The +2 NTP Binding Drives Open Complex Formation in T7 RNA Polymerase*

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Transcription initiation as catalyzed by T7 RNA polymerase consists primarily of promoter binding, strand separation, nucleotide binding, and synthesis of the first phosphodiester bond. The promoter strand separation process occurs at a very fast rate, but promoter opening is incomplete in the absence of the initiating NTPs. In this paper, we investigate how initiating NTPs affect the kinetics and thermodynamics of open complex formation. Transient state kinetic studies show that the open complex, $E_{oc}$, is formed via an intermediate $E_{oc}$, and the conversion of $E_{oc}$ to $E_{oc}$ occurs with an unfavorable equilibrium constant. In the presence of the initiating NTP that base-pairs with the template at position +2, the process of open complex formation is nearly complete. Our studies reveal that the nucleotide that drives open complex formation needs to be a triphosphate and to be correctly base-paired with the template. These results indicate that the melted template DNA in the open complex, $E_{oc}$, is required to both make the complex heparin-resistant and open promoter. Therefore, these steps can potentially modulate the $K_a$ of the initiating NTP. Kinetic studies of T7 RNAP have shown that the observed rate of promoter opening is fast ($>100$ s$^{-1}$) in the absence of initiating NTP (6–8). However, both kinetic and structural studies suggest that the equilibrium constant of the promoter opening step is unfavorable. Fluorescence studies indicate that open complex formation is incomplete even at RNAP and DNA concentrations much above the $K_a$ of the T7 RNAP-DNA complex (8). Similarly, much of the −4 to −1 TATA region of the T7 dsDNA promoter that melts in the open complex cannot be modified by KMMnO$_4$, unless initiating nucleotides are present (9–11).

Incomplete conversion of closed to open complex is also observed in the $rrn$ P1 promoters of E. coli RNAP. A closed complex between E. coli RNAP with $rrn$ P1 promoter is formed readily, but it is not stoichiometrically converted to the open complex in the absence of initiating nucleotides (1, 12, 13). The presence of ATP and CTP, the initiating nucleotides, is required to both make the complex heparin-resistant and open the −10 dsDNA region (1, 12, 13). The instability of the open complex may be the reason for this weaker $K_a$ of the initiating NTP in $rrnB$ P1 as well as T7 promoters (1, 5).

This study was undertaken to investigate the effect of initiating NTPs on the promoter opening steps, which in turn provides information on how open complex formation steps play a role in regulating initiating NTP binding. We use transient state kinetic methods to dissect the pathway of initiation and to identify the steps that are affected by the initiating NTP. The steps of DNA binding and promoter opening were monitored in real time using 2-AP-modified promoter DNAs. Such methods have been used previously to probe the promoter opening steps in T7 RNAP (2, 6–8) and in E. coli RNAP (14). The kinetic data were globally fit to derive the transcription affinity for initiating NTP, and thus rRNA synthesis is controlled by the initiating NTP concentration that varies with the cellular growth rate (1). The bacteriophage T7 RNAP is similar in that it also has a relatively weak affinity for initiating NTPs; hence, transcription is regulated by the initiating NTP concentration (2–4). It is interesting that RNAP-promoter complexes with the same initiation start sequence but different promoter core sequences respond differently to initiating NTP concentrations, because they show different affinities for initiating NTPs (3, 5). In these promoters, the template sequence at the initiation site is the same, and thus the interactions of the incoming NTP with the template and RNAP are expected to be the same; thus, it is not obvious how the core sequence regulates the affinity of initiating NTP.

Both $rrn$ P1 and T7 promoters show a common feature, and that is an unfavorable equilibrium constant for closed to open complex formation in the absence of initiating NTP. The observed $K_a$ of the initiating NTP is dependent on the efficiency of steps preceding NTP binding, such as promoter binding and opening; hence, these steps can potentially modulate the $K_a$ of the initiating NTP. Kinetic studies of T7 RNAP have shown that the observed rate of promoter opening is fast ($>100$ s$^{-1}$) in the absence of initiating NTP (6–8). However, both kinetic and structural studies suggest that the equilibrium constant of the promoter opening step is unfavorable. Fluorescence studies indicate that open complex formation is incomplete even at RNAP and DNA concentrations much above the $K_a$ of the T7 RNAP-DNA complex (8). Similarly, much of the −4 to −1 TATA region of the T7 dsDNA promoter that melts in the open complex cannot be modified by KMMnO$_4$, unless initiating nucleotides are present (9–11).

The efficiency of transcription is regulated to produce the required amount of RNA in the cell, and this can occur in numerous ways. Transcriptional efficiency is intrinsically regulated by the promoter DNA sequence during initiation through complex protein-DNA interactions. In addition, several extrinsic factors such as accessory proteins or ligands that bind to the RNAP−DNA complex regulate transcription by allosteric mechanisms. The efficiency of transcription is also regulated by the concentration of substrate NTPs when the cellular NTP concentration is close to the $K_a$ of NTPs. This is found in Escherichia coli RNAP−rRNA promoter complexes, such as the $rrnB$ P1 promoters, that have relatively weak affinity for initiating NTP, and thus rRNA synthesis is controlled by the initiating NTP concentration that varies with the cellular growth rate (1). The bacteriophage T7 RNAP is similar in that it also has a relatively weak affinity for initiating NTPs; hence, transcription is regulated by the initiating NTP concentration (2–4). It is interesting that RNAP-promoter complexes with the same initiation start sequence but different promoter core sequences respond differently to initiating NTP concentrations, because they show different affinities for initiating NTPs (3, 5). In these promoters, the template sequence at the initiation site is the same, and thus the interactions of the incoming NTP with the template and RNAP are expected to be the same; thus, it is not obvious how the core sequence regulates the affinity of initiating NTP.

