# Interaction between Wheat Germ RNA Polymerase II and Adenovirus 2 DNA. Evidence for Two Types of Stable Binary Complexes†

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ABSTRACT: Transcription of Adenovirus 2 DNA (Ad 2 DNA) by wheat germ RNA polymerase II in vitro satisfies criteria that have been used to establish that *Escherichia coli* or coliphage transcription in vitro is initiated at true promoters. (1) Wheat germ RNA polymerase forms highly stable complexes at specific sites on Ad 2 DNA, with a $K_{assoc}$ of (4–5) $\times 10^{10}$ $M^{-1}$. (2) Electron microscopic visualization of enzyme bound to Ad 2 DNA reveals the location of eight strong binding sites, at least five of which appear to correspond to promoters that have been identified in studies of Ad 2 transcription in vivo [Evans, R. M., Fraser, N., Ziff, E., Weber, J., Wilson, M., & Darnell, J. E. (1977) *Cell 12*, 733–739; Berk, A. J., & Sharp, P. A. (1977) *Cell 12*, 45–55; Weinmann, R., & Aiello, L. O. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1662–1666]. (3) Transcription of Ad 2 DNA from preformed complexes with wheat germ polymerase is capable of escaping the action of rifamycin AF/013 and is relatively resistant to polyriboinosinic acid. In addition, our results are consistent with a two-state model for the interaction of wheat germ RNA polymerase with Ad 2 DNA, indicating that the mechanisms of transcription initiation and promoter-site selection in eucaryotes may be very similar to mechanisms elucidated in procaryotic systems.

In bacterial systems, the mechanisms of transcription initiation and promoter-site selection have been well studied in vitro using purified RNA polymerase and well-defined RNA templates. Detailed studies of transcription of phage T7 DNA in vitro have shown that initiation requires at least three steps: (1) RNA polymerase holoenzyme binds stably to T7 DNA, forming "I" complexes (Mangel & Chamberlin, 1974b,c); (2) I complexes are converted into "RS" (rapid-starting) complexes, perhaps by denaturation of a short stretch of DNA at the initiation site [see Chamberlin (1974, 1976)]; (3) in the presence of ribonucleoside triphosphates, each RS complex can catalyze the rapid formation of the first phosphodiester bond in newly synthesized RNA chains (Mangel & Chamberlin, 1974a). I and RS complexes correspond to "closed" and "open" complexes (Travers, 1974), respectively [Williams & Chamberlin (1977); see Chamberlin (1976)].

RS complexes are much more stable than are complexes formed by core polymerase at nonspecific sites on T7 DNA (Hinkle & Chamberlin, 1972b). Furthermore, the two types of complexes can be distinguished by the fact that only RS complexes can initiate transcription rapidly enough to escape the action of rifampicin, and only RS complexes are resistant to inactivation by polyanions (Pfeffer et al., 1977; DeLorbe, Gussin, and Surzycki, unpublished experiments). The transition between I and RS complexes depends upon the temperature of incubation of enzyme and DNA (Mangel & Chamberlin, 1974c; Travers, 1974), the ionic strength (Mangel & Chamberlin, 1974b), and the nature of the particular promoter at which initiation occurs (Stahl & Chamberlin, 1977; Surzycki et al., 1976a,b). In fact, detailed analyses of the interaction of *Escherichia coli* RNA polymerase with promoters in T7 DNA have led Stahl & Chamberlin (1977) to favor a kinetic model for promoter-site selection and to suggest that a T7 "strong" promoter and a T7 "weak" promoter differ primarily in the rate of transition from I to RS complexes at the two sites.

Until now, corresponding information about the steps in eucaryotic transcription initiation has been lacking. Yet, in eucaryotes as in procaryotes, the relative frequency with which specific promoters are "selected" is likely to be an important factor in determining the distribution of primary gene products. To understand how this is accomplished, it will be necessary to define the steps in transcription initiation in a eucaryotic system and to determine the kinetic parameters of the interaction of a eucaryotic RNA polymerase with promoters on a defined template.

In this paper, we describe evidence that wheat germ RNA polymerase II can form stable complexes at eight specific sites on Adenovirus 2 (Ad 2) DNA. At least five of the eight sites appear to correspond to promoters that are active in the initiation of adenovirus transcription in vivo (Evans et al., 1977; Berk & Sharp, 1977; Weinmann & Aiello, 1978). In addition, we present for the first time measurements of rate constants for the association and dissociation of a eucaryotic (wheat germ) RNA polymerase and a eucaryotic (Ad 2) DNA. We show that complexes formed by wheat germ RNA polymerase II and Ad 2 DNA are similar to complexes formed by *E. coli* enzyme and phage DNAs in their stability and in their ability to initiate RNA synthesis in the presence of rifamycin AF/013 or polyriboinosinic acid (poly(I)). Furthermore, transition temperature studies indicate that the two-state (I and RS) model for bacterial transcription initiation may apply to eucaryotic transcription. Thus, in spite of obvious structural differences between eucaryotic and procaryotic RNA polymerases (Jendrisak & Burgess, 1975; Chambon, 1975), the mechanisms by which the two types of enzymes initiate transcription may be remarkably similar.

## Materials and Methods

*Enzyme Purification.* RNA polymerase II was purified from wheat germ (Pillsbury Foods or General Mills) according

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to the procedure described by Jendrisak & Burgess (1975). Purified enzyme was then precipitated with 1.5 volumes of saturated ammonium sulfate and resuspended in buffer containing 50 mM Tris, pH 7.9, 0.1 mM EDTA (sodium salt), 1 mM dithioerythritol (DTE), 25% glycerol, and 25% ethylene glycol. After ammonium sulfate was removed by using a Sephadex G-50 column, the purified enzyme was stored at −80 °C at a concentration of about 3 mg/mL. Protein concentration was assayed by using the method of Lowry et al. (1951).

The purity of the enzyme was conservatively estimated at 80–90%, based on its migration as a single band on electrophoresis in native 4% polyacrylamide gels. In a denaturing gel (Laemmli, 1970) containing an exponential 7.5–15% acrylamide gradient, 10 major bands are observed; the band pattern is in substantial agreement with those reported by Jendrisak & Burgess (1977) and Hodo & Blatti (1977). Minor differences can be attributed to differences in the proteins used as molecular weight markers and in the acrylamide content of the gels. Purified enzyme preparations typically have specific activities of 600–1200 units (Jendrisak et al., 1976) per mg of protein. Wheat germ obtained from General Mills, Minneapolis, MN, appears consistently to yield the most active enzyme. The molecular weight of purified enzyme was assumed to be 600 000 (Jendrisak & Burgess, 1975).

