We have conducted a detailed kinetic and thermodynamic analysis of open complex formation between *Escherichia coli* RNA polymerase and the A1 promoter from bacteriophage T7 by monitoring alterations in the intrinsic protein fluorescence of RNA polymerase in stopped-flow kinetic studies. The stopped-flow kinetic data are consistent with a minimal model involving four steps for the formation of the open complex. Arrhenius plots for both the association and dissociation reactions for the equilibrium binding step leading to the formation of the closed complex were linear. With a positive van’t Hoff enthalpy ($\Delta H_{\text{obs}} = 18(\pm3)$ kcal mol$^{-1}$) and a positive entropy ($\Delta S_{\text{obs}} = 94(\pm15)$ e.u.) change for the equilibrium binding process, formation of the closed complex is entropy driven. The value of the apparent association rate constant for this binding step was approximately three orders of magnitude less than that expected for facilitated binding. Thus, a minimum of two steps is required to describe the formation of the closed complex. A fast facilitated binding step appears to be followed by a conformational change in RNA polymerase which leads to the formation of the closed complex. A non-linear Arrhenius plot obtained for the isomerization step in the conversion of the closed complex to an open one indicates that there are at least two steps in the conversion of the closed complex to an open one. We have assigned the apparent activation energy of $9.1(\pm1.9)$ kcal mol$^{-1}$ to the step involving a conformational change in the protein and nucleation of strand separation and the apparent activation energy of $46(\pm12)$ kcal mol$^{-1}$ to the step involving strand separation. At $37^\circ C$, the value of the macroscopic isomerization rate constant ($0.26(\pm0.02)$ s$^{-1}$) in the conversion of the closed complex to an open one was an order of magnitude greater than the value reported in abortive initiation assays. This suggests that open complex formation is not the rate-determining step in the initiation of transcription in the case of the A1 promoter. To gain greater insight into the mechanism of initiation at the A1 promoter, we investigated the process of abortive product formation (pppApU) under conditions of non-saturating concentrations of the initiating nucleotide. A comparison of the lag times in the approach to the steady-state rate of abortive product formation when the reaction was initiated by the addition of UTP, ATP, the enzyme and the A1 promoter, respectively, indicates that the initiating nucleotide plays a key regulatory role in the initiation of transcription in the case of the A1 promoter.

**Keywords:** Escherichia coli RNA polymerase; stopped-flow kinetics; fluorescence; initiation of transcription; abortive initiation
stopped-flow Analysis of Open Complex Formation

studied extensively by a variety of techniques. Abortive initiation assays, burst kinetic experiments, gel mobility shift assays and a combination of footprinting and DNA chemical modification studies were carried out in the case of the lac UV5 promoter (Shanblatt & Revzin, 1984; Buc & McClure, 1985; Spassky et al., 1985); filter binding assays were performed in the case of the λPR promoter (Roe et al., 1984, 1985); quantitative transcriptional, and template competition assays were conducted in the case of the bacteriophage T7 A1 promoter (Kadesch et al., 1982; Rosenberg et al., 1982); and filter binding assays and DNA chemical modification studies were carried out in the case of the tetR promoter from pSC101 (Duval-Valentin & Ehrlich, 1987). On the basis of the effects of temperature and ionic strength on the interaction of RNA polymerase with the lac UV5, A1, λPR, and tetR promoters, the formation of open complexes has been postulated to occur in at least three steps as shown below.

\[
E + P \rightleftharpoons EP_c \rightleftharpoons EP_o \rightleftharpoons EP_{\alpha} \rightleftharpoons EP_{\beta} \rightleftharpoons EP_{\gamma} \rightleftharpoons EP_{\delta}
\]

(1)

The first step is the binding of the enzyme to the promoter to form a closed complex. The second step is thought to involve a conformational change in the enzyme and the nucleation of DNA strand separation. The final step has been postulated to be the local melting of the DNA and the formation of an open complex. These studies have also led to the postulation that a conformational change occurs in the protein upon the formation of the closed complex (EPc).

The various techniques listed above have provided important information regarding the thermodynamics and kinetics of open complex formation. However, none of these techniques is readily amenable to an investigation of open complex formation by rapid kinetics. Because open complex formation is a fast process, a rapid kinetic method is necessary to directly observe transient intermediates that occur on the pathway to open complex formation. Stopped-flow kinetic studies have been used previously to investigate the interaction of E. coli RNA polymerase as well as T7 RNA polymerase with promoters by using a variety of fluorescent probes. Rhodamine-labeled DNA and 2-aminopurine substituted DNA were used to investigate complex formation in the case of E. coli RNA polymerase (Dunkak et al., 1996; Sullivan et al., 1997), while 2-aminopurine substituted DNA and intrinsic protein fluorescence were used to investigate complex formation in the case of T7 RNA polymerase (Ujvari & Martin, 1996; Sastry & Ross, 1996; Jia et al., 1996). The methods involving rhodamine-labeled DNA and 2-aminopurine substituted DNA have inherent limitations in terms of the amount of information that can be obtained from data analysis. In the case of stopped-flow fluorescence anisotropy studies involving rhodamine-labeled DNA, the entire anisotropy signal change is associated with binding and not directly related to transient intermediates on the pathway to open complex formation. In the case of stopped-flow kinetic studies involving 2-aminopurine substituted DNA, fluorimetric changes are dependent on strand separation. Therefore, transient events such as closed complex formation that occur prior to open complex formation cannot be directly observed. However, by using protein intrinsic fluorescence to monitor open complex formation, transient intermediates that occur before the formation of the open complex and which are accompanied by a conformational change in the protein should be detectable. To date, such an approach has been used in the case of T7 RNA polymerase but not E. coli RNA polymerase. In the studies reported herein, we have analyzed the interaction of E. coli RNA polymerase with the A1 promoter from bacteriophage T7 by using stopped-flow kinetics to monitor changes in the intrinsic fluorescence of RNA polymerase upon open complex formation. These studies have provided estimates for the apparent association and dissociation rate constants for the formation of the closed complex, as well as for the macroscopic isomerization rate constant for the conversion of the closed complex to an open one. Furthermore, a non-linear Arrhenius plot for the step involving the conversion of EP to EPα is consistent with the presence of a transient intermediate in this process. The value of the apparent isomerization rate constant for the conversion of EP to EPα determined here is approximately an order of magnitude greater than that reported in studies in which open complex formation was analyzed by using the abortive initiation assay (Dayton et al., 1984; Johnson et al., 1991; Ozoline et al., 1993). These results have led us to postulate that there is a step after open complex formation that is the rate-determining step in the initiation of transcription in the case of the A1 promoter. To test this hypothesis, we have investigated the kinetics of abortive product formation (pppApU) from the A1 promoter. In these studies, the reaction was initiated by the addition of UTP, the A1 promoter, RNA polymerase and the initiating nucleotide (ATP), respectively, under conditions of non-saturating concentrations of the initiating nucleotide. The results indicate that the initiating nucleotide plays an important role in regulating the initiation of transcription in the case of the A1 promoter.

Results

Equilibrium binding of RNA polymerase to the A1 promoter from bacteriophage T7 as monitored by intrinsic protein fluorescence

Upon excitation at a wavelength of 280 nm, RNA polymerase (25 nM) free in solution displayed a fluorescence emission spectrum with a wavelength maximum centered at 340 nm (Figure 1). The spectrum observed with an
stopped-flow analysis of open complex formation

The interaction of RNA polymerase (25 nM) with a 529 base-pair fragment of DNA (7.5 nM) containing the A1 promoter caused a decrease of approximately 24% in the intensity of the fluorescence spectrum of RNA polymerase at a wavelength of 340 nm; however, there was no readily apparent shift in the emission wavelength maximum (Figure 1). In the titration of RNA polymerase with the DNA fragment containing the A1 promoter, there was a linear increase in the magnitude of the perturbation at a wavelength of 340 nm as a function of promoter concentration up to the point of saturation (Figure 1, inset). Saturation occurred at a molar ratio of A1 promoters to active enzyme molecules of between 0.9 and 1.0.

A perturbation in the fluorescence spectrum of RNA polymerase also occurred due to the interaction of the enzyme with a DNA fragment lacking a promoter site. Upon the addition of a 548 base-pair fragment of DNA (7.5 nM) lacking the A1 promoter to a solution containing RNA polymerase (25 nM), there was a decrease of approximately 15% in the intensity of the fluorescence at a wavelength of 340 nm with no readily apparent shift in the emission maximum (data not shown). In the titration of RNA polymerase with the non-promoter containing DNA fragment, there was a gradual increase in the magnitude of the perturbation at 340 nm as the concentration of the DNA fragment was increased (Figure 2). In this case, the data are plotted as the fraction of complex formed versus the number of binding sites. Typical values in the range of 40-50 base-pairs have been reported for the size of the RNA polymerase binding site (Gilbert, 1976; Oppenheim et al., 1980; Revzin & Woychik, 1981). The data given in Figure 2 correspond to a site size of 45 base-pairs. The data could not be approximated by two straight lines; instead, the binding isotherm was hyperbolic. Analysis of several titrations, as indicated in the legend to Figure 2, yielded a value of 1.4(±0.2) x 10^7 M^-1 for K_assoc,app. Varying the size of the RNA polymerase binding site slightly did not significantly alter the value of K_assoc,app.