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§ The abbreviations used are: RNAP, RNA polymerase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; 2-AP, 2-aminopurine; p-dsDNA, partially double-stranded DNA.
initiation pathway including the steps of promoter strand separation and initiating NTP binding. The results indicate that the promoter strand separation and initiating NTP binding steps are linked. We find that the binding of +2 NTP drives open complex formation, which in turn makes the +1 position available for +1 NTP binding, resulting in cooperative +1 and +2 NTP binding. The mechanism derived from these studies provides a better understanding of the transcription initiation pathway and provides insights into how open complex formation steps can regulate the affinity of the initiating nucleotides.

**EXPERIMENTAL PROCEDURES**

*Synthetic DNA and Other Materials—* The oligodeoxynucleotides (unmodified and 2-AP-modified) were custom-synthesized by Integrated DNA Technologies (Corvalle, IA). The oligodeoxynucleotides were purified on a 16% polyacrylamide gel containing 3 M urea. The ssDNA of desired length was excised and electroeluted, and the ssDNA was ethanol-prepared, reconstituted in water, and stored at −20 °C. The purity of ssDNA was >95%, and its concentration was determined from absorption and calculated molar extinction coefficients at 260 nm for each DNA. Complementary ssDNAs were titrated against each other to confirm both duplex formation and the absence of free ssDNA as reported previously (14). The 3′-dGTP was purchased from TriLink Biotechnologies (San Diego, CA).

*Protein—* T7 RNAP was overexpressed in *E. coli* strain BL21 (16). The enzyme was purified as previously described (2, 4, 7) with the exception that the CM-Sepharose separation step was eliminated. The purified enzyme was checked for the lack of DNA exonuclease activity and was stored at −80 °C in 20 mM sodium phosphate, pH 7.7, 1 mM trisodium EDTA, 1 mM dithiothreitol, 100 mM sodium chloride, and 50% (v/v) glycerol. The enzyme stock was diluted for each experiment, resulting in final glycerol concentrations ranging from 0.02 to 1%. The enzyme concentration was calculated from its absorbance at 280 nm and molar extinction coefficient of 1.4 × 10⁴ M⁻¹ cm⁻¹ (17). All of the experiments were carried out at 25 °C in buffer A (50 mM Tris acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol), unless stated otherwise.

**Equilibrium Fluorescence Measurements—** Experiments measuring the fraction of open complex formation were performed as follows. Using a 200-μl quartz cuvette, the fluorescence of T7 RNAP (4 μM) mixed with 1 μM dsDNA or p-dsDNA promoter containing a single 2-AP, either at (t−4) or (t−2), was measured first and then again upon the addition of 50 μM nucleotide. The sample was excited with 315-nm light (2-nm bandwidth), and the resulting fluorescence intensity at 370 nm (2 nm bandwidth) was measured on a FluoroMax-2 spectrofluorometer (Jobin Yvon-Spex Instruments S.A., Inc.) using the DataMax software program. After volume correction, the fluorescence of free T7 RNAP was corrected by carrying out a control experiment with nonfluorescent promoter DNA. The corrected fluorescence was F_{corr}(i) = F_{obs}(i) − F_{corr}(i), where F_{corr}(i) and F_{obs}(i) are the fluorescence intensities of samples containing 2-AP-modified promoter and nonfluorescent (unmodified) promoter DNA, respectively. Under these conditions, the RNAP fluorescence contributes 28% to the total signal. The signal produced from the 2-AP is therefore easily detected over this background.

The equilibrium RNAP titration binding experiment was carried out at 25 °C in a 3-m1 quartz cuvette. The titration was performed by adding small aliquots of T7 RNAP to a GGGG−dGTP-dsDNA (t−4) (0.1 μM) (Fig. 1) in the presence of 3′-dGTP (500 μM). The sample was excited with 315-nm light (3-nm bandwidth), and the resulting fluorescence intensity at 370 nm (3-nm bandwidth) was measured. The measured fluorescence intensity value was corrected for inner filter using Equation 1,

$$F_{corr} = F_{obs} \times 10^{[\log(1/Ab_{corr} + Ab_{corr})]}$$

(Eq. 1)

where \(F_{corr}\) represents the corrected fluorescence intensity, \(F_{obs}\) is the observed fluorescence intensity, \(Ab_{corr}\) is the absorbance of the solution at point i in the titration at 315 nm, and \(Ab_{corr}\) is the absorbance of the same solution at 313 nm. The concentrations of total RNAP and DNA at each point in the titration were determined from Equations 2 and 3,

$$[D] = \frac{v_{i}}{v_{i}} [D]_{i}$$

(Eq. 2)

$$[E] = \frac{e_{i}}{v_{i}} [E]_{i}$$

(Eq. 3)

where \([E]\) and \([D]\) are the concentrations of T7 RNAP and DNA, respectively, up to point i in the titration, \([E]_{i}\) and \([D]_{i}\) are the initial concentrations, \(v_{i}\) is the volume of the solution up to point i in the titration, and \(v_{0}\) is the initial volume.

