Equilibrium and Stopped-flow Kinetic Studies of Interaction between T7 RNA Polymerase and Its Promoters Measured by Protein and 2-Aminopurine Fluorescence Changes*

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The mechanism of bacteriophage T7 RNA polymerase binding to its promoter DNA was investigated using stopped-flow and equilibrium methods. To measure the kinetics of protein-DNA interactions in real time, changes in tryptophan fluorescence in the polymerase and 2-aminopurine (2-AP) fluorescence in the promoter DNA upon binary complex formation were used as probes. The protein fluorescence changes measured conformational changes in the polymerase whereas the fluorescence changes of 2-AP base, substituted in place of dA in the initiation region (−4 to +4), measured structural changes in the promoter DNA, such as DNA melting. The kinetic studies, carried out in the absence of the initiating nucleotide, are consistent with a two-step DNA binding mechanism,

\[
K_1 \quad k_2 \\
E + D \rightleftharpoons ED_a \rightleftharpoons ED_b \\
\]

where the RNA polymerase forms an initial weak ED_a complex rapidly with an equilibrium association constant \(K_1\). The ED_a complex then undergoes a conformational change to ED_b, wherein RNA polymerase is specifically and tightly bound to the promoter DNA. Both the polymerase and the promoter DNA may undergo structural changes during this isomerization step. The isomerization of ED_a to ED_b is a fast step relative to the rate of transcription initiation and its rate does not limit transcription initiation. To understand how T7 RNA polymerase modulates its transcriptional efficiency at various promoters at the level of DNA binding, comparative studies with two natural T7 promoters, Φ10 and Φ3.8, were conducted. The results indicate that kinetics, the bimolecular rate constant of DNA binding, \(k_{on}(K_{b2})\), and the dissociation rate constant, \(k_{off}(k_{-2})\), and thermodynamics, the equilibrium constants of the two steps (\(K_1\) and \(K_{b2}/k_{-2}\)) both play a role in modulating the transcriptional efficiency at the level of DNA binding. Thus, the 2-fold lower \(k_{on}\) the 4-fold higher \(k_{off}\) and the 2–5-fold weaker equilibrium interactions together make Φ3.8 a weaker promoter relative to Φ10.

Bacteriophage T7 RNA polymerase is a 98-kDa single subunit polymerase that catalyzes synthesis of RNA complemen-

tary in sequence to the template DNA (1). The phage enzymes are among the simplest RNA polymerases known, as no accessory proteins are necessary for specific initiation, elongation, or termination of transcription (2, 3). The 17 promoters of bacteriophage T7 direct specific initiation of RNA synthesis that occurs in a rapid and processive manner (4, 5). Due to their simplicity these enzymes serve as model systems to understand, in depth, the mechanisms of transcription initiation, elongation, or termination.

Initiation of transcription occurs by recognition and binding of the RNA polymerase to a promoter DNA sequence. This event is recognized as one of the important steps at which transcription and gene expression is regulated. The 17 bacteriophage T7 promoters share consensus sequence from −17 to +6 position relative to the transcription start site at +1 (6). The class III gene promoters of T7 are absolutely conserved in DNA sequence, whereas the class II gene promoters differ at a number of positions within the consensus sequence. The sequence of the promoter DNA is a primary factor that determines the strength of the promoter and the efficiency of initiation. However, the relationship between promoter DNA sequence and transcriptional efficiency is not well understood at the mechanistic level. In general, the detailed kinetics and thermodynamics of transcription are less well understood, in part, due to its complexity. The T7 promoters and the phage RNA polymerase, owing to their simplicity, should serve as a good starting model to understand the mechanism and regulation of transcription in greater detail.

The present study consists of the kinetic and thermodynamic investigations of the steps involved in promoter recognition and DNA binding. The interaction of the RNA polymerase with its promoter DNAs has not been examined previously using fast kinetic methods. Since DNA binding is a fast step, it is necessary to use rapid kinetic methods to directly observe intermediate binary species that accumulate transiently during initiation. The stopped-flow methods used in this study allow us to elucidate the mechanism of DNA binding and to determine the rate and equilibrium constants of steps leading to intermediate species. To examine DNA binding in real time we use the change in the intrinsic fluorescence of protein upon promoter binding as a signal. In addition, we have taken advantage of the fluorescent properties of 2-aminopurine (2-AP) base, an analog of dA, which can be incorporated into the promoter DNAs and used as a probe to monitor promoter opening in real time. The present studies have been carried out in the absence of the NTP substrate. Comparative studies with two natural T7 promoters, Φ10 and Φ3.8, representing strong class III and weak class II promoters, respectively, provide insights into the mechanism by which T7 RNA polymerase

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1 The abbreviations used are: 2-AP, 2-aminopurine; ds, double-stranded.

