoff enthalpy of ~50 kcal, which is similar to the value obtained by Mangel

& Chamberlin (1974) using a transcription assay and interpreted by them as resulting from the melting of approximately eight base pairs. Alternatively, this enthalpy effect may result from a conformational transition in holoenzyme or a coupled conformational change in both the enzyme and the DNA. If the van't Hoff enthalpy were attributable to DNA melting only, K_{obsd}^{RP} should continue to exhibit this temperature coefficient above 21 °C.

(f) Summary of the Model of RNA Polymerase–Promoter Interactions. deHaseth et al. (1978) concluded that the nonspecific holoenzyme–DNA complex was stabilized predominantly by the formation of electrostatic interactions ($Z_{max} = 12 \pm 2$) and the accompanying release of thermodynamically bound electrolyte ions. Since the nonspecific binding constant is insensitive to pH and temperature and contains no appreciable nonelectrostatic component, it is likely that no conformational changes in either the protein or the DNA are a part of the binding process. We view the nonspecific complex as a closed or outside complex in which interactions between the protein and the DNA involve the phosphates but not the bases.

The 0 °C promoter complex has the same ionic component as the nonspecific complex. We propose that the same binding region on polymerase is involved in this specific interaction, that the resulting complex is a closed or outside complex involving no conformational change in the DNA and that a conformational change in polymerase (accompanied by protonation) is required in order to make the specific nonionic contacts with the DNA that give rise to the greater stability of this complex as compared to the nonpromoter complex.

More ion release accompanies formation of the 37 °C complex than either the 0 °C complex or the nonspecific complex. One plausible explanation of this finding is that at least part of the additional ion release results from denaturation of a region of the DNA helix. In this model, the electrostatic interactions characteristic of the 0 °C complex would be preserved, at least in a net sense. Since the nonelectrostatic component of the binding free energy at 37 °C is small, the unfavorable contributions to the free energy from denaturation of the DNA or conformational changes in holoenzyme and the favorable contributions from formation of specific, nonionic contacts between holoenzyme and DNA bases must be of comparable magnitude. Further work is in progress to better define the thermodynamic and molecular parameters characterizing holoenzyme–promoter interactions.

Added in Proof

Very recently, Siebenlist & Gilbert (1980) have determined from chemical modification experiments that there are 11 polymerase–phosphate contacts in the 37 °C complex formed at the T7 A3 promoter and that these contacts do not extend into the 11 base pair region (−9 to +2) thought to be opened by polymerase in the 37 °C complex. The agreement between these results and our thermodynamic data is striking and supports the model proposed above in which there are 12 ± 2 ionic interactions in both the 0 and 37 °C complexes and in which the additional release of ~3.2 ions in forming the 37 °C complex results from opening (denaturing) a region of base pairing. Finally, it is interesting to note that Siebenlist & Gilbert (1980) find that only a small portion of the open region appears to interact with the polymerase, which may explain the apparent unfavorable nonelectrostatic contribution to the binding free energy that accompanies the transition from the closed to the open complex. Viewed in this manner, both formation of the closed complex and the transition from closed to open complex are driven primarily by counterion release.

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