The corrected fluorescence was plotted as a function of total T7 RNAP concentration, and the data were simultaneously fit to Equation 4 and the quadratic equation (Equation 5) to obtain the RNAP \(K_{d}\) value.

$$F_{corr} = C + [D] \times f_{D} + [E] \times f_{E} + ED \times (f_{D} - f_{D} - f_{E})$$

(Eq. 4)

where C is a constant, \(f_{D}\) and \(f_{E}\) are the fluorescence coefficients for 2-AP DNA (D), T7 RNAP (E), and T7 RNAP-DNA complex (ED), respectively. The ED is defined by the quadratic Equation 5.

$$ED = K_{d} + [E] + [D] - 1/2 \cdot K_{d} \cdot [E] + [D] \cdot [E] - 4 \times [E] \cdot [D]$$

(Eq. 5)

Selected flow Kinetics—The stopped-flow experiments were carried out using a SF-2001 instrument spectrophotometer equipped with a photomultiplier detection system from KinTek Corp. (Austin, TX). T7 RNAP (in buffer A) (with or without GTP or 3′-dGTP) was placed in one syringe, and the (t−4) 2-AP-containing (GGGG−dGTP-dsDNA promoter in buffer A was placed in the second syringe. At 25 °C, the two solutions (40 μl each from syringe) were rapidly mixed (flow rate of 6.0 ml s⁻¹), and the 2-C2A was excited with a wavelength of 315 nm. The progress of the reaction was monitored by measuring the intensity of the fluorescence emission using a cut-on filter >360 nm (WG360; Hi-Tech Scientific, serial no. 273129). Multiple traces (3–10 traces) were averaged to optimize the signal. The KinTek stopped-flow kinetic software was used to fit the stopped-flow kinetic traces to Equation 6 describing single or multiple exponential changes,

$$F = \sum A_{i} \times \exp(-k_{obs} \times t) + C$$

(Eq. 6)

where \(k_{obs}\) is the rate constant of the intermediate species (Eq. 7)

$$k_{obs} = k_{max} \times [E] / K_{d} + [E] + C$$

(Eq. 7)

where \(k_{obs}\) is the maximum rate of promoter opening, \(K_{d}\) is the concentration of \(E\) (T7 RNAP) where the rate is \(k_{max}/2\), and \(C\) is the y intercept.

The decrease in the observed rate (\(k_{obs}\)) as a function of nucleotide concentration was fit to a decreasing hyperbola, Equation 8 (19).

$$k_{obs} = k_{i} + k_{i} \times K_{d} / [GTP]$$

(Eq. 8)

where \(k_{i}\) and \(K_{d}\) are approximately equal to rate constants \(k_{i}\) and \(K_{d}\) in the reaction shown in Table I, and \(K_{d}^{-}\) is approximately equal to the intrinsic \(K_{d}\) of GTP or 3′-dGTP.

The \(K_{d}\) of +1 GTP was measured by preincubating T7 RNAP with (GACG−dGTP−dGTP) and ATP in one syringe and mixing this solution with increasing concentrations of GTP from a second syringe in a stopped flow and measuring the decrease in 2-AP fluorescence over time. The observed rate was plotted as a function of GTP concentration and was fit to a hyperbola. Equation 7, where \(k_{max}\) represents a conformational change upon GTP binding, \(K_{d}\) is the \(K_{d}\) of GTP in the presence of 200 μM ATP, and \(C\) is the y intercept.

Global Fitting of the Kinetic Data—The stopped-flow kinetic data (a total of 29 time courses) from three sets of experiments (Figs. 4, a and c, and 5a) were globally fit by the nonlinear least-squares method using the software, Inc. (Natick, MA). The overall equilibrium constant of DNA binding (\(K_{d}^{-}\)) in the presence of 500 μM 3′-dGTP (18 mM) was used as a constraint in the fitting process. Differential equations were written for each of the kinetic species in the mechanism (Table I), and the observed fluorescence \(F\) was related to the fluorescence values \(F_{obs}\) of the intermediate species (ED) and background fluorescence \(F_{bg}\) as shown in Equation 9.
An assumption was made that the fluorescence of ED, complex was the same as free 2-AP-labelled dsDNA. The differential equations (ODE) were solved using the MATLAB built-in ODE solver ode15s. Nonlinear least squares global fitting routine was carried out, using randomly chosen starting parameters, at least 33 times, to ensure that the solution represents the global minimum. The fitting converged to one set of parameters that provided the best global fit as judged by the sum of squared residuals. The correlation matrix for the fitted parameters (Table 1) was calculated from the Jacobian at the fitting solution. The 95% confidence intervals for each parameter were calculated by 257 rounds of the Monte Carlo method (20). Each of the rounds involved the generation of a new set of pseudoeperimental data by adding noise normalized by weights to the data predicted by the model. Each set of pseudoeperimental data was fit as described above to produce a new set of optimal parameters. The confidence intervals were obtained by analysis of multiple sets of parameters. The use of MATLAB for analysis of enzyme kinetics data is discussed in Ref. 21. The MATLAB code is available from S. S. P.

RESULTS AND DISCUSSION

Effect of the Initiating Nucleotides on Promoter Melting—Several studies, including crystal structural studies of the T7 RNAP-DNA complex, indicate that the promoter region from −4 to −1 is melted during preinitiation (open complex formation) (22–24). The process of promoter melting can be studied at equilibrium or followed in real time by monitoring the fluorescence of a 2-AP base incorporated in the −4 to −1 TATA region of T7 promoters (6–8). When the −4 to −1 region melts, the adenines both unpair and unstack from the double helix, and since 2-AP fluorescence is sensitive to base-stacking interactions, the fluorescence of 2-AP complexes as the promoter melts. The adenine base in position t(−4) experiences a large structural change upon open complex formation relative to other adenines in the TATA box (8). Thus, adenine at t(−4) is an ideal base to substitute with 2-AP to study the kinetics and thermodynamics of promoter opening by fluorescence.