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regulates its transcriptional efficiency at the level of DNA binding.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—T7 RNA polymerase was purified from the *Escherichia coli* BL21pAR1219 cell line (kindly provided by Alan Rosenberg and Bill Studier, Brookhaven National Laboratories) (2). The enzyme was >95% pure after three chromatography columns consisting of SP-Sephadex, CM-Sephadex, and DEAE-Sephadex purchased from Sigma (7). The polymerase was stored in 50% glycerol, and buffer containing 20 mM sodium phosphate (pH 7.7), 1 mM trisodium EDTA, 1 mM dithiothreitol, 100 mM NaCl at −80°C. The concentration of the polymerase was determined by absorbance measurement at 280 nm and from its molar extinction coefficient of 1.4 × 10^5 M^-1 cm^-1 (8).

**Synthesis of DNAs: Normal and 2-AP DNA**—The DNA promoters were synthesized on a Millipore Nucleic Acid synthesis system 899. DMT-deoxynucleoside (benzoyl or isobutyryl) β-cyanoethylphosphoramidites were purchased from PerSeptive Biosystems. 2-Aminopurine phosphoramidites were purchased from Glen Research Corp. Ac-dC-CE phosphoramidite was used for dC incorporation in 2-AP DNA. Coupling time of 15 min for 2-AP base incorporation and ultrafast cleavage and deprotection systems were used for the synthesis of 2-AP containing DNAs (deprotection was performed with a mixture of equal volumes of ammonium hydroxide and 40% methylamine aqueous solution, following the procedure provided by Glen Research). 2-AP base was incorporated at position 3, 5, 6, 11–13, and 4 for Φ10 and Φ3.8 nontemplate strands, position −4 and −2 for Φ10 template strand, and position −4 for the Φ3.8 template strand (Fig. 1).

All synthetic promoters used in this study were purified on 16% polyacrylamide, 5% urea gels. The DNA was visualized by UV shadowing, and electrosed from the gel using an Elutrap apparatus (Schleicher & Schuell). The concentration of purified DNA was determined by absorbance measurement at 260 nm using the following extinction coefficients (mM^-1 cm^-1) for the bases: dA, 15,200; dC, 7050; dG, 12,010; dT, 8400. The extinction coefficient of 2-AP, at 260 nm, equal to 1000 mM^-1 cm^-1 was used in the calculation of 2-AP DNA concentration (9). The double-stranded (ds) DNAs were prepared by annealing the individual single-stranded DNA strands. The exact ratio of the two single-stranded DNA strands to prepare the dsDNAs was determined routinely from titration experiments performed on an 18% native polyacrylamide gel that resolves dsDNA from the single-stranded DNAs.

**Fluorescence Titrations**—The fluorimetric titration experiments were performed on a Perkin Elmer LS50 Luminescence Spectrometer. The emission spectra of 2-AP containing DNAs were obtained by excitation at 348 nm (Oriel Corp., WG360, Hi-Tech Scientific, serial number 275129), after excitation at 315 nm (1–5 nm slit width). About 5–10 kinetic traces for 2-AP DNAs and 20–30 for normal DNAs were routinely averaged for each experiment.

**Stopped-flow Studies**—The stopped-flow instrument from KinTek Corp. (State College, PA) was used to measure the DNA binding kinetics. Equal volumes of protein and DNA in buffer (50 mM Tris acetic acid, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol) from separate syringes were rapidly mixed in the stopped-flow instrument at 25°C. Changes in fluorescence emission of the protein were measured using a cut-on long pass filter >348 nm (Oriel Corp., catalog number 51260) after excitation at 290 nm (1-mm slits). 2-AP fluorescence emission was measured using a cut-on filter >360 nm (WG360, Hi-Tech Scientific, serial number 275129), after excitation at 315 nm (1–5 nm slit width). About 5–10 kinetic traces for 2-AP DNAs and 20–30 for normal DNAs were routinely averaged for each experiment.

**Data Analysis**—The equilibrium binding and kinetic data were fit using SigmaPlot (Jandel Scientific) or KaleidaGraph (Abelbeck) softwares. Stopped-flow kinetic traces were fit using the KinTek stopped-flow kinetic program software to single or sum of exponential as in the equation, \( F = \sum A_n \times \exp(-k_{obs}t) + C \), where \( F \) is the fluorescence at time \( t \), \( n \) is the number of exponential terms, \( A_n \), and \( k_{obs} \) are the amplitude and the observed rate constant of the \( n \)th term, respectively, and \( C \) is the fluorescence intensity at \( t = 0 \). The error bars for \( k_{obs} \) values shown in the Figs. 5 and 7 represent errors in the fit. The errors shown for the rate constants represents the mean of deviation of the reported rate constants from the values computed from the maximum and minimum \( k_{obs} \) values.