*Growth of and Purification of Virus.* Adenovirus 2 was grown in spinner cultures of KB cells by using procedures described by Green & Piña (1963) and Green et al. (1967). Virus was purified from infected cells that had been collected by centrifugation at low speed, resuspended in about 10 mL of 0.01 M Tris, pH 8.0, and stored at −20 °C. The thawed, infected cells were disrupted by sonication, and the sonicate was homogenized in an equal volume of 1,1,2-trichloro-2,2,1-trifluoroethane in a VirTis "45" homogenizer. The aqueous phase was collected after low-speed centrifugation, and the organic phase was reextracted 3–4 times. The combined supernatants were layered onto a 5–6 mL CsCl cushion ($\rho$ = 1.43 g/cm$^3$) and centrifuged at 21 000 rpm for 1 h at 4 °C in a Beckman SW 27 rotor. The virus band above the cushion was collected and then purified by CsCl density gradient centrifugation for 21 h at 30 000 rpm in a Beckman 65 rotor, followed by CsCl centrifugation at 35 000 rpm for 24 h in a Beckman SW 50.1 rotor (CsCl density 1.34 g/mL; 10 °C). Purified virus was stored at 4 °C after dialysis against buffer I (0.02 M Tris, pH 8.0, 0.5 M NaCl, and 0.05 mM EDTA). (Ad 2 and KB cells were obtained originally from H. Raskas and M. Stinski.)

*Isolation of Ad 2 DNA.* Purified virus was diluted into a final volume of 2 mL of buffer I at an $OD_{260}$ of about 8. Proteinase K (Boehringer), EDTA, and $NaDodSO_4$ were added to final concentrations of 10 μg/mL, 0.01 M, and 0.2%, respectively. After incubation for 1 h at 50 °C, 100 μg/mL pronase (predigested 1.5 h at 37 °C) was added, and incubation was continued for an additional 0.5 h. The mixture was then extracted 3 times with 2 volumes of double-distilled phenol (saturated immediately before use with 0.1 M sodium borate). The upper (aqueous) phase was extracted with an equal volume of a 24:1 mixture of chloroform–isoamyl alcohol. The aqueous phase was dialyzed overnight against buffer II (0.05 M NaCl, 0.02 M Tris, pH 8.0, and 0.01 mM EDTA). The DNA was concentrated with Aquacide II (Calbiochem), dialyzed extensively against buffer II, and stored over chloroform at 4 °C. Purified DNA was shown by neutral and alkaline sucrose gradient centrifugation to be unit length and relatively free of single-strand breaks [less than one single-strand break per $2 \times 10^7$ daltons, or about one nick per molecule determined by the method of Daussé et al. (1972)].

DNA concentrations were determined spectrophotometrically (Thomas & Abelson, 1966). The molecular weight of purified Ad 2 DNA was taken to be $23.7 \times 10^6$ daltons (Green et al., 1967).

The *Eco*RI A fragment was obtained after treatment of Ad 2 DNA with *Eco*RI restriction endonuclease according to procedures described by Pettersson et al. (1973).

*Isolation of Radioactive Ad 2 DNA.* The method used was the same as that used for the isolation of unlabeled DNA, except that [$^3$H]thymidine was added 6 h after infection. Label was added to a concentration of 1 mCi/L and a specific activity of 15 Ci/mmol or to a concentration of 0.5 mCi/L and a specific activity of 30 Ci/mmol.

*Filter-Binding Assays.* $^3$H-Labeled Ad 2 DNA was incubated with wheat germ RNA polymerase II in binding buffer containing 10 mM Tris, pH 7.9, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM DTE, 1 mM EDTA, and 1 mg/mL bovine serum albumin (BSA) at 37 °C (or 20 °C for the experiments illustrated in Figure 7); samples were then filtered through Schleicher and Schuell BA 85 nitrocellulose filters. Enzyme and DNA concentrations, reaction volumes, and incubation periods are indicated in the figure legends. We used two types of binding experiments, which differ in the way reactions were terminated: (a) at indicated times, samples were diluted immediately to 1 mL with binding buffer and then filtered; (b) samples were diluted to 1 mL with binding buffer containing a 50- to 100-fold excess of native calf thymus DNA and incubated for an additional 5 min before filtering. Calf thymus DNA is used as a trap for enzyme bound unstably to Ad 2 DNA to prevent rebinding of dissociated polymerase to labeled DNA. Thus, only very stable binding of polymerase to Ad 2 DNA is measured. In the experiment described in Figure 2, enzyme and DNA were incubated in separate 1-mL reaction mixtures, each of which was filtered without dilution at the indicated time.

Samples were filtered without washing under a vacuum of 5 in. of mercury. Just prior to filtering, filters were washed in binding buffer at 37 °C. Filters were dried and assayed for radioactivity in Liquifluor scintillation fluid (New England Nuclear Corp.) in a Beckman liquid scintillation counter. Omission of BSA from the binding buffer did not enhance the efficiency of binding, which generally was 40–60%.

*Transcription in Vitro.* Preinitiation complexes were formed by incubating wheat germ RNA polymerase II and Ad 2 DNA for 10 min at 37 °C (or other temperatures where indicated) in a 50-μL reaction mixture containing 30 mM Tris, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM DTE, 0.4 mM $KHPO_4$, pH 7.0, 1 mg/mL bovine serum albumin (BSA), but no substrate ribonucleoside triphosphates. RNA synthesis was initiated at time zero with the addition of NTPs (0.6 mM ATP, GTP, CTP, 0.02 mM unlabeled UTP, and 2 μCi of [$^3$H]UTP, sp act. 27 Ci/mol) and either poly(I) or rifamycin AF/013 as indicated. Incorporation of labeled UMP, which was allowed to proceed for 90 s at 37 °C, was terminated by the addition of 3 mL of cold 3.5% perchloric acid (PCA). Samples were processed as described by Surzycki et al. (1976b). Rifamycin AF/013 was a gift of Dr. Francesco Parenti, Gruppo LePetit, Milan, Italy.

*Temperature Transition Experiments.* Ad 2 DNA (0.3 μg) and wheat germ RNA polymerase II (16 μg) were preincubated in 34 μL of 1.5× reaction mixture at the indicated temperatures for 10 min. Prewarmed NTPs and rifamycin

AF/013 were added at time zero to bring the final volume to 50 μL and the rifamycin AF/013 concentration to 250 μg/mL. Reactions were allowed to proceed for 90 s at 37 °C and were terminated by precipitation with PCA. On the basis of experiments of Mangel & Chamberlin (1974c), we estimate that preincubated samples at 5 °C should take about 20 s to reach 37 °C.

*Preparation of Poly(I).* Polyriboinosinic acid (poly(I)) from Miles Laboratories was subjected to partial hydrolysis with 0.1 N NaOH for 15 min at room temperature and then neutralized by the addition of 2 M $NaH_2PO_4$ to a final concentration of 0.16 M (Bautz et al., 1972). The mixture was desalted by filtration through a Sephadex G-50 column equilibrated with distilled, deionized water; the eluate was evaporated to a final poly(I) concentration of 1 mg/mL. Poly(I) concentration was determined spectrophotometrically by using an extinction coefficient at 260 nm of $5.0 \times 10^3$ $M^{-1}$.

*Electron Microscopy.* Wheat germ RNA polymerase (2.2 μg) and DNA (0.5 μg) were incubated for 10 min at 37 °C in binding buffer (minus BSA). Samples (3 μL) were added to 22 μL of deionized $H_2O$ and 25 μL of a BAC (benzyldimethylalkylammonium chloride) mixture, which contained 5 mM BAC, 0.2% glutaraldehyde, 0.06 M triethanolamine, pH 7.9, and 2.6% formamide (Matheson Coleman and Bell). The mixture was spread immediately on an aqueous hypophase of 0.1 M ammonium acetate–0.1% glutaraldehyde and incubated for 20–30 min (whole DNA) or 5–10 min (*Eco*RI fragment A). The mixture was taken up on 3.5% parlodion grids (200 mesh) and stained with 50 μM uranyl acetate in 90% ethanol. The spreading procedure is a modification of the method of Vollenweider et al. (1975).