Kinetic (8) and structural studies (9–11) of promoter opening indicate that the dsDNA promoter is not fully open in the absence of initiating nucleotide. We have proposed based on kinetic studies that both closed (ED$_c$) and open (ED$_o$) complexes exist in the absence of initiating nucleotide. One can estimate the amount of open complex in a reaction by measuring the fluorescence of 2-AP in dsDNA versus p-dsDNA promoter (Fig. 1) in complex with T7 RNAP. The p-dsDNA has a single-stranded melting and coding region. When this “open” DNA binds to T7 RNAP, the fluorescence of 2-AP increases, largely because the t(−4) base unstacks during open complex formation. The fluorescence of 2-AP in these open DNA complexes with T7 RNAP approximates the expected fluorescence of the melted dsDNA promoter in an open complex. Thus, the measurement of the fluorescence of dsDNA relative to p-dsDNA in complex with T7 RNAP provides an estimate of the fraction of open complex (ED$_o$) in the reaction.

The fluorescence of 2-AP incorporated in dsDNA or p-dsDNA increases relative to free DNA when the DNAs are bound to T7 RNAP (8). As shown in Fig. 2a, the increase in fluorescence of 2-AP in a T7 RNAP-dsDNA complex is 25–27% of the fluorescence of the RNAP-p-dsDNA complex, irrespective of the position of the 2-AP in the TATA box. The less than 100% increase in fluorescence of 2-AP in the T7 RNAP-(GGG)40-bp dsDNA promoter complex relative to the p-dsDNA complex is not due to free 2-AP DNA or T7 RNAP, because the concentrations of reacting species are 8.5 times the DNA K_d value of the (GGG)40-bp dsDNA (470 nM) (15, 25). We propose that the smaller fluorescence increase of the dsDNA complex is due to an incomplete conversion of the closed complex ED$_c$ to the open complex, ED$_o$ in the (GGG)40-bp promoter. We explored the possibility that the partial opening may be due to nonspecifically bound T7 RNAP at the noncoding region of the dsDNA. We used a t(−4) (GGG)26-bp dsDNA promoter, which contains only 5 base pairs of coding region, and observed the same results as with the (GGG)40-bp promoter (data not shown). We have also measured the binding of T7 RNAP to a predefined random sequence 20-mer DNA and found the K_d to be >100 μM. When the initiating nucleotide GTP was added to the complex of (GGG)40-bp dsDNA and T7 RNAP, a further increase in fluorescence was observed. In the presence of GTP, the fluorescence of (GGG)40-bp dsDNA reached nearly the same level as the p-dsDNA in complex. We found that the fluorescence of p-dsDNA, which is an analog of open complex, did not increase upon the addition of GTP but in fact decreased, which we believe is due to G-ladder synthesis as shown below.

We propose that the fluorescence increase upon the addition of GTP is mostly due to the shift in the population of closed complex to the open complex. Thus, GTP binding (or phosphodiester bond formation between two GTPs) stabilizes the initiation complex and drives the conversion of ED$_c$ to ED$_o$. We wished to address several questions as follows. Is it the +1 GTP, or the +2 GTP binding, or both, that drive the opening of promoter DNA? Is this effect specific to the initiating NTP? Is RNA synthesis required for stabilization of the open complex?

To investigate whether the above phenomenon was specific to the initiating nucleotide, GTP, other nucleotides were examined. The addition of GMP had no effect on the fluorescence of (GGG)40-bp dsDNA in complex with T7 RNAP (Fig. 2a). Note that GMP can initiate RNA synthesis when added with GTP (26), but it is not known whether GMP can bind to T7 RNAP-dsDNA complex in the absence of +2 NTP. The absence of an effect with GMP indicates that either GMP binding does not drive open complex formation or GMP alone binds poorly. Thus, the triphosphate moiety in GTP is important for binding and stabilizing the open complex. Similarly, ATP had no effect on the fluorescence of (GGG)40-bp dsDNA in complex with T7 RNAP, indicating that correct base-pairing interactions are necessary.

To determine whether RNA synthesis is required for stabi-
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Effect of nucleotides on the extent of promoter opening. a, the fluorescence intensity (λ_{	ext{ex}}=315 \text{ nm}; λ_{	ext{em}}=370 \text{ nm}) of 1 μM (GGG)40-bp dsDNA or (GGG)17/40 p-dsDNA modified with 2-AP at t(−4) or t(−2) as indicated) was measured at 25 °C after the addition of 4 μM T7 RNAP and either GTP, GMP, ATP, GpG, or 3′-dGTP (500 μM each). The fraction of open complex was calculated from the ratio of fluorescence of dsDNA complex to that of p-dsDNA complex in the presence or absence of nucleotide. The corrected fluorescence intensity was calculated as described under “Experimental Procedures.” b, the corrected fluorescence intensity of the (GGG)40-bp promoter (1 μM) modified with 2-AP at t(−4) was determined after the addition of T7 RNAP (4 μM [black bars]) or T7 RNAP (4 μM) plus GTP (500 μM) [gray bars] in buffer A containing the indicated [Mg(II)].