**RESULTS**

Studies in the literature (10–13), mainly using *E. coli* RNA polymerase, have shown that binding of the RNA polymerase to the promoter DNA is a multistep process, involving formation of several closed and open polymerase-DNA binary complexes. We studied the equilibrium and kinetic interactions between T7 RNA polymerase and T7 promoters with the goal of dissecting the steps involved in the process of promoter recognition during transcription initiation. The kinetics of DNA binding were measured using stopped-flow methods. The decrease in intrinsic protein fluorescence that results upon promoter-polymerase binary complex formation was used as the probe for kinetic measurements. To investigate the kinetics of promoter opening, 2-AP containing promoter DNAs were prepared. 2-AP is a fluorescent base analog of da that base pairs with dT. The fluorescence of 2-AP is sensitive to local changes that result from melting of dsDNA (9, 14, 15). This is evident from the fluorescence spectra in Fig. 2, a and b, which shows the 2–4-fold higher fluorescence of 2-AP in the single-stranded DNA form versus the dsDNA form. Both DNA binding and DNA melting during transcription initiation should be observable in real time by following the change in fluorescence of 2-AP DNA promoters.

**T7 Promoter DNAs**—The consensus sequence recognized by T7 RNA polymerase consists of bases in the region −17 to +6 relative to the transcription start site at +1 (16–18). We have synthesized promoter DNAs, 40-base pairs in length, containing the natural Φ10 and Φ3.8 sequences from positions −21 to +19 (Fig. 1). The sequence of Φ10, a class III promoter, is absolutely conserved whereas the sequence of Φ3.8, a class II promoter, differs at several positions (−2, −11, −12, and −13) from the consensus sequence. We compare here the equilibrium and kinetic interactions of these two promoters to better understand how transcriptional efficiency is regulated at the DNA binding steps during initiation.

Fluorescent promoter DNAs were chemically synthesized by incorporating 2-AP bases in place of da bases in both the template and the nontemplate DNA strands in the region between −4 and +4 (Fig. 1). We chose to incorporate the 2-AP bases in this region because the region −6 to +2 has been shown (19, 20) to be in the single-stranded form in the open binary complex. The fluorescence of 2-AP bases at those positions should be sensitive to changes in DNA structure, such as DNA melting that occurs during initiation. Changing the da bases to 2-AP at those positions does not affect transcription initiation. The steady state and pre-steady state kinetics of 2-mer to 19-mer RNA product formation were the same with the 2-AP-modified versus the unmodified promoters (data not shown). We have also synthesized partially dsDNA promoters that contain single-stranded template region from −5 onward as mimics of open promoter DNAs (Fig. 1).

**Equilibrium Binding of T7 RNA Polymerase to 2-AP-modified Promoter DNAs**—Binding of T7 RNA polymerase to 2-AP modified promoter DNAs resulted in about 6–7-fold enhancement in 2-AP DNA fluorescence, as shown in Fig. 2, a and b. This increase in fluorescence is greater than the expected change from simply melting the duplex DNA. Therefore, at least a part of the increase in fluorescence appears to be due to the interaction of the DNA with the protein active site. The increase in 2-AP DNA fluorescence change upon binary com-
plex formation was used to measure the equilibrium dissociation constant ($K_d$) of Φ10 and Φ3.8 promoters. The fluorometric titrations were carried out at constant [DNA] and increasing [polymerase] at 25 °C. The 2-AP DNA was excited at 315 nm (to minimize protein absorption) and emission was measured at 370 nm. The final equilibrium binding isotherms were obtained by subtracting the fluorescence of protein. The binding isotherms were fit to a hyperbola (Fig. 3, a and b) to calculate the apparent $K_d$ values. The measured $K_d$ values for Φ10 and Φ3.8 were 0.015 (±0.002) and 0.035 (±0.009) μM, respectively. The Φ10 promoter DNA therefore binds to the polymerase more strongly than the Φ3.8 promoter.