Grids were dried for 5 s in 2-methylbutane and then rotary-shadowed with 2.6 cm of platinum–paladium wire at an angle of 10°. Electron micrographs were taken at 14000× magnification with an accelerating voltage of 80 kV on a Phillips 300 electron microscope. For DNA length measurements, micrographs were enlarged 10× and traced by using a Numonics electronic graphics calculator interfaced with an Apple II computer for storage and analysis of the data.

## Results

*Stable Complexes between RNA Polymerase II and Ad 2 DNA.* One of the striking characteristics of the interaction between *E. coli* RNA polymerase holoenzyme and promoter sites on phage DNAs is the very high association constant ($K_{assoc}$ = $10^{12}$–$10^{14}$ $M^{-1}$) (Hinkle & Chamberlin, 1972b; Williams & Chamberlin, 1977) for the formation of open (RS) complexes. $K_{assoc}$ can be measured kinetically by separately determining rate constants for association and dissociation. Determination of both constants makes use of the fact that DNA bound to RNA polymerase is retained by nitrocellulose filters, but free DNA is not (Hinkle & Chamberlin, 1972a). The validity of these types of experiments depends on the demonstration that one molecule of RNA polymerase bound to a stable binding site on a DNA molecule is sufficient to cause the DNA to be retained to the filter.

The experiment illustrated in Figure 1 demonstrates that the binding of one wheat germ RNA polymerase II molecule to Ad 2 [$^3$H]DNA is sufficient to cause its retention by a nitrocellulose filter. In this experiment, the amount of radioactive Ad 2 DNA retained increased with the amount of enzyme added to the incubation mixture. For a DNA template with many stable binding sites, the data should approximate the equation (Hinkle & Chamberlin, 1972a)

$$R = 1 - e^{-\epsilon f u} \quad (1)$$

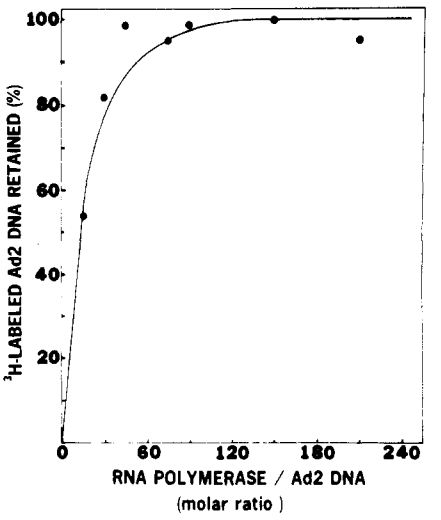


FIGURE 1: Binding of Ad 2 DNA to nitrocellulose filters as a function of enzyme concentration. Increasing amounts of wheat germ RNA polymerase II were incubated with 0.5 μg (42000 cpm) of Ad 2 [$^3$H]DNA in binding buffer at 37 °C. After 10 min, each 50-μL reaction mixture was diluted to 1 mL with binding buffer and filtered immediately. The ordinate indicates the amount of DNA retained by nitrocellulose filters, corrected for a background of 2100 cpm, and normalized to the maximum amount bound (16000 cpm). The data should fit the equation $R = 1 - e^{-\epsilon f u}$. $\epsilon$ is the fraction of enzyme–DNA complexes that does not dissociate during filtration [see Hinkle & Chamberlin (1972a)], $f$ is the fraction of enzyme molecules active in DNA binding, and $u$ is the molar ratio of enzyme to DNA (by assumption that 100% of the enzyme is active). A plot (not shown) of ln $(1 - R)$ as a function of $u$ yields a straight line with a slope ($\epsilon f$) of −0.045. This value is in agreement with the value of $\epsilon f$ determined from the value of $u$ for which $R$ = 63%. $\epsilon$ should be distinguished from the efficiency with which an (undissociated) enzyme–DNA complex is retained by the filter; in this experiment, the filter-binding efficiency was about 40% (16000/42000).

where $R$ is the fraction of DNA molecules bound to the filter (normalized to the maximum amount bound), $\epsilon$ is the fraction of RNA polymerase–DNA complexes that do not dissociate during filtration, $u$ is the molar ratio of enzyme to DNA on the assumption that all the RNA polymerase molecules are able to bind stably to the DNA), and $f$ is the fraction of the enzyme that is actually active.

A plot (not shown) of ln $(1 - R)$ as a function of $u$ is linear with a slope ($\epsilon f$) of −0.045. Therefore, one RNA polymerase molecule bound to Ad 2 DNA is sufficient to cause retention of the DNA by nitrocellulose filters. (The curve in Figure 1 was obtained by substituting −0.045 for $\epsilon f$ in eq 1.) Since we estimate[1] that only about 5% of the wheat germ RNA polymerase molecules in these preparations is able to form stable complexes, $\epsilon$ is about 0.9. The value of $\epsilon$ for complexes between T7 DNA and *E. coli* RNA polymerase, determined using slightly different conditions, varied between 0.4 and 0.8 (Hinkle & Chamberlin, 1972a).

Since one RNA polymerase molecule bound to Ad 2 DNA can cause its retention by the filter, filter binding can be used as an assay for the rate of binding of the enzyme to DNA. In this experiment (Figure 2), enzyme and radioactive DNA were incubated at 37 °C, and at various times aliquots were filtered to determine the extent of binding. Under the con-

[1] Enzyme prepared according to the method of Jendrisak & Burgess (1975) is a homogeneous enzyme based on native polyacrylamide gel electrophoresis. However, our preliminary electron microscope measurements of equilibrium binding to Ad 2 DNA fragments (Williams & Chamberlin, 1977) indicate that only about 5% of the enzyme molecules bind with $K_{assoc}$ in the range of $10^{10}$–$10^{11}$ $M^{-1}$; the remainder of the molecules bind several orders of magnitude less tightly or not at all.

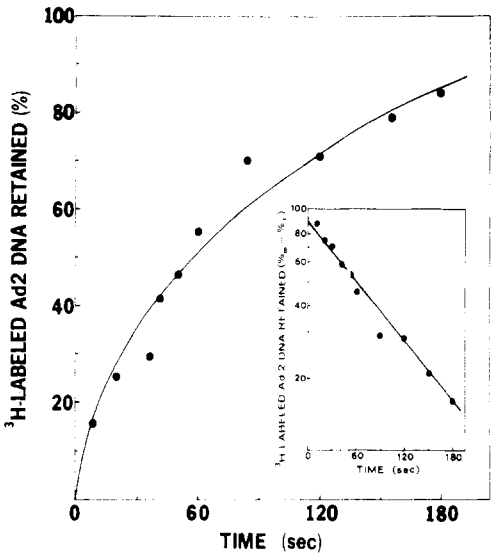


FIGURE 2: Rate of formation of binary complexes between wheat germ RNA polymerase and Ad 2 DNA. Wheat germ RNA polymerase (1.5 μg) was incubated with 0.75 μg (65 000 cpm) of Ad 2 [$^3$H]DNA at 37 °C in 1 mL of binding buffer; at the indicated times, separate 1-mL incubation mixtures were filtered as described under Materials and Methods. After subtracting the background (3300 cpm), data were normalized to the level of DNA bound at $t = \infty$ (14 000 cpm). Assuming 5% of the polymerase is active in the binding reaction[1] and that there are eight stable binding sites on Ad 2 DNA, the ratio of active enzyme molecules to DNA sites is 1:2. Inset: first-order plot of data. A linear regression fit to the data yielded at $t_{1/2}$ of 72 s and $k_{obsd}$ of $(0.96 \pm 0.12) \times 10^{-2}$ s$^{-1}$ (95% confidence limits of the regression coefficient).