FIG. 2. Effect of nucleotides on the extent of promoter opening. a, the fluorescence intensity (λ_{	ext{ex}}=315 \text{ nm}; λ_{	ext{em}}=370 \text{ nm}) of 1 μM (GGG)40-bp dsDNA or (GGG)17/40 p-dsDNA modified with 2-AP at t(−4) (or t(−2) as indicated) was measured at 25 °C after the addition of 4 μM T7 RNAP and either GTP, GMP, ATP, GpG, or 3′-dGTP (500 μM each). The fraction of open complex was calculated from the ratio of fluorescence of dsDNA complex to that of p-dsDNA complex in the presence or absence of nucleotide. The corrected fluorescence intensity was calculated as described under “Experimental Procedures.” b, the corrected fluorescence intensity of the (GGG)40-bp promoter (1 μM) modified with 2-AP at t(−4) was determined after the addition of T7 RNAP (4 μM [black bars]) or T7 RNAP (4 μM) plus GTP (500 μM) [gray bars] in buffer A containing the indicated [Mg(II)].

To dissect the elementary steps of DNA binding and promoter opening, we used the stopped-flow technique to measure the kinetics of promoter opening. The stopped-flow traces were computer-fit to equation 6, and the observed rate increases in a hyperbolic manner with increasing [T7 RNAP] with or without 3′-dGTP, indicating that DNA binding and promoter opening occurs by a minimal two-step mechanism outlined below.
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**Mechanism of transcription initiation: Kinetic and equilibrium parameters from global fitting**

| Parameters \(^a\) | 95% confidence intervals | Correlation matrix |
|-----------------|--------------------------|-------------------|
| \(k_1 (\mu M^{-1} s^{-1})\) | 255 | -32, +24 | 1 |
| \(k_2 (s^{-1})\) \(^b\) | 9.4 | -1.5, +1.4 | -0.5 | 1 |
| \(k_3 (s^{-1})\) | 15.8 | -1.4, +1.6 | -0.37 | 0.93 | 1 |
| \(K_{dGTP}(+d) (\mu M)\) | 69 | -12, +5 | -0.13 | -0.14 | -0.26 |

\(^a\) Determined using three types of experiments, a total of 29 time courses shown in Figs. 4, a and c, and 5a.

\(^b\) Fitting was constrained by fixing \(k_4 = k_5 \cdot K_{dGTP} \cdot (1 + k_2/k_3 \cdot (1 + [3'-dGTP]/K_{dGTP}(+d)))\), where DNA binding \(K_{dGTP} = 0.018 \pm 0.002 \mu M\) at [3'-dGTP] = 500 \(\mu M\) measured directly using fluorescent equilibrium titrations (Fig. 6).

Extract the intrinsic rate constants is to globally fit the kinetics of DNA binding to a model. Global fitting was performed (described in detail below), and the solid lines going through the kinetic data in Fig. 4, a and c (residuals shown in Fig. 4, b and d), are fits to Reaction 2 (see below) with intrinsic constants listed in Table I.

The Observed Rate of Open Complex Formation Depends on GTP Concentration—To understand why the observed rate of promoter opening in the presence of 3'-dGTP (30 s\(^{-1}\)) is lower than the rate in the absence of nucleotide (180 s\(^{-1}\)), we measured the stopped-flow kinetics of open complex formation at increasing concentrations of GTP or 3'-dGTP. T7 RNAP plus an increasing concentration of GTP or 3'-dGTP was mixed with 2-AP-modified (GGG)-40-bp dsDNA. The time-dependent increase in fluorescence of DNA at various 3'-dGTP concentrations is shown in Fig. 5a. We found that the observed exponential rate decreased with increasing [3'-dGTP] or [GTP] (Fig. 5c), and the corresponding fluorescence amplitude increase (Fig. 5d).

The decrease in the observed rates with increasing GTP or 3'-dGTP concentration is consistent with the mechanism shown in Reaction 2. In this mechanism, the DNA binding steps precede the GTP binding step. If ED, formation is a fast step relative to ED, formation and GTP binding is a rapid equilibrium step, the rate dependences can be fit to Equation 8 (19) to obtain approximate values of GTP or 3'-dGTP \(K_{dGTP} = 26 \mu M\) for GTP and 111 \(\mu M\) for 3'-dGTP. The observed \(K_{dGTP}\) is lower than 3'-dGTP \(K_{dGTP}\) most likely because the ED_G intermediate reacts to give additional species resulting from RNA synthesis, and the observed \(K_{dGTP}\) is a function of the equilibrium constant of all of the subsequent species. In addition, it is also likely that the \(K_{dGTP}\) value of 3'-dGTP is higher because it lacks the 3'-OH group. The [GTP] or [3'-dGTP] dependence also provided estimates of the intrinsic rate constant \(k_3\) or the promoter opening rate (between 16 and 19 s\(^{-1}\)) and a lower estimate of \(k_4\) or the promoter closing rate (between 70 and 80 s\(^{-1}\)). The derived values indicate that the rate of promoter closing is at least ~5 times faster than the rate of promoter opening. Thus, the equilibrium constant between closed and open forms is <1, in agreement with the conclusions from the fluorescence equilibrium binding studies shown in Figs. 2 and 7.