For a site binding size of 40 base-pairs, K_assoc,app was 1.2(±0.2) x 10^7 M^-1; whereas, for a site binding size of 50 base-pairs, K_assoc,app was 1.5(±0.2) x 10^7 M^-1. When RNA polymerase was titrated with an equimolar mixture of non-promoter and A1 promoter containing DNA fragments, there was a linear increase in the magnitude of the perturbation in the fluorescence spectrum at a wavelength of 340 nm as a function of the A1 promoter concentration up to the point of saturation (data not shown). In this case, saturation occurred at a ratio of A1 promoters to active enzyme molecules between 0.9 and 1.0. This demonstrates that non-readable accessible to acrylamide molecules. Because of the greater sensitivity displayed with an excitation wavelength of 280 nm, we used this wavelength to carry out studies on the interaction of RNA polymerase with the A1 promoter.

The interaction of RNA polymerase (25 nM) with a 529 base-pair fragment of DNA (7.5 nM) containing the A1 promoter caused a decrease of approximately 24% in the intensity of the fluorescence spectrum of RNA polymerase at a wavelength of 340 nm; however, there was no readily apparent shift in the emission wavelength maximum (Figure 1). In the titration of RNA polymerase with the DNA fragment containing the A1 promoter, there was a linear increase in the magnitude of the perturbation at a wavelength of 340 nm as a function of promoter concentration up to the point of saturation (Figure 1, inset). Saturation occurred at a molar ratio of A1 promoters to active enzyme molecules of between 0.9 and 1.0.

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promoter containing regions in the DNA fragments do not interfere with our ability to detect the specific binding of active enzyme molecules to the A1 promoter as monitored by intrinsic protein fluorescence.

Heparin has been used extensively to characterize the interaction between RNA polymerase and promoters (Pfeffer et al., 1977; Cech & McClure, 1980). Preincubation of a solution of RNA polymerase and heparin results in a complex that does not bind to promoters. Furthermore, addition of heparin to a solution containing RNA polymerase bound to a promoter results in the displacement of the promoter. We attempted to establish a correlation between the observed changes in the intrinsic fluorescence of RNA polymerase and formation of open complexes by investigating these alterations in the presence of heparin. However, these experiments were complicated by the fact that heparin (25-100 μg/ml) caused a perturbation in the fluorescence spectrum of RNA polymerase (25 nM) that was comparable to that observed due to the binding of the A1 promoter (7.5 nM). Thus, there was no way to distinguish between protein-heparin complexes and protein-A1 promoter complexes. Over a time period of 90 minutes, there was no appreciable change in the fluorescence intensity of RNA polymerase when heparin was added to a solution containing protein-A1 promoter complexes. Previous studies have demonstrated that heparin should disrupt protein-A1 promoter complexes over this time span (Pfeffer et al., 1977). Presumably, the absence of a change in fluorescence intensity can be attributed to the fact that after heparin traps free protein that has dissociated from the A1 promoter, it causes a similar decrease in the fluorescence intensity of the protein as that observed with the A1 promoter. There was also little if any perturbation of the fluorescence spectrum of RNA polymerase when the A1 promoter was added to a solution containing protein-heparin complexes. In and of itself, this does not demonstrate the absence of binding of RNA polymerase to the A1 promoter in the presence of heparin. The data are also consistent with a mechanism in which RNA polymerase binding to heparin is replaced by binding to the A1 promoter. The replacement of heparin by the A1 promoter would cause a similar perturbation in the fluorescence spectrum of RNA polymerase as observed for the protein-heparin complexes. Overall, the heparin studies do not provide definitive information regarding the specific interaction of RNA polymerase with the A1 promoter as monitored by intrinsic protein fluorescence.

**Figure 2.** Fluorimetric titration of RNA polymerase (25 nM total protein concentration) with the non-promoter containing DNA fragment. The concentrations of the DNA fragment used in the titration were 1.8, 2.6, 5.4, 7.2, 10.8, 13.6, 18, and 21 nM. The data were fitted to the equation: [fraction complex formed] = [binding sites]/(1/K_assoc_app + [binding sites]). Fits were conducted assuming various degrees of saturation for the experimental data over the range of 100 to 65%. In all titrations that were conducted, saturation values between 80 and 70% gave the best fits. For the data in this Figure, the theoretical line through the data points corresponds to 77% saturation.

**Equilibrium binding of RNA polymerase to the A1 promoter from bacteriophage T7 as monitored by gel electrophoresis**

In the fluorimetric titration of RNA polymerase with the DNA fragment containing the A1 promoter, there appeared to be no detectable signal change due to inactive enzyme molecules. There are two possible explanations for this observation. Either inactive enzyme molecules do not bind to the A1 promoter or binding does occur but without a perturbation in the fluorescence spectrum. To investigate this further, we analyzed the interaction of RNA polymerase with the promoter and non-promoter containing DNA fragments by non-denaturing gel electrophoresis.

In electrophoretic analysis in non-denaturing gels, RNA polymerase displayed a distinct complex with the DNA fragment containing the A1 promoter that was clearly separated from free DNA (Figure 3(a)). Upon the titration of the DNA fragment containing the A1 promoter with RNA polymerase, there was a progressive decrease in the intensity of the band due to free DNA and a corresponding increase in the intensity of the band due to the protein-DNA complex. At molar ratios of total protein to DNA fragment of four or greater, a second complex appeared that apparently has a stoichiometry greater than 1:1. In the analysis of the interaction of RNA polymerase with the 548 base-pair fragment lacking a promoter, there was a progressive decrease in the intensity of the band corresponding to free DNA as the amount of protein was increased; but no distinct protein-DNA complexes were observed (data not shown). As pointed out by Revzin et al. (1986), whether a band due to complex formation is seen depends on the
strength of the DNA-protein interaction. If the complex is weak and short-lived, then dissociation can occur during gel electrophoresis. The dissociated DNA molecules will migrate under the influence of the electric field. Eventually, this leads to a decrease in the intensity of the band corresponding to the complex and a smear of DNA that moves ahead. This smear of DNA may or may not be sufficiently intense to be readily detected. In the case of the interaction of RNA polymerase with the non-promoter containing DNA fragment, it appears that the expected smear is not intense enough to be observed.

When the data in Figure 3(a) were analyzed further, it was found in the case of the DNA fragment containing the A1 promoter that there was a linear increase in the amount of protein bound to the DNA up to the point of saturation. (Figure 3(b)). Saturation occurred at a value between 1.0 and 1.1 for the ratio of active enzyme molecules to A1 promoters. Therefore, the absence of a detectable signal change due to inactive enzyme molecules in fluorimetric titrations appears to be due to the fact that inactive enzyme molecules do not form stable complexes with the A1 promoter under the current experimental conditions.

**Stopped-flow kinetic analysis of the interaction of RNA polymerase with the T7 A1 promoter over the temperature range of 25 to 37°C**

The interaction of RNA polymerase with the A1 promoter was monitored in real time by the decrease in the intrinsic protein fluorescence of RNA polymerase upon open complex formation. Over the temperature range of 25 to 37°C, the fluorimetric decrease in each case was best described by a double exponential fit. Double exponential fits were statistically better than single exponential fits. In the case of triple exponential fits, either the data could not be fitted with three exponentials or the resulting fit did not yield unique values for the three rate constants. At temperatures of 30°C and above, the data were analyzed over the time range of ten seconds; whereas between temperatures of 25 and 28°C, the data were analyzed over the time range of 20 seconds. A typical kinetic trace for the fluorimetric decrease that occurred upon open complex formation at 37°C is given in Figure 4. The continuous curve through the experimental data corresponds to a double exponential fit. The amplitude of the fluorimetric change for the fast phase was independent of the concentration of the DNA fragment over the range of 20 to 50 nM; and the average value for the amplitude was $1.2(\pm 0.2) 	imes 10^{-3}$. The value of the observed rate constant ($k_{obs,1}$) for the fast phase displayed a linear increase as a function of the concentration of the A1 promoter (Figure 5(a)). This dependency of $k_{obs,1}$ on promoter concentration is consistent with a bimolecular process (Bernasconi, 1976); i.e. RNA polymerase binding to the promoter site. This binding can be represented as a single step:

$$E + P \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} EP_c$$

where $P$ represents the A1 promoter, $E$ is the enzyme and $EP_c$ is the closed complex formed between RNA polymerase and the A1 promoter.