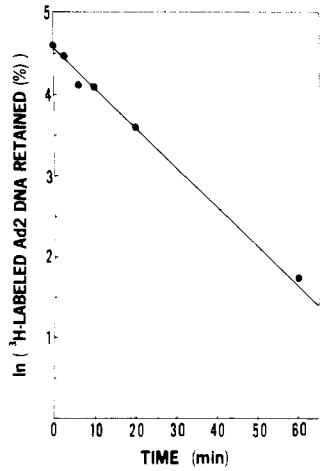


FIGURE 3: Dissociation of binary complexes between wheat germ RNA polymerase and Ad 2 DNA. Wheat germ RNA polymerase (7 μg) and Ad 2 [$^3$H]DNA (10 μg; 130 000 cpm) were incubated in 0.1 mL of binding buffer for 10 min at 37 °C. At time zero, 0.4 mL of binding buffer containing 0.5 mg of native calf thymus DNA was added. At the indicated times, 50-μL aliquots were withdrawn, diluted to 1 mL in binding buffer, and filtered. The background of 2400 cpm was subtracted from each data point; the amount of [$^3$H]DNA bound at zero time was 14 600 cpm. $t_{1/2}$ is 15 min and $k^{-1}$ is $8.0 \times 10^{-4} \pm 0.59 \times 10^{-4}$ s$^{-1}$; calculation of $k_{-1} \pm$ 95% confidence limits was based on a linear regression fit of the data to a first-order plot.

ditions used (molar ratio of active enzyme to DNA sites of about 1:2), the association reaction was pseudo first order, with an association half-time of 72 s; $k_{obsd}$, the pseudo-first-order rate constant, determined by linear regression analysis of the data in Figure 2, was $(0.96 \pm 0.12) \times 10^{-2}$ s$^{-1}$ (95% confidence limits). $k_1$, the true second-order rate constant, is obtained by dividing $k_{obsd}$ by the molar concentration of DNA binding sites.[2] On the assumption (see Figure 8) that there are eight strong binding sites for wheat germ RNA polymerase on Ad 2 DNA, the calculated value for $k_1$ is $3.8 \times 10^7$ M$^{-1}$ s$^{-1}$. In a second experiment (not shown), in which the ratio of active enzyme to DNA sites was about 1:3, $t_{1/2}$ was 69 s, $k_{obsd}$ was $(0.99 \pm 0.21) \times 10^{-2}$ s$^{-1}$, and $k_1$ was calculated to be $3.0 \times 10^7$ M$^{-1}$ s$^{-1}$. Both values for $k_1$ are similar to those calculated for the association of *E. coli* RNA polymerase holoenzyme with T7 DNA (Hinkle & Chamberlin, 1972b).

To determine the first-order rate constant for dissociation, we incubated RNA polymerase and radioactive Ad 2 DNA for 15 min at 37 °C to allow the formation of stable complexes. At time zero, excess calf thymus DNA was added to prevent further binding of polymerase to radioactive Ad 2 DNA, and the incubation mixture was sampled periodically thereafter to assay the number of radioactive DNA molecules still capable of being retained by nitrocellulose filters. In one experiment (Figure 3), the initial molar ratio of active enzyme to DNA was about 1.4:1. As expected, the number of molecules retained by nitrocellulose filters decreased with first-order kinetics. The dissociation half-time was about 15 min and $k_{-1}$, the slope of the decay curve, was $(8.0 \pm 0.59) \times 10^{-4}$ s$^{-1}$ (95% confidence limits). In a second experiment (data not shown), the active enzyme to DNA molar ratio was about 8:1. In this case, the half-time was about 16 min and $k_{-1}$ was $(7.2 \pm 2.5) \times 10^{-4}$ s$^{-1}$. $K_{assoc}$, the ratio of $k_1$ to $k_{-1}$, is thus about $(4–5) \times 10^{10}$ M$^{-1}$.

In spite of uncertainties about the actual percentage of active enzyme molecules in our RNA polymerase preparations, the calculated constant is likely to be a reasonable approximation. Although $K_{assoc}$ is about 20-fold lower than the value determined in the same way for the formation of stable complexes between *E. coli* RNA polymerase holoenzyme and T7 DNA (Hinkle & Chamberlin, 1972b; Williams & Chamberlin, 1977), it is clear that, like *E. coli* RNA polymerase, the wheat germ enzyme very rapidly forms highly stable complexes with Ad 2 DNA.

*Evidence for Two Types of Stable Complexes.* Investigation of the mechanism of bacterial transcription initiation has been facilitated by the use of initiation inhibitors; these include rifamycin derivatives, which bind specifically to the β subunit of RNA polymerase [see Zillig et al. (1976)], and polyanions, which inactivate transcription complexes by displacing polymerase from DNA (Hirschbein et al., 1967; Hinkle & Chamberlin, 1972a). Both types of inhibitor preferentially block transcription from closed (I) complexes between *E. coli* RNA polymerase holoenzyme and phage DNAs (Dubert & Hirschbein, 1969; Hinkle & Chamberlin, 1970; Hinkle et al., 1972; Bautz et al., 1972; Mangel & Chamberlin, 1974c).

Figures 4 and 5 illustrate the effects of rifamycin AF/013 and poly(I), respectively, on initiation of transcription by wheat germ RNA polymerase II with Ad 2 DNA as a template. In both cases, the inhibitors drastically limit transcription; however, significant transcription occurs if the enzyme and DNA are preincubated in the absence of nucleoside triphosphates (NTPs) prior to the addition of inhibitor. These

[2] For the association reaction $d[E{\cdot}D]/dt = k_1[E][D]$, where [E·D], [E], and [D] are the concentrations of enzyme–DNA complexes, enzyme, and DNA binding sites, respectively. When [D] is sufficiently greater than [E], the reaction becomes pseudo first order, and $k_1[E][D] = k_{obsd}[E]$. In the experiment in Figure 2, the molar ratio of enzyme to DNA was about 4:1 (by assumption that 5% of the enzyme molecules is active in binding to Ad 2 DNA). Since electron microscope studies (Figure 9) suggest that there are eight strong binding sites for wheat germ RNA polymerase on Ad 2 DNA, these conditions are sufficient to approximate *pseudo*-first-order kinetics (see Hinkle & Chamberlin, 1972a,b).