**FIG. 4. Stopped-flow kinetics of open complex formation with and without 3'-dGTP.** a and c show, respectively, the stopped-flow kinetic traces in the absence and in the presence of 3'-dGTP (500 \(\mu M\)) measured using a constant amount of the (GGG)-40-bp dsDNA (0.15 \(\mu M\)) and increasing amount of T7 RNAP (dots). The solid lines are the global fit to Reaction 2 and intrinsic rate constants shown in Table I. b and d show, respectively, the residuals obtained from the simulated versus the experimental data for a and c. e, exponential rates of the time courses in a and c in the absence (filled circles) or in the presence of 3'-dGTP (open circles) are plotted against [T7 RNAP]. The dependences were fit to Equation 7 with a T7 RNAP \(K_i = 1.0 \pm 0.4 \mu M, k_{max} = 183 \pm 14 \text{ s}^{-1}, \text{ and } C\) close to 0 (without 3'-dGTP) and T7 RNAP \(K_i = 1.0 \pm 0.3 \mu M, k_{max} = 32 \pm 2 \text{ s}^{-1}, \text{ and } C\) of 16.3 \(\pm 2.5 \text{ s}^{-1}\) (with 3'-dGTP).

\[
E + D \xrightarrow{k_1} ED \xrightarrow{k_2} ED_G
\]

(Reaction 1)

where \(E\) represents T7 RNAP, and \(D\) is the dsDNA promoter that forms an initial complex that we propose is the closed complex \(ED_k\) (with intrinsic rate constants, \(k_1\) and \(k_2\)). The closed complex undergoes a conformational change to form the open complex \(ED_c\) (with intrinsic rate constants, \(k_3\) and \(k_4\)). We assume that the formation of \(ED_c\) is a rapid equilibrium step, then the rate constants of the hyperbolic fit (Equation 7) can be interpreted (18). With a rapid equilibrium assumption, the maximum observed rate of promoter opening in the absence of 3'-dGTP, \(k_{max} = k_3 + k_4\), is 180 s\(^{-1}\); the concentration of T7 RNAP required to reach the half-maximal rate, \(K_{i} = k_4/k_2\), is 1 \(\mu M\) and \(C\) is \(k_2\), which the fit indicated was close to 0. In the presence of 500 \(\mu M\) 3'-dGTP, the \(k_{max}\) is about 6-fold slower (30 s\(^{-1}\)) with no change in the T7 RNAP \(K_{i} = 1 \mu M\). If \(ED_c\) formation is not a rapid equilibrium step, then the meaning of \(k_{max}\) and \(K_{i}\) is complicated, because the observed rate at any given concentration of \(E\) and \(D\) is dependent on all four intrinsic rate constants (18). In this case, the only way to
The fluorescence amplitudes obtained from fitting increased as a function of [GTP]-dGTP and were fit to a hyperbola with an observed $K_D$ of 588 µM. This $K_D$ of [GTP]-dGTP is about 5 times weaker than the $K_D$ obtained from the rate dependence in Fig. 5c. If [GTP]-dGTP binds to the $E_D$ species, which due to the unfavorable equilibrium constant of the $E_D$ to $E_D$ step, is present in substoichiometric amounts, then the observed $K_D$ of [GTP]-dGTP is the intrinsic $K_D$ of 3'-dGTP estimated to be 110 µM from the rate dependence, and $K_D$ is the equilibrium constant of $E_D$ to $E_D$, conversion estimated to be 0.2 (16 s⁻¹) and 0.8 s⁻¹. Thus, the observed $K_D$ of [GTP]-dGTP is 110 (1 + 1/2) = 660 µM, which is close to the experimentally measured $K_D$ of 588 µM from amplitude dependence. Thus, the kinetics of DNA binding in the presence of initiating GTP are consistent with the mechanism shown in Reaction 2. Note that the above treatment provides only estimates of intrinsic rate constants. To obtain accurate values of the intrinsic rate constants, the data were globally fit to Reaction 2 by numerical methods, as described below.

**Global Fitting of the Kinetic Data**—The goal of globally fitting the data is to test the model and derive a set of intrinsic parameters that satisfactorily explains all of the data. Previous work (8) examining T7 RNAP-promoter binding provided evidence for an intermediate before open complex formation and suggested that the closed to open complex formation step was unfavorable. In that work, it was assumed that the formation of the intermediate, the closed complex, occurred by a rapid equilibrium step. In order to account for these observations, promoter opening was proposed to occur via a three-step mechanism whereby the T7 RNAP and promoter DNA first formed a closed complex, which formed two open complex species sequentially. The RNAP-promoter binding data were globally fit to this three-step mechanism and yielded an intrinsic equilibrium constant of 0.13 and overall equilibrium constant of 0.8 µM for the promoter opening step.

The current studies of promoter opening in the presence of initiating nucleotide provide additional details in the process of promoter opening, enabling the refinement of the previous model. Here three sets of kinetic data (Figs. 4, a and c, and 5a), representing 29 time courses, including the data that were fit previously (8), were globally fit to the solution of the differential equations describing Reaction 2. This is a minimal mechanism that satisfactorily describes the given experimental data sets. In contrast to the previous fittings, promoter binding was not assumed to be a rapid equilibrium. The fitting was constrained using the observed $K_D$ of the RNAP-(GGG)40-bp dsDNA complex formed in the presence of 3'-dGTP (18 ± 2 nM) obtained from fluorescence equilibrium titration (Fig. 6). Global fitting of the kinetic data was performed using MATLAB software to obtain a consistent set of intrinsic rate and equilibrium constants. The set of intrinsic rate constants that fit the described data sets is shown in Table I, and simulated lines that describe the data are shown as solid lines in Figs. 4, 5a and c, and 5a with the corresponding residuals in Figs. 4, b and d, and 5b. These parameters are considered to be more accurately determined than those previously reported, since the model in Reaction 2 was subject to more rigorous global fitting constraints.