Analysis of the data in Figure 5(a) according to the equation $k_{obs,1} = k_{1}[P] + k_{-1}$ (Bernasconi, 1976),

![Figure 3. Electrophoretic titration of the A1 containing DNA fragment (12.5 nM) with RNA polymerase was performed as detailed in Experimental Procedures. (a) Lane 1 shows the migration of the free DNA fragment. The concentrations of RNA polymerase from lanes 2 to 10 were 5, 10, 15, 20, 25, 30, 40, 50 and 60 nM, respectively. (b) Quantification of the formation of specific RNA polymerase-A1 promoter complexes as monitored by native gel electrophoresis. The autoradiograph shown in (a) was scanned and then the intensities of the respective bands were analyzed by using ImageQuant. The extent of complex formation was determined by measuring the decrease in the band corresponding to free DNA fragment. The percentage of active enzyme molecules, as determined as indicated in Experimental Procedures by the abortive initiation assay, was approximately 39%.](image)
yielded values of $4.0(\pm 0.6) \times 10^7$ M$^{-1}$ s$^{-1}$ and $4.0(\pm 1.3) \times 10^{-1}$ s$^{-1}$ for $k_1$ and $k_{-1}$, respectively, at 37°C. Although this reaction is written as a simple one-step process, the rate constants are macroscopic constants that describe the overall kinetics of promoter binding and dissociation. Determination of values for the association and dissociation rate constants also provided an estimate of $1.0(\pm 0.4) \times 10^8$ M$^{-1}$ for the apparent association equilibrium constant ($K_{assoc, app} = k_1/k_{-1}$) at 37°C.

The amplitude of the fluorimetric change for the slow phase was independent of the concentration of the DNA fragment over the range of 20 to 50 nM; and the average value for the amplitude was 0.4(±0.1) × 10$^{-2}$. The value of $k_{obs,2}$ for the slow phase was also independent of the concentration of A1 promoter over this range (Figure 5(b)); and the average value of $k_{obs,2}$ was 0.26(±0.02) s$^{-1}$.

We have assigned the unimolecular process associated with the slow phase to the isomerization of the closed complex to an open one which can be represented as:

$$\text{EP}_c \rightarrow \text{EP}_o$$

(3)

where $k_{isom}$ (which is equivalent to $k_{obs,2}$) is the apparent macroscopic rate constant for this process and EP$_o$ is the open complex. In this case, $k_{isom}$ describes all the steps involved in the isomerization and it is assumed that the rate of the reverse reaction is negligible with respect to the forward reaction.

Similar analyses were performed for experimental data obtained over the temperature range of 25 to 34°C. For the fast phase, plots of $k_{obs,1}$ versus the concentration of A1 promoter displayed linear increases; whereas, for the slow phase, $k_{obs,2}$ was independent of the concentration of A1 promoter. Also, the amplitudes of the fluorimetric changes associated with each phase were found to be independent of the concentration of A1 promoter over the range of 20 to 50 nM. This indicates that the system was analyzed under conditions of saturation. Values for the respective constants ($k_1$, $k_{-1}$, $k_{isom}$ and $K_{assoc, app}$) at various temperatures are listed in Table 1.

Figure 4. Time-course for complex formation between RNA polymerase and the A1 promoter at 37°C. The kinetic trace shows the time-dependent decrease in the intrinsic protein fluorescence after rapidly mixing equal volumes of solutions of RNA polymerase and the A1 promoter. The final concentrations after mixing were 5 nM for RNA polymerase in terms of active enzyme molecules and 50 nM for the A1 promoter. The data represent the average of five measurements.

Figure 5. (a) Linear variation in the values of the observed rate constant for the fast phase of complex formation as a function of A1 promoter concentration at 37°C. (b) The invariance in the values of the observed rate constant for the slow phase of open complex formation as a function of A1 promoter concentration at 37°C. The concentration of RNA polymerase was held constant at 5 nM in terms of active enzyme molecules for both (a) and (b).
In control studies, the interaction of RNA polymerase (25 nM) with a DNA fragment (30 nM) lacking the A1 promoter was monitored in real time by the decrease in the protein fluorescence of RNA polymerase upon complex formation. At 37°C, the fluorimetric decrease was best described by a single exponential (data not shown). The amplitude of this change was 0.44(±0.15) × 10⁻². Double exponential fits were poorly defined. They yielded values for the two rate constants and corresponding amplitudes with errors equal to or in some cases four times greater than the estimated values. These results further establish that the stopped-flow kinetic studies involving the DNA fragment containing the A1 promoter represent the specific interaction of active enzyme molecules with promoters.

Table 1. Estimates of constants for complex formation between RNA polymerase and the A1 promoter at different temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>(k₁, app) × 10⁷ (M⁻¹ s⁻¹)</th>
<th>(k₋₁, app) × 10⁻¹ (s⁻¹)</th>
<th>(K_assoc, app = k₁/k₋₁) × 10⁷ (M⁻¹)</th>
<th>k_iso, app (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>4.0 ± 0.6</td>
<td>4.0 ± 1.3</td>
<td>10 ± 4</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>34</td>
<td>2.2 ± 0.8</td>
<td>3.5 ± 1.8</td>
<td>6.2 ± 4.0</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>30</td>
<td>1.5 ± 0.3</td>
<td>2.8 ± 0.6</td>
<td>5.1 ± 1.5</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>28</td>
<td>1.4 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>7.0 ± 0.5</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>26</td>
<td>0.42 ± 0.52</td>
<td>1.6 ± 1.2</td>
<td>2.7 ± 3.8</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>25</td>
<td>0.30 ± 0.02</td>
<td>0.94 ± 0.05</td>
<td>3.2 ± 0.3</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>20</td>
<td>0.05 ± 0.03</td>
<td>0.28 ± 0.07</td>
<td>1.8 ± 1.2</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>0.02 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>1.1 ± 0.6</td>
<td>–</td>
</tr>
</tbody>
</table>

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Stopped-flow kinetic analysis of the interaction of RNA polymerase with the T7 A1 promoter at 15 and 20°C

At temperatures of 15 and 20°C, the fluorimetric decrease upon complex formation between RNA polymerase and the A1 promoter was best described by a single exponential in each case. For double and triple exponential fits, the resulting fits did not yield unique values for the rate constants. Also, in some cases, the data could not be fitted with three exponentials. The data at both temperatures were analyzed over the time range of 50 seconds. A typical kinetic trace of the fluorimetric decrease at 20°C is given in Figure 6(a). The continuous curve through the experimental data corresponds to a single exponential fit. The amplitude of the fluorimetric change increased as a function of A1 promoter concentration over the range of 20 to 50 nM. There was also a linear increase in the value of the observed rate constant (kobs) as a function of the A1 promoter concentration at 20°C (Figure 6(b)). This dependency of kobs on promoter concentration is consistent with a bimolecular process in the binding of RNA polymerase to the promoter site. This can be represented as a single step process leading to the formation of a closed complex between RNA polymerase and the A1 promoter as shown in equation (2). Analysis of the data according to the equation kobs = k₁[P] + k₋₁ (Bernasconi, 1976), yielded values of 5.0(±3.0) × 10⁵ M⁻¹ s⁻¹ and 2.8(±0.7) × 10⁻² for k₁ and k₋₁, respectively, at 20°C. A value of 1.8(±1.2) × 10⁷ M⁻¹ for K_assoc, app at 20°C was calculated from the values of k₁ and k₋₁. A similar analysis was performed for the experimental

Figure 6. (a) Time-course for complex formation between RNA polymerase and the A1 promoter at 20°C. The kinetic trace shows the time-dependent decrease in intrinsic protein fluorescence after rapidly mixing equal volumes of solutions of RNA polymerase and the A1 promoter. The final concentrations after mixing were 5 nM for RNA polymerase in terms of active enzyme molecules and 30 nM for the A1 promoter. The data represent the average of four measurements. (b) Linear variation in the values of the observed rate constant for complex formation as a function of A1 promoter concentration at 20°C. The concentration of RNA polymerase was held constant at 5 nM in terms of active enzyme molecules.

Stopped-flow Analysis of Open Complex Formation
data at 15°C. There was a linear increase in the value of the observed rate constant \(k_{\text{obs}}\) as well as an increase in the amplitude of the fluorimetric change as a function of the A1 promoter concentration. Table 1 contains a complete list of the values for the respective constants \(k_1, k_{-1}\) and \(K_{\text{assoc, app}}\) for the interaction of RNA polymerase with the A1 promoter at 15 and 20°C. Once again, it should be noted that \(k_1\) and \(k_{-1}\) are macroscopic rate constants that describe the overall kinetics of RNA polymerase association and dissociation.