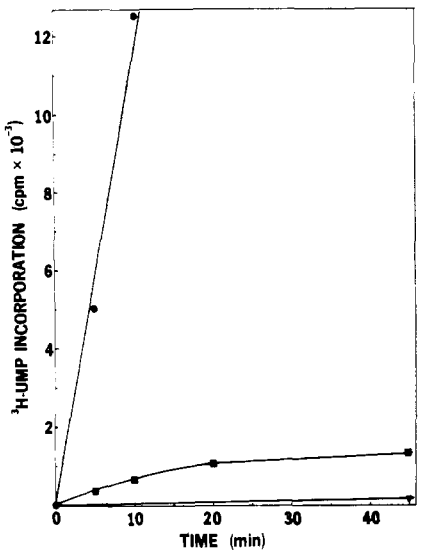


FIGURE 4: Transcription of Ad 2 DNA in the presence and absence of rifamycin AF/013. Wheat germ RNA polymerase (30 $\mu$g) and Ad 2 DNA (1 $\mu$g) were preincubated 10 min at 37 °C in 0.15-mL reaction mixtures to allow the formation of binary complexes. At time zero, rifamycin AF/013 and substrate NTPs were added simultaneously and incorporation was allowed to proceed at 37 °C. At the indicated times, 25-$\mu$L aliquots were withdrawn and assayed for incorporation of [$^3$H]UMP into PCA precipitable material. 4500 cpm = 1 pmol. (■) Rifamycin AF/013 (final concentration 0.2 mg/mL) and NTPs were added simultaneously at time zero; (●) NTPs were added without rifamycin AF/013 at time zero; (▼) rifamycin AF/013 and NTPs were added to enzyme and DNA without a 10-min preincubation period.

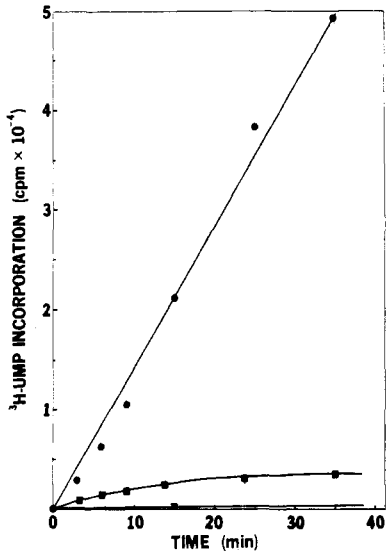


FIGURE 5: Transcription of Ad 2 DNA in the presence and absence of poly(I). Wheat germ RNA polymerase (20 $\mu$g) and Ad 2 DNA (0.3 $\mu$g) were preincubated for 10 min at 37 °C in 0.15-mL reaction mixtures to allow formation of binary complexes. At time zero, poly(I) (final concentration 240 $\mu$g/mL) and substrates were added; at the indicated times, 25-$\mu$L aliquots were taken and assayed for incorporation of [$^3$H]UMP into PCA precipitable material. 1 pmol = 1700 cpm. (■) Poly(I) and NTPs were added simultaneous at time zero; (●) NTPs without poly(I) were added at time zero; (▼), poly(I) and NTPs were added to enzyme and DNA without a 10-min preincubation period.

results are similar to those obtained when *E. coli* RNA polymerase holoenzyme is used to transcribe phage DNAs (Chamberlin, 1976) or Ad 2 DNA (Surzycki et al., 1976a; DeLorbe, Surzycki, and Gussin, unpublished experiments). Thus, at least some stable complexes between wheat germ

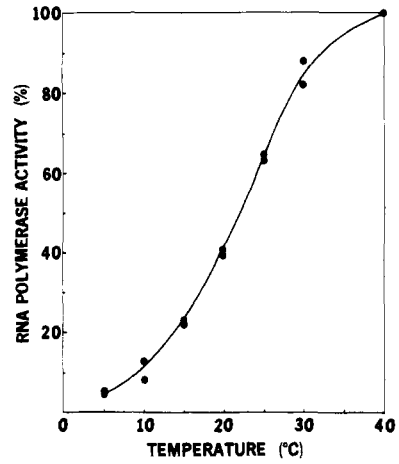


FIGURE 6: Transcription initiation in the presence of rifamycin AF/013 as a function of preincubation temperature. Wheat germ RNA polymerase (16 $\mu$g) and Ad 2 DNA (0.3 $\mu$g) were preincubated for 10 min at each temperature in the absence of NTPs. At time zero, rifamycin AF/013 (final concentration 250 $\mu$g/mL) and NTPs were added simultaneously, incorporation was allowed to proceed for 90 s at 37 °C, and each 50-$\mu$L reaction mixture was assayed for PCA precipitable [$^3$H]UMP. Maximum incorporation (after preincubation at 37 °C) of 16000 cpm (1700 cpm/pmol) was taken as 100%.

RNA polymerase II and Ad 2 DNA are able to initiate transcription in the presence of the inhibitors.

The formation of bacterial complexes able to escape inactivation by rifampicin is dependent upon the temperature at which polymerase and DNA are preincubated prior to addition of substrate NTPs and inhibitor. Similar temperature dependence is observed when Ad 2 transcription catalyzed by wheat germ RNA polymerase is plotted as a function of temperature of preincubation, as is illustrated in Figure 6. Since incorporation is allowed to proceed for only 90 s at 37 °C after addition of inhibitor (in this case rifamycin AF/013) and NTPs, the amount of RNA synthesized should reflect the frequency of transcription initiation.

The dependence of incorporation on temperature can be interpreted in terms of a two-state model for transcription initiation similar to the model proposed for procaryotic initiation (Travers, 1974; Mangel & Chamberlin, 1974a,c): at low preincubation temperatures, most of the stable complexes formed are "I" complexes, which are unable to initiate transcription in the presence of rifamycin AF/013; at higher temperatures, the percentage of stable complexes that are RS complexes increases to the point that most or all of the complexes formed at temperatures above 37–40 °C are able to initiate in the presence of the drug. Similar results are obtained when poly(I) is used to inhibit initiation (data not shown).

Proof of this model requires a demonstration that the binding of RNA polymerase to Ad 2 DNA is not significantly affected by low preincubation temperatures. An experiment designed to investigate this question indirectly (Chamberlin et al., 1976) is illustrated in Figure 7. The rate of stable binding of wheat germ RNA polymerase to Ad 2 DNA is compared with the rate at which the enzyme forms putative RS complexes capable of rapid chain initiation in the presence of rifamycin AF/013. In these experiments, wheat germ RNA polymerase and Ad 2 [$^3$H]DNA were incubated at either 20 (Figure 7A) or 37 °C (Figure 7B). Samples were withdrawn at various times and incubated for 5 min at 37 °C with a 100-fold excess of unlabeled calf thymus DNA prior to filtration. This method measures the rate at which the enzyme becomes "permanently" bound to Ad 2 DNA (Chamberlin