**Stopped-flow Kinetics of RNA Polymerase Binding to Unmodified Promoter DNAs**—The kinetic mechanism of RNA polymerase binding to DNA was investigated using the stopped-flow method. The binding of Φ10 and Φ3.8 promoter DNAs to the polymerase leads to quenching of protein tryptophan fluorescence. This intrinsic change in protein fluorescence was used to measure the kinetics of DNA binding, under conditions of excess polymerase over DNA as well as excess DNA over polymerase. Except for an extra kinetic phase under excess DNA conditions, the kinetics under the two conditions were comparable. Fig. 4 shows representative kinetic traces under excess [DNA] conditions, where 0.3 μM Φ10 DNA (Fig. 4a) or Φ3.8 DNA (Fig. 4b) was mixed with 0.05 μM polymerase. The kinetics fit best to two exponentials. To elucidate the mechanism of DNA binding and determine the bimolecular rate constant of DNA binding, the kinetics were measured as a function of increasing [DNA]. As shown in Fig. 5, a and b, the observed rate constant ($k_{obs}$) of the fast phase increased linearly with increasing [DNA]. If DNA binding occurred with a simple one-step mechanism:

$$E + D \rightleftharpoons ED$$

Reaction 1

then, $k_{obs} = k_{on}[S] + k_{off}$, where $S$ is DNA or polymerase, $E$.

The slope of $k_{obs}$ versus [DNA] provided the bimolecular rate constant, $k_{on}$, and the intercept provided the dissociation rate constant, $k_{off}$ (21). DNA binding is most likely a multistep process. Therefore, the derived $k_{on}$ and $k_{off}$ are macroscopic rate constants that describe the overall kinetics of DNA binding and dissociation and do not necessarily represent the intrinsic rate constants. The stopped-flow kinetic results show that Φ10 dsDNA promoter binds to the polymerase with a $k_{on}$ of 72 ± 7 μM$^{-1}$ s$^{-1}$ (slope), and dissociates from the binary complex with a $k_{off}$ of 4.0 ± 0.3 s$^{-1}$ (intercept). The Φ3.8 dsDNA promoter binds to the polymerase with about 2-fold slower $k_{on}$ (42 ± 4 μM$^{-1}$ s$^{-1}$) and also dissociates faster from the complex with a 4-fold higher $k_{off}$ (17.7 ± 1.0 s$^{-1}$). Control experiments with a dsDNA containing a non-promoter DNA sequence (Fig. 1), that is a DNA with random sequence, showed no measurable fluorescence changes confirming that the above rate constants measure specific interactions of the polymerase with the respective promoter sequences.

The presence of a second slow phase in the DNA binding kinetics (Fig. 4) suggested a second step in the DNA binding
mechanism (21). Curiously, this slow phase was observed only when [DNA] was in excess of the polymerase. In addition, the observed rate constant of the second phase decreased with increasing [DNA] because of the following relationship between the observed rate constant and [DNA] (22).

\[ k_{obs} = k_1 + \frac{k_{-1} \times K_d}{K_d + [DNA]} \]  
(Eq. 1)

At very low [DNA], the observed rate constant will be close to \( k_1 + k_{-1} \), whereas at very high [DNA], the observed rate will plateau at \( k_2 \). Thus, Reaction 3 predicts that the observed rate constant will decrease from the sum of the rate constants \( k_1 + k_{-1} \) to \( k_2 \) with increasing [DNA]. This is clearly the case with the 3.8 promoter as shown in Fig. 5d. In case of the 30 promoter, the decrease is small (Fig. 5c). This is both because of the tighter \( K_d \) of 30 DNA relative to 3.8, and since the kinetics were not measured at very low [DNA]. The nature of this slow conformational change in the polymerase required for DNA binding is not known. It may represent movement of one of the polymerase domains such as the thumb region that has been postulated to be flexible and involved in DNA binding (23). Judging from the relative amplitudes of the fluorescence changes in the kinetic experiments, we estimate that \( E' \) represents about 30% of the population of RNA polymerase. Thus, approximately 70% of the RNA polymerase is in a conformation that binds DNA with fast kinetics. The rest of the discussion in this paper is concerned with the kinetics of the fast form of the polymerase.

**Stopped-flow Kinetics of the Polymerase Binding to 2-AP-modified Promoter DNAs**—To measure the kinetics of DNA melting, the stopped-flow experiments were repeated with the 2-AP-modified promoter DNAs. The binding of 2-AP promoter DNAs to the polymerase resulted in a time-dependent increase in 2-AP DNA fluorescence (Fig. 6). Similar to the results with the non-fluorescent DNAs, the kinetics of DNA binding at excess [polymerase] were monophasic, whereas the kinetics at excess [DNA] were multiphasic. Fig. 6, a and b, show some of the stopped-flow kinetic traces resulting from 2-AP10 and 2-AP3.8 DNA binding under the conditions of excess [polymerase] (0.3 \( \mu M \)) over the [DNA] (0.05 \( \mu M \)). Similarly, Fig. 6, c and d, show the multiphasic kinetics under conditions of excess [DNA] (0.3 \( \mu M \)) over [polymerase] (0.05 \( \mu M \)). The observed rate constants of the fast phase were comparable under both conditions.