**Thermodynamic analysis of the interaction of RNA polymerase with the T7 A1 promoter**

Arrhenius plots over the temperature range of 15-37°C were linear in the case of both the association and dissociation reactions for the formation of EPc (Figure 7(a) and (b)). Over this temperature range, the association rate constant \(k_1\) displayed a significantly greater dependency on temperature than the dissociation rate constant \(k_{-1}\). The apparent activation energies for the association and dissociation reactions were found to be 46.0(±0.4) and 28(±3) kcal mol\(^{-1}\), respectively. Over the temperature range of 25-37°C, the Arrhenius plot for the isomerization reaction in the conversion of the closed complex to an open one was non-linear (Figure 7(c)). There was a large change in the activation energy from 46(±12) kcal mol\(^{-1}\) between 25 and 28°C to 9.1(±1.9) kcal mol\(^{-1}\) between 28 and 37°C. A van’t Hoff plot was constructed over the temperature range of 15-37°C for the association equilibrium reaction in the formation of the closed complex (Figure 8). This plot is linear and yielded a value of 18(±3) kcal mol\(^{-1}\) for the van’t Hoff enthalpy for this process. It should be noted that the enthalpy change for this step can also be calculated from the activation energies of the forward and reverse reactions; i.e., \(\Delta H_{\text{obs}} = E_{\text{a, assoc}} - E_{\text{a, dissoci}} = 18(±3)\) kcal mol\(^{-1}\). A value of \(-12(±5)\) kcal mol\(^{-1}\) was calculated for the standard free energy change, \(\Delta G_{\text{obs}}\), for the association reaction at 37°C. From the values of \(\Delta G_{\text{obs}}\) and \(\Delta H_{\text{obs}}\), a value of 94(±15) e.u. was calculated for the entropy change, \(\Delta S_{\text{obs}}\), for the equilibrium binding step.

**Analysis of initiation of transcription by using the abortive initiation assay**

The value of the apparent macroscopic isomerization rate constant for the conversion of the closed complex to an open one as determined in stopped-flow kinetic studies \((0.26(±0.02)\) s\(^{-1}\)) at 37°C was approximately an order of magnitude greater than that reported in studies in which abortive initiation assays were used to analyze the system (Dayton et al., 1984; Johnson et al., 1991; Ozoline et al., 1993). This disparity has led us to postulate that there is a step between open complex formation and formation of the abortive product that is the rate-determining step in the initiation of transcription in the case of the A1 promoter. To test this hypothesis, we used the
[pppApU] = V_0 - V_{obs}[1 - \exp(-t/\tau_{obs})] where V is the steady-state rate of abortive synthesis, t is time and \tau_{obs} is the lag time. The estimate for V was obtained from the linear portion of the reaction profile and the estimate for \tau_{obs} was obtained by extrapolating the linear portion of the reaction profile to the x-axis. For this data set, the estimated value of \tau_{obs} was found to be 2.8 minutes and the estimated value for the steady-state production of the abortive product was found to be 1.7 \times 10^{-1} \mu M \text{ min}^{-1}. Enzyme, ATP and A1 promoter were preincubated for ten minutes at 37°C for the control (\textcircled{a}). The reaction was initiated by the addition of UTP. A linear least-squares analysis of the data yielded a value of 1.7 \times 10^{-1} \mu M \text{ min}^{-1} for the steady-state rate of abortive synthesis for this data set. (b) A1 promoter, ATP and UTP were preincubated at 37°C for ten minutes (\textcircled{b}). The reaction was initiated by the addition of enzyme. The theoretical curve was generated as detailed above. For this data set, the estimated value of \tau_{obs} was found to be 2.1 minutes and the estimated value for the steady-state production of the abortive product was found to be 1.6 \times 10^{-1} \mu M \text{ min}^{-1}. Enzyme, ATP and A1 promoter were preincubated for ten minutes at 37°C for the control (\textcircled{a}). The reaction was initiated by the addition of UTP. For this data set, a linear least-squares analysis of the data yielded a value of 1.6 \times 10^{-1} \mu M \text{ min}^{-1} for the steady-state rate of abortive synthesis. (c) RNA polymerase, ATP and UTP were preincubated at 37°C for ten minutes (\textcircled{b}). The reaction was initiated by the addition of the A1 promoter. (\textcircled{c}) Enzyme, ATP and A1 promoter were preincubated for ten minutes at 37°C. The reaction was initiated by the addition of UTP for the control. A linear least-squares analysis of all of the data yielded a value of 1.7 \times 10^{-1} \mu M \text{ min}^{-1} for the steady-state rate of abortive synthesis.

Abortive initiation assay (McClure, 1980) to analyze initiation from the A1 promoter under conditions in which the reagent that was used to initiate the reaction was varied. The concentrations of RNA polymerase (6.25 nM), A1 promoter (3.2 nM), ATP (0.04 mM) and UTP (50 \mu M) were kept constant. Because the percentage of active enzyme molecules for this preparation of RNA polymerase was approximately 39%, the ratio of A1 promoters to active enzyme molecules corresponded to 1.3. Moreover, with a lower limit of 10^{13} M^{-1} for the overall equilibrium constant for the formation of an open complex between RNA polymerase and the A1 promoter (Dayton et al., 1984), all of the active enzyme molecules should be capable of binding to A1 promoters and forming open complexes. If open complex formation is the rate-determining step in the initiation of transcription, then the lag times (i.e. the times required to reach the steady-state rate of abortive product formation) for the protein and A1 promoter initiated reactions should be comparable. On the other hand, if the initiating nucleotide plays a crucial role in regulating initiation, then the lag times for the ATP and RNA polymerase initiated reactions should be comparable. In Figure 9 we present representative time-courses for abortive product (pppApU) formation under conditions in which the reaction was initiated by the addition of ATP (Figure 9(a)), enzyme (Figure 9(b)) and A1 promoter (Figure 9(c)). In each case, there is a control in which the reaction was initiated by the addition of UTP. No detectable lag was observed when the reaction was initiated by the addition of UTP; i.e. steady-state levels of abortive product formation occurred over the entire time range beginning at zero time. The lag times observed when the reaction was initiated by the addition of either enzyme (1.9 (±0.8) minutes) or ATP (2.3 (±0.8) minutes) were comparable. In stark contrast, there was no discernible lag upon the initiation of the reaction by the addition of the A1 promoter under these conditions. These results indicate that open complex formation is not the rate-determining step in the initiation of transcription from the A1 promoter. Instead, it appears that the initiating nucleotide plays a crucial role in regulating this process.

Discussion

Steady-state fluorescence studies

The fluorimetric titrations and gel mobility shift studies establish that the alteration observed in the fluorescence spectrum of RNA polymerase in the presence of the A1 promoter represents the specific binding of active enzyme molecules to promoters. With the distribution of tryptophan residues throughout the various subunits, the observed perturbations represent global changes that occur in RNA polymerase upon open complex formation. Because of the strong interaction between RNA
polymerase and the A1 promoter, a value for the apparent overall association equilibrium constant corresponding to the formation of an open complex could not be determined. A lower limit of $10^6 \text{M}^{-1}$ can be estimated for the value of the apparent overall association equilibrium constant for this process. Values of $10^{12}-10^{14} \text{M}^{-1}$ were observed by Hinckle & Chamberlin (1972a) for the apparent overall association equilibrium constant in the formation of open complexes with promoters found in bacteriophage T7 DNA by using filter binding assays (at 37°C and in 10 mM Tris-HCl (pH 7.9), 10 mM MgCl$_2$, 50 mM NaCl). Dayton et al. (1984) reported a lower limit of $10^{11} \text{M}^{-1}$ for the apparent equilibrium constant for the formation of an open complex between RNA polymerase and the A1 promoter by using the abortive initiation assay (at 37°C and in 40 mM Tris-HCl (pH 8), 10 mM MgCl$_2$, 80 mM KCl). The value of the apparent association equilibrium constant found in this study for the interaction of RNA polymerase with the non-promoter containing DNA fragment was $1.3(\pm 0.3) 	imes 10^7 \text{M}^{-1}$ at 25°C and at a salt concentration of 0.05 M. This value is in good agreement with estimates of $10^6-10^7 \text{M}^{-1}$ and at a salt concentration of 0.05 M. This value is consistent with estimates of $10^6-10^7 \text{M}^{-1}$ and at a salt concentration of 0.05 M. This value is consistent with estimates of $10^6-10^7 \text{M}^{-1}$.