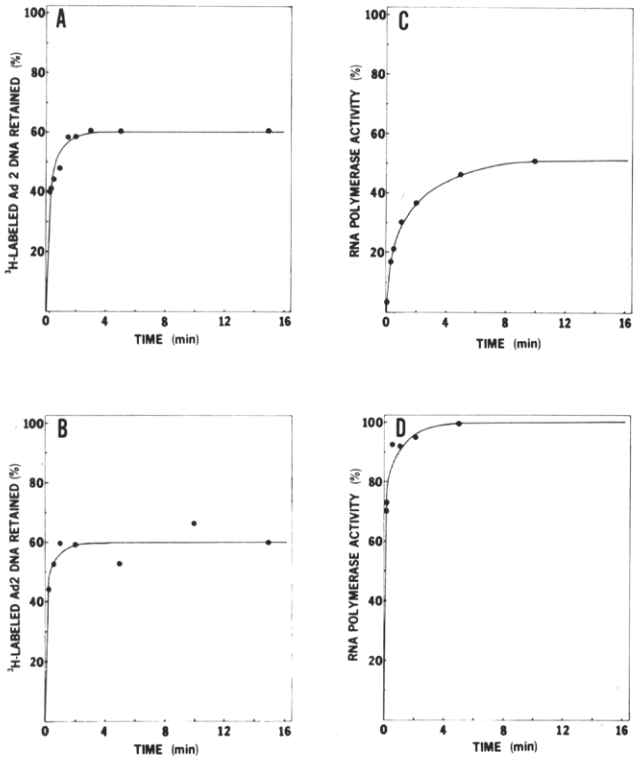


FIGURE 7: Comparison of rate of formation of stable binary complexes and rate of formation of RS complexes at 20 and 37 °C. (A and B) Kinetics of formation of binary complexes. Wheat germ RNA polymerase (20 μg) and Ad 2 [$^3$H]DNA (0.5 μg, 33 000 cpm) were incubated either at 20 (A) or at 37 °C (B) in 50 μL of binding buffer. At the indicated times, individual reaction mixtures were diluted to 1 mL with binding buffer containing 0.5 mg/mL calf thymus DNA, incubated an additional 5 min at 37 °C, and filtered. Levels of binding were calculated after subtraction of the background (2100 cpm). (C and D) Kinetics of formation of RS complexes. Wheat germ RNA polymerase (16 μg) and unlabeled Ad 2 DNA (0.3 μg) were incubated in the absence of NTPs at either 20 (C) or 37 °C (D). At the indicated times, NTPs and rifamycin AF/013 (final concentration 250 μg/mL) were added simultaneously to separate reaction mixtures. Incorporation of [$^3$H]UMP was allowed to proceed for 90 s at 37 °C before assaying for PCA precipitable radioactivity. Maximum incorporation of 16 000 cpm (1700 cpm/pmol) after preincubation at 37 °C was taken as 100%.

et al., 1976). To measure the formation of putative RS complexes (Figure 7C,D), we withdrew samples from parallel preincubation mixtures at various times and assayed them for the ability to catalyze transcription upon addition of NTPs and rifamycin AF/013.

Figure 7A and Figure 7B show that the kinetics of binding Ad 2 DNA to nitrocellulose filters are almost identical at 20 and 37 °C. However, more of the stable complexes formed at 37 °C are able to initiate transcription in the presence of rifamycin AF/013 (Figures 7C and 7D). Furthermore, putative RS complexes are formed much more rapidly at 37 than at 20 °C. Thus, even though RNA polymerase is able to bind efficiently to Ad 2 DNA at 20 °C, the stable complexes formed at this temperature are unable to initiate transcription efficiently when subsequently challenged by NTPs and rifamycin AF/013. In this respect, they are similar to bacterial I complexes.

On the basis of very similar experiments with *E. coli* holoenzyme and T7 DNA, Chamberlin et al. (1976) concluded that two types of complexes (I and RS) existed and that I complexes were precursors to RS complexes. However, in our experiments, the enzyme to DNA ratio was chosen to optimize RNA synthesis upon addition of rifamycin AF/013 and NTPs; thus, it was roughly 10 times the ratio necessary to achieve

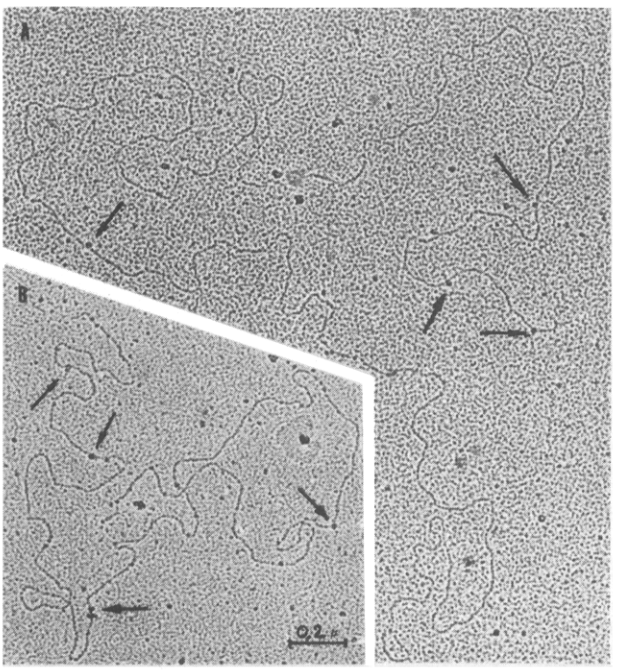


FIGURE 8: Visualization of wheat germ RNA polymerase II bound to Ad 2 DNA. Sample electron micrographs show RNA polymerase bound to sites on intact Ad 2 DNA (A) or *Eco*RI fragment A (B). Arrows indicate polymerase molecules bound to RNA. Samples were prepared and visualized in the electron microscope as described under Materials and Methods.

maximal binding of DNA to nitrocellulose filters. Since the filter-binding assay cannot distinguish the actual number of enzyme molecules bound to a DNA molecule that is retained by the filter, the results in Figure 7 do not by themselves demonstrate the existence of two kinds of stable complexes.

We have, however, repeated the binding experiment at a molar ratio of active enzyme to DNA of 7:1 (data not shown). As in the experiment described in Figures 7A and 7B, the kinetics of binding were identical at 20 and 37 °C, although, as expected, the half-times for binding of the DNA to filters are somewhat higher ($t_{1/2}$ about 75 s compared with $t_{1/2}$ values of about 20 s in the experiments illustrated in Figures 7A and 7B). The important point is that at this lower enzyme to DNA ratio, the kinetics of binding of DNA to nitrocellulose filters and, by inference, the kinetics of binding of the enzyme to DNA are unaffected by temperature. If we take this result as evidence that the rate of binding of polymerase to DNA is temperature independent even at high enzyme to DNA ratios, then the experiments illustrated in Figure 7C and Figure 7D lead us to conclude that there are at least two types of enzyme–DNA complexes: (1) those that form at low temperature are predominantely I complexes, incapable of escaping rifamycin AF/013 inactivation (Figure 7C); (2) those that form at high temperature are mostly RS complexes, capable of escaping the action of rifamycin AF/013 (Figure 7D).