To investigate the steps involved in DNA binding and to measure their rate constants, the stopped-flow kinetics of 2-AP DNA binding were measured at increasing [polymerase]. The kinetics fit best to a single exponential and as shown in Fig. 7, a and b, the observed rate constants increased linearly as a function of [polymerase], analogous to the dependence measured by protein fluorescence change. Both protein and DNA fluorescence changes therefore appear to measure essentially the same process. The \( k_{on} \) and \( k_{off} \) rate constants (Table I) for 2-AP10 are 65 \( \pm \) 8 \( \mu M^{-1} \) s\(^{-1}\) and 3.4 \( \pm \) 0.4 s\(^{-1}\), respectively, and these are also comparable to those obtained from protein fluorescence changes. The \( k_{on} \) (84 \( \pm \) 9 \( \mu M^{-1} \) s\(^{-1}\)) and \( k_{off} \) (8.7 \( \pm \) 0.3 s\(^{-1}\)) rate constants of 2-AP3.8 are about 2-fold different from those of the non-fluorescent 3.8 DNA. The 2-AP modification does not affect the DNA binding kinetics of 30 promoter, but it seems to affect the kinetics of 3.8 promoter to a small extent. No separate phase due to DNA melting was observed. Thus, if the increase in 2-AP fluorescence measures DNA melting, this step is fast and appears to occur concomitantly with DNA binding. The second slow phase observed under excess [DNA] conditions is due to the population of RNA polymerase that binds DNA with slower kinetics, since the observed slow rate constants of the slow phase decreased with [DNA].

Measurement of the kinetic rate constants also provides an estimate of equilibrium constants such as \( K_{d1} \) (\( K_d = k_{off} / k_{on} \))
which provides the strength of the binary complex. Consistent with the $K_d$ values obtained from the equilibrium fluorescence titrations (Table I), the $K_d$ value for the 2-AP modified Φ3.8 DNA was about 2-fold higher relative to the Φ10 promoter. The equilibrium $K_d$ values measured from the fluorimetric titrations are, however, lower than those derived from the kinetic rate constants. The different $K_d$ values from the two methods suggest the presence of an additional step after the bimolecular DNA binding step. This step must be very slow since it was not observed by stopped-flow kinetic measurements. Additional studies need to be carried out to understand the differences in the $K_d$ values from the two methods.

Stopped-flow Kinetics of the Polymerase Binding to Partially dsDNA—To further understand the process of open binary complex formation, the kinetics of polymerase binding to partially dsDNA promoters were investigated. These promoters are double-stranded in the promoter binding region (−21 to −5), but they mimic open promoters because the template DNA in the initiation and coding regions (from −5 onward) is single-stranded. The DNA binding kinetics were measured by following both the protein fluorescence change and the 2-AP fluorescence change in the DNA. As with the fully dsDNA promoters, binding of partially dsDNA to the polymerase resulted in a decrease in protein fluorescence and an increase in 2-AP fluorescence (data not shown). The kinetics of DNA binding under all conditions, excess [polymerase] or excess [DNA], fit to a single exponential. The absence of the second slow phase suggested that both $E$ and $E'$ forms of the polymerase in the proposed mechanism (Reaction 1) were capable of binding the partially dsDNAs. The $k_{on}$ and $k_{off}$ values were derived from...

FIG. 4. Stopped-flow kinetics of RNA polymerase binding to ds Φ10 and Φ3.8 DNAs. The kinetics of DNA binding to the polymerase were measured in a stopped-flow instrument at 25 °C. The kinetic traces show the time-dependent decrease in intrinsic protein fluorescence (excitation at 290 nm and emission >348 nm) after mixing 0.3 μM promoter DNA and 0.05 μM polymerase (final concentrations). Each kinetic trace shown is an average of about 25 measurements. The observed kinetics are biphasic under the conditions of excess DNA over polymerase. The small panels above the plot of the time course display the regression analysis of the curve fitting. a, the fast and slow kinetic phases of ds Φ10 promoter DNA binding to the polymerase fit to first-order rate constants, 27.9 ± 1.8 and 0.27 ± 0.08 s⁻¹. b, the kinetics of ds Φ3.8 promoter binding fit to rate constants of 27.0 ± 1.4 and 1.14 ± 0.11 s⁻¹.