Stopped-flow kinetic analysis of open complex formation

Over the temperature range of 25 to 37°C, the interaction of RNA polymerase with the A1 promoter was investigated under conditions of saturation. Under these conditions, stopped-flow kinetic experiments on the interaction of RNA polymerase with the A1 promoter displayed a fast concentration dependent initial phase followed by a slower concentration independent phase. These results are consistent with a minimal model in which a fast bimolecular protein-promoter association to form a closed complex is followed by a slow unimolecular isomerization of the closed complex to form an open one. At temperatures of 15 and 20°C, stopped-flow kinetic experiments showed a single concentration dependent phase which is consistent with a minimal model in which a bimolecular protein-promoter association occurs to form a closed complex. On the basis of the analyses of the stopped-flow kinetic data at 15 and 20°C, there was no indication of a concentration independent phase which would correspond to the conversion of a closed complex to an open one. This is consistent with footprinting and DNA chemical modification studies that indicate that open complex formation does not occur readily at temperatures of 20°C and below (Becker & Wang, 1984; Spassky et al., 1985; Duval-Valentini & Ehrlich, 1987).

The apparent association rate constant ($k_1$) for the initial binding step is a macroscopic rate constant that describes all of the events leading to the formation of the closed complex EPc. These events include non-specific binding, counterion displacement from the DNA, promoter search, conformational transitions, productive binding and at low temperatures release of water. The value of $k_1$ at 37°C is less than that expected for a diffusion controlled process ($\sim 10^8 \text{M}^{-1} \text{s}^{-1}$) for molecules of the size of RNA polymerase and the promoter (von Hippel et al., 1984). Thus, $k_1$ does not represent the rate constant for the formation of a collision complex. Moreover, the value of $k_1$ is considerably less than that expected for the facilitated binding ($\sim 10^{10} \text{M}^{-1} \text{s}^{-1}$) of RNA polymerase to a promoter (von Hippel et al., 1984). This indicates that the formation of EPc is preceded by another step

$$E + P \xrightarrow{\text{quench}} EP' \xrightarrow{k_1} EP_c$$

where $k_F$ is the equilibrium association constant due to facilitated binding of RNA polymerase to the promoter site. Apparently, the formation of EPc is not accompanied by a detectable perturbation in the intrinsic fluorescence of RNA polymerase. The rate-determining step in the formation of EPc is the conversion of EP' to EPc; i.e. $[A1]k_{F,\text{forward}} + k_{F,\text{reverse}} \gg k_F, k_{F,\text{reverse}}$. The apparent association rate constant ($k_1$) as well as the apparent dissociation rate constant ($k_{-1}$) displayed significant temperature dependencies. Over the temperature range of 15 to 37°C, the Arrhenius plots were linear in both cases. The apparent activation energy for the association step was $46.0(\pm 0.4)$ kcal mol$^{-1}$; whereas for the dissociation step it was $28(\pm 3)$ kcal mol$^{-1}$. Due to the complexity of this step (i.e. the conversion of EPc to EPc', i.e. $k_{F,\text{forward}} + k_{F,\text{reverse}} \gg k_F, k_{F,\text{reverse}}$, it was not possible to unambiguously assign these activation energies to a single process. However, with the DNA undergoing only partial unwinding upon the formation of the closed complex (Wang et al., 1977; Hsieh & Wang, 1978; Spassky et al., 1985; Amouyal & Buc, 1987), it is unlikely that these large activation energies are associated with a conformational change in the DNA. It is more likely that a structural alteration in the protein is the major contributor to these large activation energies. Structural alterations in the protein may represent movement of RNA polymerase domains such as the thumb region that has been postulated to be involved in DNA binding (Darst et al., 1989; Polyakov et al., 1995).

Estimates for the apparent association equilibrium constant at various temperatures were calculated from the values of the individual association and dissociation rate constants. At 37°C the value of $K_{\text{assoc}}$, which obtained in this study was $1.0(\pm 0.4) 	imes 10^5 \text{M}^{-1}$. In previous studies performed under identical experimental conditions by using the abortive initiation assay, a value of $1.0(\pm 0.5) 	imes 10^5 \text{M}^{-1}$ was reported (Johnson et al., 1991). Comparable values for $K_{\text{assoc,app}}$ were also observed in abortive initiation assays conducted by Dayton et al. (1984) ($3 \times 10^5 \text{M}^{-1}$), and Ozoline et al.
(1993) (1.6 x 10^8 M^{-1}) under somewhat different experimental conditions. Over the temperature range of 15-37°C, the van’t Hoff plot for this process was linear. With a van’t Hoff enthalpy of 18(±3) kcal mol^{-1}, the initial binding step over the temperature range of 15 to 37°C is endothermic. Buc & McClure (1985) reported a similar temperature dependence for \( K_{\text{assoc, app}} \) in the interaction of RNA polymerase with the lac UV5 promoter by using abortive initiation assays (in 25 mM Hepes (pH 8.0), with 10 mM MgCl\(_2\) and 100 mM KCl). Over the range of 19-42°C, the van’t Hoff plot was linear and the equilibrium binding reaction was found to be endothermic with a value of 30 kcal mol^{-1} for \( \Delta H_{\text{obs}} \). Bertrand-Burggraf et al. (1984) reported an increase in \( K_{\text{assoc, app}} \) with increasing temperature for the interaction of RNA polymerase with the tet promoter of pBR322 over the range 25-37°C by using a filter binding assay (in 40 mM Tris-HCl (pH 8.0), 10 mM MgCl\(_2\), 50 mM KCl). The van’t Hoff plot was linear and a value of 29 kcal mol^{-1} was found for \( \Delta H_{\text{obs}} \). Roe et al. (1985) observed that the apparent association equilibrium constant for the interaction of RNA polymerase with the \( \lambda Pr \) promoter was insensitive to temperature over the range of 13-37°C by using a filter binding assay (in 50 mM Hepes (pH 8.0), 10 mM MgCl\(_2\), 120 mM KCl). The observation that \( K_{\text{assoc, app}} \) for closed complex formation is temperature insensitive for the \( \lambda Pr \) promoter but not for the lac UV5, tet or A1 promoters may reflect differences due to different promoter site sequences, differences in experimental conditions under which binding was measured or differences inherent in the techniques that were used to monitor binding. In the cases of the lac UV5, tet and A1 promoters, however, the positive van’t Hoff enthalpy changes indicate that formation of the closed complex (EP) is entropy driven. The value of \( \Delta S_{\text{obs}} \) for the A1 promoter was 94(±15) e.u. Bertrand-Burggraf et al. (1984) reported a value of 130 e.u. for \( \Delta S_{\text{obs}} \) in the case of the tet promoter from pBR322. Based on the values of \( K_{\text{assoc, app}} \) and the van’t Hoff enthalpy determined by Buc & McClure (1985), one can calculate a value of 134 e.u. for \( \Delta S_{\text{obs}} \) in the case of the lac UV5 promoter. The large positive entropy change associated with this process can be attributed, as has been done in other studies, to counterion release from the DNA molecule upon closed complex formation (Record et al., 1976, 1978). It should also be noted that the linear van’t Hoff plots obtained in the case of the lac UV5, tet and A1 promoters for the formation of the closed complex indicate that burial of non-polar surface residues upon closed complex formation does not make a major contribution to the net free energy of association. A curved van’t Hoff plot would have indicated that both the burial of non-polar surface residues (the hydrophobic effect) and the release of cations (the polyelectrolyte effect) made major contributions in driving the thermodynamically unfavorable process of closed complex formation between RNA polymerase and promoters (Ha et al., 1989).

The isomerization rate constant (\( k_{\text{obs}} \)) for the conversion of EP \(_o\) to EP \(_c\) is a macroscopic rate constant that describes all of the events for the conversion of the closed complex to an open one. The Arrhenius plot for this process was found to be non-linear. There are several phenomena that can account for this non-linearity (Han, 1972; Silvius & McElhaney, 1981). These include (1) parallel reaction pathways with different activation energies, (2) a temperature-induced change in the conformation of one or more components that results in a different activation energy for the process and (3) a change in the rate-determining step as a function of temperature. The possibility of parallel reaction pathways with different activation energies can be eliminated because an Arrhenius plot of this type is always concaved upward, and the Arrhenius plot obtained in this study is concaved downward. Although it may not be possible to totally eliminate the possibility of temperature-induced conformational changes in either RNA polymerase or DNA as the origin of the non-linear Arrhenius plot, there is evidence that there is no significant alteration in the structure of either RNA polymerase or DNA over the temperature of 5-37°C. Sen & Dasgupta (1994) demonstrated in acrylamide fluorescence quenching studies that the structure of RNA polymerase does not vary significantly over the temperature range of 5-37°C. Also, Vesnavaer & Breslauer (1991) demonstrated in UV spectral studies that the structure of DNA does not vary significantly over the temperature range of 5-37°C. Thus, the non-linear Arrhenius plot observed for the isomerization step is most likely due to a change in the rate-determining step over the temperature range of 25-37°C. This indicates that there are at least two steps in this conversion.