*Location of Stable Complexes at Specific Sites on Ad 2 DNA.* Electron microscopic visualization of wheat germ RNA polymerase II bound to Ad 2 DNA (Figure 8) reveals that there are preferred sites at which the enzyme binds to the DNA. We have used a computer program (details to be published elsewhere), which is based on modification of the methods of Giacomoni et al. (1977), to orient individual DNA molecules with respect to each other. In addition, the distribution of binding sites on Ad 2 DNA (Figure 9B) has been correlated with the physical map of the DNA by comparison with the distribution of binding sites on *Eco*RI restriction fragment A (Figure 9A). The distribution of binding sites on

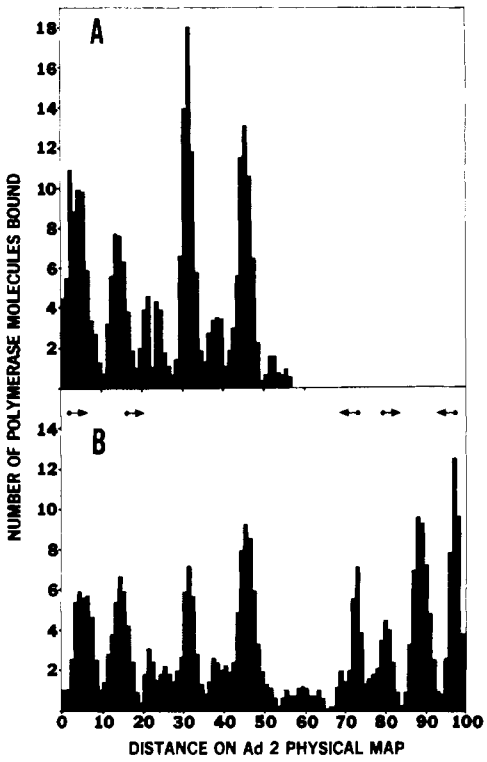


FIGURE 9: Distribution of wheat germ RNA polymerase binding sites on Ad 2 DNA. The positions of RNA polymerase molecules bound to Ad 2 DNA were measured from electron micrographs of (A) 63 *Eco*RI fragment A molecules or (B) 50 intact Ad 2 DNA molecules. The orientation of individual molecules and the distribution of binding sites were determined by using a computer program which was based on a modification of the method of Giacomoni et al. (1977). Each distribution is "integrated" to take into account possible errors in measurement and to compensate for the relatively small numbers of bound enzyme molecules. A map unit corresponds to 1% of the Ad 2 DNA molecule. In the integrated distribution an enzyme bound at position $n$ is assigned a value of 1.0 at position $n$, 0.61 at position $n \pm 1$, and 0.135 at position $n \pm 2$. Arrows in (B) indicate origins and directions of synthesis of known Ad 2 transcripts (Evans et al., 1977; Berk & Sharp, 1977).

fragment A, which constitutes the left 58% of the Ad 2 DNA molecule, coincides with the distribution of sites on the whole molecule in only one orientation—the orientation shown in Figure 9.

The correspondence between the distribution shown in Figure 9B and the positions of in vivo promoters (Evans et al., 1977; Berk & Sharp, 1977; Weinmann & Aiello, 1978) is striking. The distribution contains eight major peaks located at 5, 15, 32, 45, 72–73, 79–80, 89, and 97% on the Ad 2 physical map. In addition, where are two minor peaks located at 25 and 37%. Four of the major peaks correspond to the reported positions of Ad 2 in vivo transcription start points at 16, 73, 80, and 97% (Evans et al., 1977). Evans et al. also identified a fifth in vivo promoter, located at 2%, which may correspond to our peak at 5% or may have been obscured in our distribution because of its near symmetry with the peak at 97%.

## Discussion

In spite of the structural differences between eucaryotic RNA polymerases and RNA polymerases from procaryotes, our experiments demonstrate striking similarities between the two classes of enzymes. (1) In both cases, binding is very rapid with a second-order rate constant of about $(3.1–3.8) \times 10^7$ $M^{-1}$ $s^{-1}$, and the complexes once formed are relatively stable. The $K_{assoc}$ for the binding of wheat germ RNA polymerase to Ad 2 DNA is about $(4–5) \times 10^{10}$ $M^{-1}$. (2) Stable complexes formed after 10–15 min preincubation at 37 °C with Ad 2 DNA in the absence of substrate NTPs are capable of initiating transcription when challenged with the rifamycin derivative AF/013 (Figure 4) or poly(I) (Figure 5). Both inhibitors prevent initiation by preincubation complexes between *E. coli* RNA polymerase core enzyme and phage DNAs and limit initiation by holoenzyme to that catalyzed by RS (open) complexes. (3) There is evidence (Figures 6 and 7) that wheat germ RNA polymerase II forms two types of stable complexes with Ad 2 DNA.

We have not shown directly that the sites of binding of the enzyme to DNA are those sites at which transcription of Ad 2 DNA is initiated in vivo. Ultimate proof would require the demonstration that the 5′-terminal sequences of RNAs synthesized in vitro and in vivo were the same. However, the distribution of enzyme–DNA complexes observed in the electron microscope is consistent with the existence of eight stable binding sites in vitro, and the positions of at least five of these sites appear to correspond to the positions of five promoters for Ad 2 transcription in vivo (Berk & Sharp, 1977; Evans et al., 1977; Weinmann & Aiello, 1978). It is very unlikely that polymerase is binding only to nicks in Ad 2 DNA, since analysis of DNA preparations in alkaline sucrose gradients indicates that there is at most one nick per Ad 2 DNA molecule.

The nonrandom distribution of binding sites and the correlation between the positions of the binding sites and the location of known Ad 2 promoters indicate that the interaction between wheat germ RNA polymerase II and Ad 2 DNA in vitro may reflect the in vivo interaction. The ability of the enzyme to form rifamycin AF/013 resistant preinitiation (RS) complexes is also consistent with this conclusion. In bacterial systems, RS complexes are formed only at promoters known to be active in vivo and are formed only by RNA polymerase holoenzyme [see Chamberlin (1974, 1976)]. Such complexes escape inactivation by rifampicin because they initiate transcription very rapidly (Mangel & Chamberlin, 1974a). If we assume the same mechanism is involved in the escape of eucaryotic RS complexes from inactivation by rifamycin AF/013, it would be reasonable to conclude that (1) at least some fraction of purified wheat germ RNA polymerase II molecules corresponds functionally to "holoenzyme" and (2) many of the sites at which the enzyme forms stable complexes in vitro should be bona fide Ad 2 promoters.

*Determination of Kinetic Parameters.* The value for $K_{assoc}$ $[(4–5) \times 10^{10}$ $M^{-1}]$ is roughly 20-fold lower than the value determined by Hinkle & Chamberlin (1972b) for the interaction of *E. coli* RNA polymerase holoenzyme and T7 DNA. Nevertheless, binding is fairly tight, especially considering the fact that Ad 2 DNA is not a natural template for wheat germ RNA polymerase.

Calculated values of $K_{assoc}$ depend on separate measurement of $k_1$ and $k_{-1}$, rate constants for the association and dissociation of enzyme and DNA, respectively. These measurements are subject to some degree of error because only about 5% of the enzyme molecules in our purified preparations is able to bind stably to Ad 2 DNA; this uncertainty makes it difficult to determine exactly the enzyme to DNA ratio at which the binding reactions are performed. In the case of $k_1$ (Figure 2), the actual molar ratio of enzyme to DNA was 80:1, but the assumed ratio of active enzyme to DNA was about 4:1. The fact that the kinetics of binding were pseudo first order under these conditions suggests that our estimates of the fraction of active enzyme are reasonable.[1] Implicit in the

measurement of $k_1$ are several assumptions about the actual mechanism of interaction between the enzyme and the DNA, which may involve more than a simple second-order reaction [see Hinkle & Chamberlin (1972b)]. In addition, the filter-binding assay cannot distinguish between DNA molecules bound by only one RNA polymerase molecule and those DNA molecules to which more than one polymerase molecule is bound.