FIG. 5. Φ10 and Φ3.8 dsDNA concentration dependence of the stopped-flow kinetics. The stopped-flow kinetics of DNA binding were measured at constant [polymerase] and increasing [DNA]. The biphasic kinetics (as shown in Fig. 4) were fit to sum of two exponentials. The observed rate constant of the fast phase was plotted versus [DNA] as shown in a for Φ10, and b for Φ3.8 dsDNAs, and fit to a straight line. The linear fit provided the bimolecular rate constant, $k_{on}$ (slope) and the dissociation rate constant, $k_{off}$ (intercept). The $k_{on}$ of Φ10 dsDNA is 72 ± 7 μM⁻¹ s⁻¹ and the $k_{off}$ is 4.0 ± 0.3 s⁻¹. The ratio $k_{on}/k_{off}$ provided the equilibrium $K_d$ of 0.055 ± 0.010 μM. The $k_{on}$ of Φ3.8 dsDNA (b) obtained by similar analysis is 42 ± 4 μM⁻¹ s⁻¹ and the $k_{off}$ is 18 ± 1.0 s⁻¹. The ratio $k_{on}/k_{off}$ provided a $K_d$ of 0.421 ± 0.022 μM. The observed rate constant of the slow phase decreased with [DNA] as shown in c for Φ10 and d for Φ3.8 DNAs, whereas the amplitudes increased with [DNA] (inset). The solid lines were fit to Equation 1 described in the text which provided: $k_1$ = 0.17 s⁻¹, $k_2$ = 46 s⁻¹, and $K_d$ = 0.0007 μM for Φ10; and $k_1$ = 0.14 s⁻¹, $k_2$ = 26 s⁻¹, and $K_d$ = 0.011 μM for Φ3.8 DNA.
the slopes and the intercepts of the linear increases in the observed rate constant versus [DNA] or [polymerase] (Table I).

Interestingly, the partially dsDNAs bind to the polymerase with much higher \( k_{on} \) and dissociate with much lower \( k_{off} \) values relative to the fully dsDNAs. For instance, the partially dsF10 DNA binds with a 5-fold higher \( k_{on} \), and a 30–200-fold lower \( k_{off} \) relative to the fully dsDNA (Table I). Similarly, the partially dsF3.8 DNAs bind with a 5-fold higher \( k_{on} \), but the \( k_{off} \) values are 2–3-fold lower than those of the fully dsDNA.

**DISCUSSION**

We have investigated the equilibrium and kinetic interactions between T7 promoters and T7 RNA polymerase to dissect the steps in the mechanism of DNA binding during initiation of transcription. The polymerase-DNA binary complex formation was quantitated by following both the decrease in protein tryptophan fluorescence and the increase in 2-AP DNA fluorescence. Since the fluorescence of 2-AP dsDNA increases upon DNA melting, this change was used to probe the kinetics of promoter opening. The 2-AP base was introduced in place of dA bases in the region shown to be in the open form during initiation (19, 20). Replacement of dA bases with 2-AP in this region did not affect transcription initiation, since the measured steady state and pre-steady state kinetics of 2-mer to 19-mer RNA formation with 2-AP-modified DNAs as templates were comparable to those with the unmodified DNAs.

We compare here the mechanism of polymerase binding to two natural T7 promoters: F10, a strong class III promoter, and F3.8, a weaker class II promoter that has base changes at positions 22, 211, 212, 213 from the consensus sequence. The base changes in class II promoters affect their efficiency of transcription, however, the regulatory mechanisms are unclear. There are several ways by which transcriptional efficiency can be regulated. A promoter may have weaker equilibrium (\( K_d \)) or unfavorable kinetic interactions with the polymerase, that is, low \( k_{on} \) and high \( k_{off} \) values. Thus, both kinetic and thermodynamic interactions can play a role in dictating the efficiency of transcription. Efficiency of transcription is also controlled by steps following initial DNA binding, such as open complex formation, binding of initiating nucleotides, phosphodiester bond formation, and processivity of RNA synthesis.