A two step mechanism for the conversion of the closed complex to an open one has been proposed previously for the A1 promoter (Kadesh et al., 1982; Rosenberg et al., 1982) as well as for the \( \lambda Pr \) (Roe et al., 1984) and lac UV5 (Buc & McClure, 1985) promoters. It was not possible to obtain estimates of the rate constants for the individual steps in this isomerization reaction. However, analysis of the Arrhenius plot for this process yielded estimates for the activation energies. Between 25 and 28°C, the apparent activation energy was 46(±12) kcal mol^{-1}; whereas between 28 and 37°C, it was 9.1(±1.9) kcal mol^{-1}. These activation energies may be associated with conformational changes in either the protein or the DNA. It should be noted that these putative conformational changes are not temperature-induced; instead, they are conformational changes that occur as a part of the mechanism for the conversion of the closed complex to an open one. In previous studies (Buc & McClure, 1985; Roe et al., 1985), the first step in the conversion of the closed complex to an open one was postulated to be a conformational change in RNA polymerase and nucleation of strand sep-
aration. The second step was postulated to be strand separation and formation of an open complex. A priori, nothing can be stated about the thermodynamics of the step involving the conformational change in RNA polymerase and nucleation of strand separation. It may or may not be associated with a large activation energy. In the case of strand separation, however, it has been demonstrated that DNA denaturation is accompanied by a large positive enthalpy change (Vesnaver & Breslauer, 1991). Moreover, the opening of base-pairs by RNA polymerase at the lac UV and tac promoters has been shown to be accompanied by a very steep temperature dependence (Kirkegaard et al., 1983). On this basis, we have assigned the apparent activation energy of 46(±12) kcal mol⁻¹ to the step involving strand separation and the apparent activation energy of 9.1(±1.9) kcal mol⁻¹ to the step involving a conformational change in the protein and nucleation of strand separation. If we assume that the rates of the reverse reactions are insignificant with respect to the forward reactions, we can describe the effect of temperature on the conversion of the closed complex to an open one over the temperature range of 25 to 37°C. At temperatures above 28°C, the rate-determining step is that corresponding to the conformational change in the protein and nucleation of strand separation; whereas at temperatures below 28°C, the rate-determining step is strand separation. It should be noted that Buc & McClure (1985), observed a non-linear Arrhenius plot for k_{iso} in the case of the lac UV5 promoter; whereas, Bertrand-Burggraf et al. (1984) reported a linear Arrhenius plot for the tet promoter of pBR322. The observation of a linear Arrhenius plot for the tet promoter and non-linear plots for the A1 and lac UV5 promoters may reflect differences in promoter site sequences, differences in experimental conditions under which binding was measured or differences inherent in the techniques used to monitor binding. In the case of the lac UV5 promoter, Buc & McClure (1985) assigned the apparent activation energy of 5.5(±1.5) kcal mol⁻¹ to the step involving a conformational change in the protein and nucleation of strand separation and the apparent activation energy of 60(±7) kcal mol⁻¹ to the step involving strand separation. These values are comparable to those obtained in the current study for the A1 promoter.

In the stopped-flow kinetic studies conducted by Sullivan et al. (1997), they used alterations in the fluorescence intensity of 2-aminopurine fluorescent probes in synthetic promoters to investigate open complex formation in the case of E. coli RNA polymerase. They reported a value of 0.8(±0.3) x 10⁷ M⁻¹ s⁻¹ for k_{assoc, app} and a value of 2.5(±0.7) s⁻¹ for the apparent macroscopic isomerization rate constant in the conversion of the closed complex to an open one (in 30 mM Hepes (pH 7.6), with 10 mM MgCl₂, 0.1 mM DTT and 100 mM KCl). Values for k₁ and k⁻¹ could not be resolved with the model that they used to obtain estimates of k_{assoc, app} and k_{iso}. The second-order rate constant for the formation of the open complex, which is given by k₂ = (k_{assoc, app} k_{iso}) was 2 x 10⁷ M⁻¹ s⁻¹. In the stopped-flow kinetic study conducted by Dunkak et al. (1996), the interaction between RNA polymerase and a synthetic DNA fragment containing a consensus sequence promoter with a probe attached to one end was monitored by fluorescence anisotropy. They reported values of 1.5 x 10⁷ to 7.4 x 10⁷ M⁻¹ s⁻¹ for the second-order association rate constant (in 50 mM Tris-HCl (pH 7.9), with 10 mM MgCl₂, 0.1 mM DTT, 0.1 mM EDTA and 150 mM KCl). Estimates for k_{assoc, app} and k_{iso} were not resolved from the fluorescence anisotropy data. There is remarkably good agreement between the values of k₂ obtained in the studies conducted by Sullivan et al. (1996) and Dunkak et al. (1997) with the value of 2.6(±0.8) x 10⁷ M⁻¹ s⁻¹ calculated for the A1 promoter from the data in Table 1. This suggests that the same overall process was monitored by all three methods. Upon comparing the values of k_{assoc, app} and k_{iso} obtained by Sullivan et al. (1997) with a variant P_rm promoter to the values obtained here with the A1 promoter (Table 1), we see that k_{assoc, app} for the A1 promoter is approximately an order of magnitude greater than that observed for the variant P_rm promoter and that k_{iso} for the A1 promoter is approximately an order of magnitude less than that observed for the variant P_rm promoter. The difference in k_{assoc, app} can be explained, in part, by the higher salt concentration that was used in the studies by Sullivan et al. (1997); i.e. the interaction between RNA polymerase and DNA decreases as a function of salt concentration. The difference in k_{iso} indicates that the variant P_rm promoter is intrinsically a better promoter than the A1 promoter in terms of the conversion of the closed complex to an open one.

A direct comparison between the data for E. coli RNA polymerase and T7 RNA polymerase is complicated by the fact that little is known about the mechanism of open complex formation for T7 RNA polymerase. A closed complex has not been trapped. There is no direct experimental data for a melted region in open complexes. There are no estimates for the forward or reverse steps for a two step minimal model for the formation of the open complex. Also, it is not clear whether the equilibrium binding constant of T7 RNA polymerase to a T7 promoter reflects R_{p, o} R_P or a mixture of both states. In the three studies conducted by using 2-aminopurine fluorimetric changes to monitor the interaction between T7 RNA polymerase and promoters, there were increases in the intensity of the fluorescence due to 2-aminopurine upon complex formation (Ujvari & Martin, 1996; Sastry & Ross, 1998; Jia et al., 1996). Because fluorimetric changes in 2-aminopurine substituted DNA are dependent on strand separation, these data suggest strand separation and open complex formation. In all three studies, the values of the overall second-order association rate constant are com-
parable to those obtained for *E. coli* RNA polymerase. They ranged in value from 0.6 × 10^7 to 6.5 × 10^7 M⁻¹ s⁻¹. In the studies conducted by Jia *et al.* (1996) in which the intrinsic protein fluorescence of T7 RNA polymerase was used to monitor promoter binding, the values obtained for the second-order association rate constant and the first order dissociation rate constant were comparable to those obtained in the studies in which 2-aminopurine was used. However, there was no evidence of a slow step that could be attributed to the conversion of a closed complex to an open one. This contrasts with the studies reported herein, in which the data were consistent with transient closed complex formation for *E. coli* RNA polymerase. Jia *et al.* (1996) suggested that although there are two steps in the formation of the open complex, the stopped-flow kinetic studies show only one phase because of the rapid formation of the transient closed complex.

**Analysis of the initiation of transcription by the abortive initiation assay**

The value of the macroscopic isomerization rate constant determined here at 37°C is an order of magnitude greater than the value determined in other studies by using the abortive initiation assay to analyze open complex formation. In the current study involving stopped-flow kinetic analyses, we obtained a value of 0.26(±0.02) s⁻¹; whereas, in abortive initiation assays, Johnson *et al.* (1991) reported a value of 0.02(±0.01) s⁻¹ under identical experimental conditions. Also, Dayton *et al.* (1984) obtained a value of 0.09 s⁻¹ and Ozoline *et al.* (1993) reported a value of 0.008 s⁻¹ under somewhat different experimental conditions. A similar disparity was observed in the case of the λPR promoter. The value of the isomerization rate constant as determined in filter binding assays (Roe *et al.*, 1984) was at least an order of magnitude greater than the value obtained in abortive initiation assays (Hawley & McClure, 1980, 1982). These results indicate that the direct methods (i.e. filter binding assays and stopped-flow kinetics) that were used for analyzing open complex formation monitored a different step than in the case of the abortive initiation assay which is an indirect method for analysis of open complex formation. Insight into a possible reason for these disparities may be obtained by examining the results of Spassky *et al.* (1985). They investigated the footprints and DNA chemical modification patterns for the lac UV5 promoter-RNA polymerase open complex in the absence and presence of substrate. ApA and UTP produced a slight modification of the footprint. However, the reactivities of the bases were drastically modified when ApA and UTP were added together. This indicates that there is an alteration in the conformation of the open complex in the presence of substrates. Therefore, the isomerization rate constant obtained in abortive initiation assays may correspond to the conformational change that occurs upon substrate binding; whereas, the isomerization rate constant obtained in stopped-flow kinetic studies may represent the conversion of the closed complex to an open one.