To measure $k_{-1}$ (Figure 3), the initial incubation of enzyme and DNA was carried out at a molar ratio of enzyme to DNA of about 1.4:1. The measured dissociation rate constant in this case was $(8.0 \pm 0.59) \times 10^{-4}$ s$^{-1}$. Somewhat lower values were obtained when higher enzyme to DNA ratios were used (data not shown). Since the filter-binding assay detects the dissociation of the last enzyme molecule to dissociate from each DNA molecule, the experiment shown in Figure 3, in which the initial molar ratio of enzyme to DNA was lowest, should provide the most reliable estimate of $k_{-1}$.

Since there are at least eight binding sites for wheat germ RNA polymerase II on Ad 2 DNA (Figure 9), both $k_1$ and $k_{-1}$ represent a weighted average of the corresponding constants for association and dissociation at each site. [The same considerations apply to studies of binding of *E. coli* RNA polymerase to T7 DNA (Hinkle & Chamberlin, 1972a).] It may be possible, for example, that binding to a particular site is much more rapid than to the other sites; in this case $k_1$ would reflect the binding to only one site on the DNA. However, the electron microscopic binding data do not suggest the existence of one dominant binding site. Therefore, it seems likely that $k_1$ and $k_{-1}$ can be used with some validity for the determination of an "average" $K_{assoc}$.

*Existence of I and RS Complexes.* The existence of two types of stable complexes between wheat germ RNA polymerase and Ad 2 DNA is indicated by the results in Figures 6 and 7. The same temperature transition curve is obtained whether rifamycin AF/013 (Figure 6) or poly(I) (data not shown) is used to limit initiation in spite of the fact that the two inhibitors act by very different mechanisms, at least on *E. coli* RNA polymerase. [Rifamycin AF/013 has the same effect as rifampicin on transcription of Ad 2 DNA by *E. coli* RNA polymerase (De Lorbe, Gussin, and Surzycki, unpublished experiments).] The transition curve in Figure 6 is most likely a composite, reflecting transitions occurring at as many as eight separate promoters. The temperature at which 50% incorporation is observed, the *transition temperature*, is thus an average transition temperature. In the experiment illustrated in Figure 6, the transition temperature was 22–23 °C, which is similar to values for the transition temperatures of procaryotic promoters (see Mangel & Chamberlin, 1974c; Surzycki et al., 1976b; Travers, 1974).

In procaryotic systems, the transition curve is explained in the following way. Presumably, at temperatures below the transition temperature, RNA chain initiation is limited by the rate of DNA strand separation required for conversion of I complexes to RS complexes. At temperatures above the transition temperature, this conversion is rapid, and the binding of the enzyme to the DNA becomes rate limiting. We expect the rate of binding to be much less dependent on temperature than the rate of conversion of I complexes to RS complexes; therefore, the observed temperature transition (Figure 6) is interpreted to reflect the temperature dependence of the I to RS conversion. Consistent with this interpretation is the observation that the kinetics of formation of RS complexes between Ad 2 DNA and wheat germ RNA polymerase II are dependent on the temperature of preincubation (Figure 7C,D).

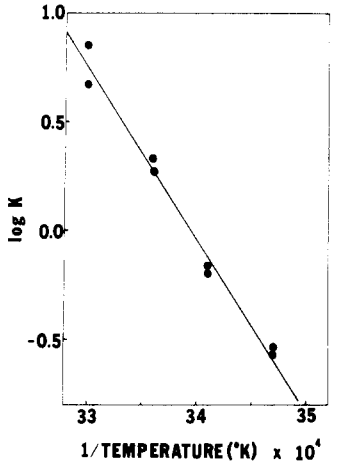


FIGURE 10: van't Hoff plot of putative transition between two states (I and RS) of enzyme–DNA binary complexes. The data from Figure 6 are rearranged to fit the equation $K$ = [RNA polymerase activity (%)]/[100 − RNA polymerase activity (%)] and plotted as a function of the reciprocal of the absolute temperature [see Mangel & Chamberlin (1974c)].

As expected, the formation of RS complexes (able to escape inactivation by rifamycin AF/013) is much slower and is less extensive at 20 than at 37 °C. On the other hand, the rate of formation of stable, nonfilterable enzyme–DNA complexes (Figure 7A,B) appears to be unaffected by the change in temperature from 37 to 20 °C.

We have already pointed out the difficulty in our interpretation because the enzyme–DNA ratios were so high in the experiments illustrated in Figure 7. However, in a similar binding experiment at a molar enzyme/DNA ratio of only 7:1 (data not shown), there was no effect of temperature on the rate of binding of enzyme to DNA. Thus, unless the enzyme to DNA ratio itself affects the rate of binding of the enzyme to DNA, our results are consistent with the bacterial two-state (I and RS) model.

On the basis of very similar experiments with *E. coli* enzyme and T7 DNA, Chamberlin et al. (1976) concluded that bacterial I complexes were in fact precursors to RS complexes. Such a conclusion was possible because in those experiments the enzyme/DNA ratio was only about 2:1. Because of the necessity of using higher enzyme/DNA ratios in our experiments (Figure 7C,D), we can conclude only that there are two kinds of complexes; the question of a precursor–product relationship between them is unanswered.

Based on the two-state model and the additional assumption and that the two types of complexes (I and RS) are in equilibrium, the enthalpy change ($\Delta H$) for the transition between the two states can be calculated by transforming the data in Figure 6 into a van't Hoff plot. On the basis of such a transformation (Figure 10), we calculate $\Delta \bar{H}$ to be about 38 kcal/mol. Mangel & Chamberlin (1974c) calculated $\Delta \bar{H}$ for the transition between I and RS complexes of *E. coli* enzyme and T7 DNA to be about 57 kcal/mol. In several experiments involving *E. coli* RNA polymerase holoenzyme and T7, λ, or Ad 2 DNA, we have obtained values ranging from 30 to 60 kcal/mol. These values are consistent with the energy required to separate between five and eight base pairs of DNA during the formation of RS (open) complexes [see Mangel & Chamberlin (1974c)].

*Relative Inactivity of Purified Wheat Germ RNA Polymerase.* How can inactivity of our wheat germ RNA polymerase II preparations in binding to double-stranded DNA be explained? The most satisfying explanation would be that there are two classes of enzyme corresponding to *E. coli* RNA

polymerase core and holoenzyme. As yet there is no evidence that this is the case. However, wheat germ RNA polymerase prepared as described under Materials and Methods contains some subunits in less than equimolar amounts (Jendrisak & Burgess, 1977; Hodo & Blatti, 1977; our data, not shown). Possibly one or more of these subunits corresponds to the *E. coli* $\sigma$ factor.

Another possibility is that there acutally are two classes of enzyme that are functionally distinct, as has been suggested to account for the behavior of RNA polymerase B from Ehrlich ascites cells (Dreyer & Hausen, 1978). For example, the bulk of the enzyme may specifically bind and transcribe only single-stranded DNA. This would explain the observed preference of eucaryotic RNA polymerases for single-stranded templates [see Roeder (1976)].

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