The equilibrium interactions of F10 and F3.8 promoters with the polymerase were measured using fluorimetric titrations. The increase in fluorescence of 2-AP-modified promoters upon binding to the polymerase was used to determine the apparent \( K_d \) values. The derived \( K_d \) values indicate that T7 RNA polymerase does discriminate against F3.8 promoter at the DNA binding step. F3.8 promoter interaction with the polymerase was at least 2-fold weaker relative to the strong F10 promoter. Investigation of the stopped-flow kinetics of DNA binding showed that the weaker interactions of the F3.8 DNA were due to slower \( k_{on} \), the bimolecular rate constant of DNA binding, as well as faster \( k_{off} \), the dissociation rate con-
stant. The $K_d$ values calculated from the ratio of $k_{off}/k_{on}$ for the various $\Phi$3.8 promoters are also consistently weaker relative to the $\Phi$10 promoters (see Table I). The kinetics of protein-DNA interactions (the $k_{on}$ and $k_{off}$ values) must play a significant role in promoter discrimination in vivo. The slow $k_{on}$ and faster $k_{off}$ translates into unfavorable kinetic interactions that could decrease promoter utilization. The 2-fold lower $k_{on}$ and the 2-fold higher $k_{off}$ together make $\Phi$3.8 a weaker promoter relative to $\Phi$10. Exactly which base change in $\Phi$3.8 is responsible for the weaker binding cannot be determined from this study. Most likely candidates are bases at positions −11, −12, and −13 because base changes at these positions occur in T7 promoters at lower frequencies (6–18%) than changes at, say, position −2 (30%) (6).

The initial steps of DNA binding, including closed and open binary complex formation, have been studied to a large extent with $E.\ coli$ RNA polymerase (13). Techniques such as DNA footprinting and nitrocellulose membrane binding have been used to measure the kinetics of DNA binding. Similarly, sensitivity of open single-stranded DNA to KMnO$_4$ (24, 25), and nitrocellulose filter binding (26) and polyacrylamide gel-retardation assay with heparin chase (27, 28) have been used to probe open complex formation steps. All of these methods are manual and cannot measure transient complexes formed with rapid kinetics. The stopped-flow method, especially with promoter DNAs containing the fluorescent base 2-AP, is ideal for measuring the kinetics of DNA binding as well as the kinetics of promoter opening in real time. The fluorescence of 2-AP is sensitive to changes in the state of the DNA. Thus, stopped-flow kinetic studies with promoter DNAs containing 2-AP bases placed in the initiation region allow measurement of local conformational changes, such as DNA melting, that occur during initiation. Additionally, these experiments provide both kinetic and equilibrium information necessary to elucidate the detailed mechanism of DNA binding during transcription.

To dissect the various steps in the mechanism of promoter binding, the kinetics of DNA binding were measured by following both the changes in the fluorescence of protein and the 2-AP DNA. These experiments indicated that both protein and DNA fluorescence changes measured the same bimolecular process of DNA binding to the protein. No distinct kinetic phase due to promoter opening was observed with the fluorescent 2-AP DNAs. Stopped-flow study of both protein and DNA fluorescence changes provided the $k_{on}$ and $k_{off}$ rate constants that describe the overall rate of binary complex formation and dissociation. Comparison of the kinetic constants for different promoters shows that the strength of binary complex is determined by both the $k_{on}$ and the $k_{off}$ values (Table I). Partially dsDNA promoters bind to the polymerase with the highest affinity. The strong binding of partially dsDNAs is due to both a higher $k_{on}$ (5–10-fold) and a lower $k_{off}$ (2–200 fold) compared to the fully dsDNAs. Furthermore, it is also seen, that the ds $\Phi$10 promoter binds to the polymerase strongly, and with a higher $k_{on}$ (about 2-fold) and a lower $k_{off}$ (about 2-fold) relative to the weaker ds $\Phi$3.8 promoter. The lower $k_{off}$ values indicate that the binary complex is kinetically stable. Thus, the partially dsDNAs form the most kinetically stable binary complexes whereas the $\Phi$3.8 dsDNAs the least.

The lower than diffusion limited $k_{on}$ and the differences in $k_{on}$ values between various promoter DNAs suggest that DNA binding occurs by a two-step mechanism,

$$K_d \cong \frac{E + D \rightleftharpoons ED_a}{k_{-2}}$$

**REACTION 3**

The first step in the above mechanism represents the diffusion-controlled binding of the DNA to the polymerase to form $ED_a$, with an equilibrium association constant $K_d$. The interactions of the DNA with polymerase in this complex are weak, and the free polymerase, DNA, and the $ED_a$ species are in rapid equi-

### Table I

Kinetic constants of T7 RNAP binding to T7 promoters

| Promoter   | $k_{on}$ (s$^{-1}$) | $k_{off}$ (s$^{-1}$) | $K_d$ (m$^{-1}$) | $K_d^s$ (m$^{-1}$) |
|------------|---------------------|----------------------|-----------------|-------------------|
| $\Phi$10   | 72 ± 7              | 4.0 ± 0.3            | 0.055 ± 0.010   | 0.015 ± 0.002     |
| 2AP-10     | 65 ± 8              | 3.4 ± 0.4            | 0.055 ± 0.013   | 0.002 ± 0.002     |
| $\Phi$10-17/40 | 364 ± 28            | 0.02 ± 0.6           | 0.000005 ± 0.002 | 0.003 ± 0.006     |
| 2AP-10-17/40 | 329 ± 47            | 0.1 ± 0.7            | 0.000005 ± 0.002 | 0.005 ± 0.009     |
| 2AP-17/40  | 54 ± 2              | 17.7 ± 1.0           | 0.42 ± 0.022    | 0.035 ± 0.009     |
| 2AP-3.8    | 84 ± 12             | 8.7 ± 0.3            | 0.104 ± 0.010   | 0.018 ± 0.0045    |
| 2AP-3.8-17/40 | 312 ± 4             | 5.6 ± 0.6            | 0.018 ± 0.0045  | 0.018 ± 0.001     |