To test this hypothesis, we analyzed the kinetics of abortive initiation under conditions in which the reaction was initiated by the addition of enzyme, A1 promoter, ATP and UTP, respectively. Also the initiating nucleotide (ATP) was present at non-saturating concentrations. The concentration of each component was the same in the four different initiation experiments. Lags in the approach to the steady-state rate of formation of pppApU occurred when the reaction was initiated by the addition of ATP or the enzyme, but not when it was initiated by the addition of UTP or the A1 promoter. Furthermore the lag times observed in the case of the enzyme and ATP initiated reactions were comparable. The lack of a lag phase for the A1 promoter initiated reaction and the presence of a significant lag phase for the enzyme initiated reaction indicates that open complex formation is not the rate-determining step in the initiation of transcription for the A1 promoter. Moreover, because the lag times in the approach to steady-state formation of the abortive product for the enzyme and ATP initiated reactions were nearly identical, the rate-determining step in the initiation of transcription from the A1 promoter appears to be associated with an ATP-dependent phenomenon. These results contrast with the reported behavior of the T7 D promoter (McClure, 1980). For the T7 D promoter, it was observed that there was no lag phase when the reaction was initiated by the addition of the initiating nucleotide; whereas, comparable lag times were observed when the reaction was initiated by the addition of either protein or the promoter. Thus, it appears in the case of the T7 D promoter that the rate-determining step in the initiation of transcription is open complex formation. Although the abortive initiation reaction has been used in the analysis of other promoters, systematic analyses in which the reaction was initiated by different reagents have not been performed. Therefore, it is not clear which step is rate-determining for other promoters. However, recently, Gaal *et al.* (1997) demonstrated that rRNA synthesis in bacteria is regulated by the concentration of the initiating nucleotide within the cell. A key question to address in the case of the T7 A1 promoter as well as the rRNA promoters is whether the initiating nucleotide is acting through the active site or an allosteric site. If the initiating nucleotide is acting through the active site by first binding and then causing a slow conformational change in the enzyme to convert it to a transcriptionally active complex, then one would expect to observe burst kinetics in the abortive initiation reaction when the reaction is initiated by the addition of UTP in the case of the A1 promoter. That is, the preincubation of the enzyme with the initiating nucleotide (ATP) should result in the conversion of the open complex to a transcriptionally
active open complex. When UTP is added, a burst of pppApU is produced. After the first turnover, the rate of product formation is slower because another ATP molecule has to bind to the active site and convert it back to the transcriptionally active open complex. This corresponds to a steady-state rate of abortive product formation. If the reaction is initiated by the addition of the initiating nucleotide (ATP), then the rate of abortive product formation should be linear from time zero. Moreover, the steady-state rates of abortive product formation should be comparable for both the ATP and UTP initiated reactions. Clearly, the data for the A1 promoter given in Figure 9 do not display burst kinetics and are not consistent with this mechanism involving the active site. Because AMP displayed the same general behavior as ATP in terms of the relative magnitudes of the lag times (R.S.J., unpublished results), the effect of the initiating nucleotide (ATP) is not a result of protein phosphorylation. In the case of a mechanism in which the rate-determining step is the release of the product, there should be steady-state production of pppApU from zero time regardless of whether the reaction is initiated by ATP or UTP. Clearly, this is not the case for the A1 promoter. The data shown in Figure 9 appear to be consistent with a mechanism in which the initiating nucleotide is acting through an allosteric site. In this case, ATP binds to an allosteric site and causes a slow conformational change in the enzyme to convert it to a transcriptionally active open complex. Therefore, the initiation of the reaction by the addition of ATP should generate a lag phase as the steady-state level of the transcriptionally active open complex is reached. Because the ATP is acting at an allosteric site, turnover at the active site should not convert the enzyme back to a less active or inactive state. Steady-state levels of abortive product formation should continue for multiple turnovers. When the reaction is initiated by the addition of UTP, steady-state levels of abortive product formation should be observed from time zero. Equilibrium dialysis binding studies by Wu & Goldthwait (1969) have established that RNA polymerase contains two binding sites for the purine nucleotides and only one site for the pyrimidine nucleotides. Therefore, an allosteric mechanism is consistent with the number of purine nucleotide binding sites.

Conclusions

The data that we have obtained here are consistent with the following model for the initiation of transcription in the case of the A1 promoter:

The first step involves the facilitated reversible binding of RNA polymerase to the promoter to form the EPc complex. This is followed by the formation of the EPc complex. The formation of EPc from EPo appears to involve a structural alteration in the protein. The EPc complex is converted to an open complex EPo in two steps. The first step corresponds to a conformational change in the protein and nucleation of strand separation. This is followed by strand separation and formation of the open complex. After open complex formation, the initiating nucleotide (in this case ATP) binds to the allosteric site and causes the conversion of the open complex to a transcriptionally active conformational state ATP-EP TA. Next, ATP and UTP at the active site rapidly react to form the abortive product (pppApU). At 37°C, the rate-determining step in the initiation of transcription appears to be the conversion of ATP-EPo to ATP-EP TA. It should be noted, however, that although we have shown open complex formation occurring before the binding of ATP in equation (5), the experimental data indicate that ATP can bind to RNA polymerase in the absence of DNA. Therefore, there appear to be two pathways for the formation of the ATP-EP TA complex.

The model presented in equation (5) is not inconsistent with the data reported for the T7 D promoter. The rate of open complex formation would be expected to vary with promoters. For promoters such as the A1 promoter (k = 2.6 x 10^7 M^-1 s^-1) and presumably the rRNA promoters (k = 10^8 M^-1 s^-1; Rao et al., 1994) that undergo rapid open complex formation, the levels of the initiating nucleotides would serve to regulate the initiation of transcription. For promoters such as T7 D (k = 9.6 x 10^7 M^-1 s^-1) that undergo slow open complex formation relative to the effect of the initiating nucleotide, it would be expected that regulation of transcription lies at the level of open complex formation.

**Experimental Procedures**

**Reagents and materials**

[γ-32P]ATP and [α-32P]UTP were obtained from ICN. E. coli K12 cell paste (3/4 log phase, enriched medium) was purchased from the University of Wisconsin. All restriction enzymes were obtained from New England BioLabs. T4 polynucleotide kinase and calf intestinal alkaline phosphatase (CIP) were purchased from Promega. All other reagents were of the highest purity available from commercial sources.

\[
E + P \quad \rightarrow \quad EP^* \quad \rightarrow \quad EP_c \quad \rightarrow \quad EP' \quad \rightarrow \quad EP_o \quad \rightarrow \quad ATP-EP_o \quad \rightarrow \quad ATP-EP TA
\]

(5)

ATP
Purification of RNA polymerase and isolation of holoenzyme

For the purification of RNA polymerase, the method of Burgess & Jendrisak (1975) as modified by Lowder & Johnson (1982) was used. Separation of the core polymerase and holoenzyme was accomplished by chromatography on a Bio-Rex-70 (Bio-Rad) column. Purified RNA polymerase was dialyzed against a solution containing 50 mM Tris (pH 8.0), 50 mM DTT, 10 mM MgCl₂, 0.1 mM EDTA and 50% (v/v) glycerol. A Bio-Rex 70 column (20 ml bed volume) was prepared and washed with 150 ml of P5 buffer (40 mM K₂HPO₄, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 5% glycerol). The column was then equilibrated with P50 buffer (40 mM K₂HPO₄, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% glycerol). Next, the mixture of core polymerase and holoenzyme was applied to the column and elution was carried out with P50 buffer. Contaminating proteins eluted in the first peak. After the absorbance at 280 nm returned to baseline, the holoenzyme was eluted with P50 buffer containing 0.2 M KCl. The core polymerase was eluted with P50 buffer containing 0.5 M KCl. As the final step in the purification of the holoenzyme, a modification of the method of Hager et al. (1990) was used involving chromatography with Q Sepharose FF. The holoenzyme was dialyzed against TGED (10 mM Tris-HCl (pH 7.9), 0.1 mM DTT, 5% glycerol) containing 0.25 M NaCl. The column (10 ml bed volume) was equilibrated with approximately 50 ml of TGED containing 0.3 M NaCl. After the sample was loaded onto the column, it was washed with TGED containing 0.3 M NaCl until the absorbance at 280 nm returned to baseline. The column was then eluted with a gradient (1000 ml total volume) from 0.30 M to 0.45 M NaCl in TGED. Trace amounts of the core polymerase eluted first, followed by the holoenzyme. The fractions containing the holoenzyme were pooled, concentrated, dialyzed against storage buffer (10 mM Tris-HCl (pH 7.9), 0.1 mM DTT, 50% glycerol, 0.1 M NaCl) and stored at −10°C.