Several interesting features are revealed from the intrinsic parameters obtained by global fitting. The derived rate constants reveal that T7 RNAP binds the dsDNA promoter to form a closed complex $E_D$ with a bimolecular rate constant of 255 $M^{-1} s^{-1}$, which is close to diffusion-limited. The $E_D$ isomerizes to free RNAP and DNA at a relatively slow rate of 9.4 s⁻¹. The $E_D$ isomerizes to $E_D$ with a rate constant of 15.8 s⁻¹. The $E_D$ species is not stable and reverses back to $E_D$ with a faster rate constant close to 125 s⁻¹. Thus, $E_D$ to $E_D$ conver-
concentration of GTP in a stopped-flow instrument at 25°C. The dependence of fluorescence intensity was calculated as described under “Experimental Procedures.” The S.E. value in data acquisition was less than 2%.

Fig. 7. Effect of nucleotides on the extent of promoter opening in the GAC promoter. The fluorescence intensity (λexcitation 315 nm; λemission 370 nm) of 1 μM (GAC)40-bp dsDNA (gray bars) or (GAC)17/40 p-dsDNA (black bars) modified with 2-AP at t (−4) was measured after the addition of T7 RNAP (4 μM) or T7 RNAP in the presence of GTP, ATP, ATP + GTP, 3’-dGTP, or GMP (500 μM each). The corrected fluorescence intensity was calculated as described under “Experimental Procedures.”

Fig. 8. Kinetics of GTP binding to the +1 site of a GAC promoter. T7 RNAP (1.35 μM) was preincubated with (GAC)40-bp dsDNA (t = −2) (0.45 μM) and ATP (200 μM) and rapidly mixed with increasing concentrations of GTP in a stopped-flow instrument at 25°C. The time-dependent decrease in 2-AP fluorescence was measured at λ ≥ 360 nm upon excitation at 315 nm. For each experiment, 4–7 traces were collected and averaged. The dependences were fit to Equation 7 with a T7 RNAP Kd of 422 ± 184 μM, kmax of 26 ± 3 s⁻¹, and C of 14 ± 3 s⁻¹. The observed amplitude versus [GTP] fit to Equation 7 with an apparent Kd of GTP equal to 364 ± 167 μM.

Fig. 9. Free energy profile of the pathway of transcription initiation. The free energies of activation were calculated from the intrinsic rate constants shown in Table I using the relationship 

\[ \Delta G = \Delta G^0/R + RT \ln(\text{products/substrates}), \]  

assuming that +1 GTP binds with a Kd of 422 μM. The activation energies shown for these steps are arbitrary. The schematic diagram shows the hypothetical structures of the intermediate species in the pathway of initiation.

The Binding of +2 NTP Drives Open Complex Formation—The consensus promoter used in the above studies starts with the sequence -1GGG, where both +1 and +2 template positions base-pair with GTP; hence, we cannot determine using the (GGG)40-bp promoter which GTP binding step, the +1 or +2, drives open complex formation. We made a DNA starting with the coding sequence -1GG (Fig. 1) to investigate whether +1, +2, or both initiating NTPs play a role in stabilizing the open complex. In the GAC promoter, the +1-position base-pairs with G, the +2 base-pairs with A, and +3 base-pairs with C; thus, we can study the effects of GTP, ATP, or CTP binding on open complex formation.

Experiments similar to those in Fig. 2 were carried out with the GAC promoters. The fluorescence of 2-AP at t (−4) in both dsDNA and p-dsDNA was measured in an equilibrium experiment under various conditions (Fig. 7). Similar to the GGG promoter, the fluorescence of T7 RNAP-(GAC)40-bp dsDNA complex in the absence of initiating NTPs is about 30% of the fluorescence of (GAC)40-bp dsDNA complex. Interestingly, when GTP or 3’-dGTP or GMP that base-pairs with the +1-position was added to the (GAC)40-bp complex, no change in fluorescence was observed. On the other hand, when ATP that base-pairs with the +2-position was added, the fluorescence of dsDNA complex increased and reached the same level as that of the p-dsDNA complex. The addition of CTP alone that base-pairs with +3 had no effect (data not shown). These results indicate that the NTP that base-pairs with the template at the +2-position drives open complex formation. When ATP was
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,... added with GTP, conditions under which phosphodiester bond formation occurs, no additional increase of fluorescence over the level with ATP alone was observed. These results indicate that ATP binding is sufficient and that RNA synthesis is not necessary for ED<sub>1</sub> to ED<sub>2</sub> conversion. In addition, the t(−4) base unstacking reaction is complete in the absence of RNA synthesis. These results are somewhat different from the reported KMnO<sub>4</sub> modification experiments (10, 30), where the reactivity of −3 and −1 Ts in the template strand was found to increase significantly only when RNA synthesis occurred. A possible explanation is that the reactivity of KMnO<sub>4</sub> is influenced by the accessibility of MnO<sub>4</sub><sup>−</sup> to the DNA bound to RNAP (31), and −3 and −1 Ts become reactive to KMnO<sub>4</sub> only when a 4- or 5-mer RNA is made.