*Equilibrium constant from fluorimetric titrations.*
librium (that is, the rates of binding and dissociation are faster relative to \( k_2 \) and \( k_{-2} \)). Therefore, although there are two steps in the DNA binding mechanism, due to fast formation of \( E_D a \), the stopped-flow kinetics shows only one phase. The second step leading to the formation of \( E_D b \) is a conformational change, wherein the interactions between the polymerase and the DNA are more stable. If we compare the above mechanism to that proposed for DNA binding to \( E. coli \) RNA polymerase (13), the \( E_D \) complex would be analogous to one of the closed binary complexes, and the \( E_D \) species may be analogous to one of the open complexes. Since the stopped-flow kinetics of DNA binding measured by protein or DNA fluorescence changes were identical, the isomerization step may be global. In other words, both polymerase and DNA may change their conformation concomitantly during the isomerization step leading to formation of \( E_D b \).

Studies with partially dsDNA promoters (both 2-AP and unmodified DNAs) provide clues as to the nature of this second step. Since the partially dsDNAs are already open, we did not expect the 2-AP fluorescence to change. However, the 2-AP fluorescence in the partially dsDNAs increased in an analogous manner as in the fully dsDNAs. These results can be explained in two ways. The 2-AP fluorescence increase may be due to a conformational change that occurs in both fully double-stranded and partially dsDNAs such as DNA twisting or DNA bending, distortions which could lead to promoter opening (29). Thus, DNA bending or twisting may represent the second step in mechanism (Reaction 3), and the resulting \( E_D b \) complex in the fully dsDNA promoters may be an intermediate open binary complex. Alternatively, the increase in 2-AP fluorescence may simply be due to the change in the protein conformation which may result in changes in the local environment around the DNA.

According to the proposed mechanism (Reaction 3), the bi-molecular rate constant, \( k_{on} \) listed in Table I is equal to \( K_f k_2 \) and the measured dissociation rate constant, \( k_{off} \) is equal to \( k_{-2} \). The lower-than-diffusion-limited \( k_{on} \) values for the fully dsDNA promoters can result both from a weaker \( E_D a \) complex or a slower conformational change (\( k_2 \)). The \( \Phi 3.8 \) promoter, which has a lower \( k_{on} \), therefore forms a weaker \( E_D a \) complex or undergoes a slower conformational change, or both, relative to the \( \Phi 10 \) promoter. Since the partially dsDNAs are already open, they do not have to go through a “closed” complex, and therefore the \( k_{on} \) values of partially dsDNAs are higher and close to diffusion-limited. The above two-step mechanism of DNA binding predicts that the observed rate constants should saturate at \( k_2 + k_{-2} \) at high DNA or polymerase concentrations. Since no saturation was observed in our measurements up to rate constant of 100 s\(^{-1}\), the second step in the above mechanism must be fast (\( >100 \) s\(^{-1}\)) for both the \( \Phi 10 \) and \( \Phi 3.8 \) promoters. Relative to the rates of transcription initiation, \( E_D a \), the intermediate open binary complex, is formed with fast kinetics that cannot limit transcription initiation. This does not mean that regulation cannot occur at the DNA binding step. Although promoter opening is not rate-limiting, the equilibrium constants of DNA binding and isomerization steps can modulate the efficiency of transcription initiation. For instance, the faster \( k_{on} \) or \( k_{-2} \) of \( \Phi 3.8 \) makes the isomerization of \( E_D a \) to \( E_D b \) to occur with an unfavorable equilibrium relative to the \( \Phi 10 \) promoter. Thus, efficiency of transcription initiation is modulated kinetically by the macroscopic rate constants \( k_{on} \) and \( k_{off} \), and thermodynamically by the equilibrium constants (\( K_f \) and \( k_{2/k_{-2}} \) of the two steps during DNA binding.

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* In T7 RNA polymerase, transcription initiation occurs at rate constants ranging from 1 to 5 s\(^{-1}\) (Y. Jia and S. S. Patel, unpublished results).