Characterization of holoenzyme

Protein samples were analyzed by performing electrophoresis on sodium dodecyl sulfate/8.75% polyacrylamide gels as described by Laemmli (1970). Analyses of the Coomassie blue stained gels by using ImageQuant indicated in all cases that the holoenzyme was at least 90% saturated with σ. The procedure of Oen et al. (1979), as modified by Solaiman & Wu (1984) was used to estimate the number of active enzyme molecules present in the samples. In these analyses, the A1 promoter (5 nM) was titrated with RNA polymerase (0-150 nM). The rate of abortive product synthesis (pApU) was determined as a function of protein concentration. The reaction mixtures (30 µl) contained 40 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 50 mM KCl, 1 mM DTT, 2 mM AMP, 7.8 µM [γ⁻³²P]ATP at a specific radioactivity of 360 cpm/pmol, 5 nM A1 promoter and the appropriate amounts of RNA polymerase. After a 30 minute incubation at 37°C, each reaction mixture was terminated by the addition of 10 µl of EDTA. The mixtures were then spotted on Whatman 3 MM paper in 5-6 µl aliquots with interdrying and were resolved with ascending chromatography in WASP solvent (water/saturated (NH₄)₂SO₄ (pH 8.0)/2-propanol, 18:80:2 (by vol.), containing 5 mM EDTA). The chromatograms were dried at room temperature, then developed into strips and analyzed for radioactivity by Cerenkov counting. The Rₛ value of the product (pApU) was approximately 0.30. The rate of product synthesis increased linearly with increasing protein concentration up to the point of saturation. The rate of product synthesis plateaued sharply and remained essentially constant following subsequent addition of protein. The point of intersection of these two straight lines gives the protein concentration required to saturate the A1 promoter that is present, and allows one to calculate the percentage of active enzyme molecules present. In the studies conducted herein, the percentage of active enzyme molecules was approximately 39%.

Isolation of plasmids and DNA fragments

Plasmid pAR1707 was generously provided by Drs W. Studier and A. H. Rosenberg (Biology Department, Brookhaven National Laboratory, Upton, NY). This plasmid contains the A1 promoter as well as the Tₐ termination site from bacteriophage T7 cloned into the BamHI site of pBR322. Bacteria (DH5α containing either pAR1707 or pBR322) were grown in LB medium (1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl) containing 40 µg/ml ampicillin according to standard protocol (Maniatis et al., 1982). Plasmids were isolated by using Qiagen columns according to the protocol in the manufacturer’s manual. After restriction enzyme digestion of the plasmids with the appropriate restriction enzymes, the fragments were separated on native 5% polyacrylamide gels. The electrophoresis buffer was TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). The DNA fragments of interest were then isolated by electroelution from the gel. This was followed by concentration with sec-butanol, phenol extraction and then precipitation with ethanol. The precipitated fragment was then resuspended in Hepes buffer (10 mM Hepes, 0.05 M KCl, 10 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.1 mM DTT at pH 8.0). Treatment of pAR1707 with Smal and RsaI yielded a 529 base-pair fragment containing the A1 promoter along with the Tₐ termination site; whereas, treatment of pBR322 with RsaI and PshA1 yielded a 548 base-pair fragment lacking a promoter site.

Radioactive labeling of DNA fragments

The Smal-RsaI fragment from pAR1707 and the RsaI-PshA1 fragment from pBR322, respectively, were dephosphorylated by using CIP as described by Maniatis et al. (1982). Labeling with ³²P was performed by mixing the dephosphorylated DNA fragment with [γ⁻³²P]ATP in the presence of T4 polynucleotide kinase as described by Maniatis et al. (1982). Unincorporated [γ⁻³²P]ATP was removed by electrophoresis on a native 5% polyacrylamide gel. TBE was the electrophoresis buffer. The labeled DNA fragment was then isolated by electrophoresis from the gel. This was followed by concentration with sec-butanol, phenol-extraction and then precipitation with ethanol. The precipitated fragment was then resuspended in Hepes buffer.

Steady-state fluorescence studies

All fluorescence studies were conducted by using a Spex Model 1681 spectrophotometer that was interfaced to a Spex DM3000 computer. Measurements were made in the steady state, by using front-face illumination (Eisinger & Flores, 1979). Excitation and emission bandwidths were 1.8 and 4.5 nm, respectively. With front-
face illumination, a linear dependence of fluorescence intensity on protein concentration was observed for RNA polymerase concentrations up to at least 2000 nM. Therefore, no corrections were required for inner filter effects. For fluorescence emission spectra, data were recorded every 1 nm with an integration time of one second. The excitation wavelength was either 280 or 295 nm. Each spectrum was corrected for blank emission and wavelength-dependent effects. All studies were conducted at 25 °C.

In the fluorimetric titration of RNA polymerase by the respective DNA fragments, 11 measurements were made at 340 and 500 nm, respectively, at each concentration of DNA fragment. (An integration time of two seconds was used in recording each data point.) These measurements were then averaged, and the differences between the measurements at 340 and 500 nm were determined and corrected for dilution.

Gel-mobility shift titrations

All binding studies were conducted in Hepes buffer. The reaction solutions (15 μl) were incubated at 37 °C for ten minutes. Next 5 μl of tracking dye (two parts 50% glycerol and one part 0.1% bromophenol blue) was added, and the entire sample was loaded onto a native 5% polyacrylamide gel. (Gels were prerun at 100 V for at least 90 minutes prior to loading.) A total of 1000 counts was added to each lane, and the samples were subjected to electrophoresis at 100 V. The electrophoresis buffer was TBE. After electrophoresis, the gels were dried and then subjected to autoradiography by using Fuji XR film. The autoradiographs were scanned and then analyzed by using ImageQuant.

Stopped-flow measurements

Rapid kinetic measurements were performed by using an Applied Photophysics SF17MV stopped-flow spectrometer. The excitation wavelength was 280 nm and the slit width was 5 mm. Light emitted from the sample was monitored after passing through a 324 nm cut-off filter (Oriel). A series of stopped-flow experiments were performed in Hepes buffer (10 mM Hepes, 0.05 M KCl, 10 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.1 mM DTT at pH 8.0) at different temperatures. In each experiment 400 data points were recorded, and the sets of data from four to ten traces at identical conditions were averaged. Each averaged set of stopped-flow data was then fitted to either single or multiple exponentials according to the equation \( F = \sum A_n \exp(-k_{obs,t}) + C \) where \( F \) is the fluorescence at time \( t \), \( n \) is the number of exponential terms, \( A_n \) and \( k_{obs,t} \) are the amplitude and the observed rate constants of the \( n \)th term, respectively, and \( C \) is the fluorescence intensity at \( t = 0 \). As is apparent by the lack of a perturbation over the time span of 50 seconds at 37°C, the protein does not undergo photo-bleaching during the time-course over which the interaction of RNA polymerase with the A1 promoter is monitored.

Abortive initiation assay

The procedure of McClure (1980) was used to analyze the kinetics in the formation of the abortive product. Reaction mixtures contained 10 mM Hepes (pH 8.0), 0.05 M KCl, 10 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.1 mM DTT, RNA polymerase (6.25 nM), A1 promoter (3.2 nM), [γ-32P]UTP (50 μM) at a specific radioactivity of 360 cpm/pmol and various amounts of ATP. Reactions were initiated by the addition of protein, A1 promoter, ATP or [γ-32P]UTP, respectively. Prior to the initiation of the reactions, the solutions were preincubated at 37°C for ten minutes. Aliquots (30 μl) were removed at timed intervals after initiation, the reaction was stopped by the addition of EDTA and the samples were placed on ice. The mixtures were spotted on Whatman 3 MM paper in 5-6 μl aliquots with interim drying and resolved with ascending chromatography in WASP (solvent water/saturated (NH₄)₂SO₄ (pH 8.0)/2-propanol, 18:80:2 (by vol.), containing 5 mM EDTA). The chromatograms were dried at room temperature, cut into strips and analysed for radioactivity by Cerenkov counting. The \( R^* \) value of the product (pppApU) was approximately 0.44.

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