The kinetics of open complex formation were measured with the (GAC)-40-bp dsDNA promoter, both in the absence and in the presence of ATP, the +2 nucleotide. Essentially the same rate dependences as shown in Figs. 4 and 5 were observed with the GAC promoter in the presence of ATP (data not shown). The observed rate of DNA binding decreased with increasing [ATP], and the fluorescence amplitude increased with increasing ATP. The hyperbolic fits of rate versus [ATP] provided a K<sub>d</sub> of 30 μM, and amplitude versus [ATP] provided a K<sub>d</sub> around 220 μM. These data also indicate that the intrinsic equilibrium constant of the promoter opening step is close to 0.16, similar to the equilibrium constant of 0.11 for the (GGG)-40-bp promoter (Table I). We also attempted to directly measure the K<sub>d</sub> of +1 GTP using the (GAC)-40-bp promoter. In the absence of ATP, no detectable fluorescence change was observed even at high millimolar concentrations of GTP. However, when a solution of T7 RNAP, (GAC)-40-bp dsDNA, and ATP (200 μM) was mixed with GTP (see scheme in Fig. 8), a small decrease in fluorescence was observed. Both the rate and amplitude increased with increasing [GTP] as shown in Fig. 8. The rate dependence provided a GTP K<sub>d</sub> of 422 ± 183 μM, which is an estimate of the GTP K<sub>d</sub> in the presence of 200 μM ATP. Thus, the K<sub>d</sub> of +1 NTP is 10 times weaker than the K<sub>d</sub> of +2 NTP.

Pathway of Transcription Initiation and Initiating NTP Binding—The kinetic and equilibrium binding studies described above provide a detailed pathway of initiation in T7 RNAP. The kinetics of DNA binding in the presence of initiating NTP provide direct evidence for the unfavorable equilibrium constant of the promoter opening step. It is clear from the free energy diagram (Fig. 9) shown at 1 μM, 1 mM, and 1 mM DNA and initiating NTP concentrations that the binding energy of the initiating nucleotide is required to stabilize the preinitiation open complex in T7 RNAP. Our results show that phosphodiester bond formation is not necessary and that simply high concentrations of initiating nucleotide (>K<sub>d</sub> of +2 NTP) provides sufficient energy to drive the ED<sub>2</sub> to ED<sub>1</sub> conversion. The results reported here also show that the initiating NTP that base-pairs with the template at the +2-position is responsible for stabilizing the open complex. These results indicate that the template DNA in the preinitiation complex is positioned in the active site to bind the +2 NTP and that the +1 NTP binding step is not required for +2 NTP binding.

It has been postulated that the stacking interactions of the −1 T in the template with Trp<sup>422</sup> and the +1 base play a role in positioning the melted template strand within the T7 RNAP active site for correct initiation at the +1-position (24, 32, 33). It also appears that the interactions of T7 RNAP with the upstream promoter binding region are important in the correct positioning of the template strand (29). The binding of the incoming NTP is directed both by the positioning of the −1 base and possible interactions with H<sup>31</sup> (34). The studies reported in this paper indicate that +2 NTP binds before +1 NTP and that +2 NTP binding plays a role in start site selection by directing the binding of +1 NTP at the T7 RNAP active site. The affinity of +1 NTP is about 10 times higher than that of the +1 NTP, most likely because the +2 NTP and the Mg(II) ion bound to the β-γ phosphate of +2 NTP interacts extensively with the amino acids at the active site of the RNAP-DNA complex. The tighter interactions of +2 Mg-NTP complex can therefore lock the template DNA in place to facilitate +1 NTP binding.

How Does the Promoter Opening Step Modulate the K<sub>d</sub> of the Initiating NTP?—Although the intrinsic K<sub>d</sub> of the +2 NTP (in this case 3′-dGTP) is 50 μM, the observed K<sub>d</sub> is close to 10 times weaker. This is because the initiating NTP binds to the ED<sub>0</sub> complex, which is present in substoichiometric level. The observed K<sub>d</sub> of the initiating NTP is a function of both its intrinsic K<sub>d</sub> and the equilibrium constant of ED<sub>1</sub> to ED<sub>2</sub> conversion, which, being unfavorable, limits the binding of initiating NTP. These observations reveal an interesting mechanism by which promoter bases upstream from the start site (bases in the promoter specificity region) can influence the apparent K<sub>d</sub> values of the initiating NTPs. A promoter can have the same sequence at the start site, but the apparent K<sub>d</sub> of the initiating NTPs can be different, depending on the equilibrium constant of the promoter opening step. For example, T7 promoters Φ3.8 and Φ10 both have the same initiation sequence, 5′GGGAGA, but the Φ3.8 promoter with nonconsensus base pairs at positions −2, −11, −12, and −13 has a weaker affinity for the initiating GTPs (29). The nonconsensus base pairs alter the protein-DNA interactions decreasing the intrinsic equilibrium constant of the promoter opening step, which in turn affects the K<sub>d</sub> of the initiating NTPs. The same reason may explain why E. coli rRNAP P1 promoters have a high K<sub>m</sub> for the initiating NTP and why base changes in the promoter core region affect the K<sub>m</sub> of the initiating NTP (5). Thus, base changes upstream from the start site can modulate initiating NTP binding and make transcription respond to cellular NTP pools. In general, any accessory factor that binds to the RNAP-DNA complex, such as transcription factors that change the conformation of the RNAP or the DNA, can regulate the intrinsic parameters of DNA binding and promoter opening steps, which in turn can regulate the efficiency of transcription.

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