Extended Upstream A-T Sequence Increases T7 Promoter Strength*

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Bacteriophage T7 promoters contain a consensus sequence from −17 to +6 relative to the transcription start site, +1. In addition, the strong class III promoters are characterized by an extended AT-rich region upstream of −17, which is often interrupted by one or more GC base pairs in the weaker class II promoters. Herein we studied the role of the AT-rich region upstream of −17 in transcription regulation of T7 RNA polymerase. Equilibrium DNA binding studies with promoter fragments of consensus sequence truncated at various positions between −17 and −27 showed that the polymerase-promoter complex is significantly stabilized as the upstream AT-rich sequence is extended to and beyond −22. Similarly, promoters in which the AT-rich region from −17 to −22 is interrupted by several GC base pairs showed weak binding. Kinetic studies indicated that the presence of extended AT-rich sequence slows the dissociation rate constant of the polymerase-promoter complex and slightly stimulates the association rate constant, thereby increasing the stability of the complex. Measurement of the transcription activity revealed that the extended AT-rich region does not affect the kinetics of abortive synthesis up to the formation of 8-nucleotide RNA but causes accumulation of longer abortive products between 9 and 13 nucleotides. The observed effects of the upstream DNA region were AT-sequence-specific, and the results suggested a larger role for the extended AT-rich sequence that has been unappreciated previously. We propose that the AT-rich DNA sequence upstream of −17 plays a role in modulating the efficiency of transcription initiation by affecting both the affinity of T7 RNA polymerase for the promoter and the efficiency of promoter clearance.

Bacteriophage T7 RNA polymerase (RNAP) and T7 promoters constitute a model system for studying the protein-DNA interactions that occur during transcription as well as to understand the basic catalytic mechanism of DNA transcription. The 99-kDa single subunit enzyme of phage T7 shares many functional characteristics of transcription catalysis with multisubunit RNAPs despite lacking proteins structural similarities. T7 RNAP, without the assistance of accessory proteins, is capable of catalyzing all the fundamental transcription activities. The various crystal structures of T7 RNAP-promoter DNA complex provide deeper insights into the interactions that occur with the promoter as well as conformational changes in T7 RNAP that occur during promoter clearance (1–4). The structure of T7 RNAP bound to a minimal promoter fragment shows that the specific recognition of T7 promoter involves both base-specific and nonspecific contacts with the 13 conserved promoter base pairs from −17 to −5. These include upstream contacts in the major groove of the specificity region of the promoter (−11 to −5) and in the minor groove of the AT-rich region of the promoter (−17 to −13) (1, 2). The base pairs from −4 to −2 that include the initiation site (+1) are melted, and the single-stranded template DNA is plunged into the activity cleft of the T7 RNAP (1).

The well defined class II and class III promoters of phage T7 share a consensus sequence from −17 to +6. Numerous studies have explored the role of promoter regions or specific base pairs within the highly conserved region (−17 to +1) of the T7 RNAP promoter (5–9). In contrast, the role of the DNA region upstream of base pair −17 is less understood, although some experimental evidences suggest the existence of polymerase-DNA contacts in the near region upstream of −17 (10–12). Enzymatic and chemical footprinting assays indicate that the upstream boundary of protection by T7 RNAP may extend up to −22 on the template strand and −17 on the non-template strand (10–12). This protection of the upstream AT-rich region around −20 by T7 RNAP is maintained in the initiation complex and at least up to the synthesis of 7–9-mer RNA (12, 13). The conserved upstream DNA region from −17 to −20 is AT-affuent, and the region from −21 to −27 is homologous in the strong class III promoters (14). In contrast, the sequence of this region is variable among the weaker class II promoters, with one or more GC bp interrupting the AT run (14). By observing the salt-regulated transcriptional activity of T7 RNAP in vitro and the usage of promoters in vivo, McAllister and colleagues (15, 16) found that shortening this AT-rich region from 9 to 4 bp but keeping the integrity within the −13 to −17 region was sufficient to make a class III promoter act more like a weaker class II promoter. Promoter ϕ83.8 is the only class II promoter with uninterrupted AT runs from −13 to −22, and it behaves more like a class III promoter (14, 15). These studies suggest that the AT-rich region upstream of −17 may play a role in the control of promoter selection during T7 transcription in vitro.

To investigate whether the AT-rich region upstream of −17 has an influence on the interactions of the promoter with T7 RNAP, we constructed a series of promoter fragments based on the class III promoter ϕ99 sequence with its upstream end truncated at different positions from −27 to −17 while leaving the consensus sequence downstream of −17 identical. The binding affinity of T7 RNAP for the promoter DNA fragments was measured by three different methods. Equilibrium binding was monitored using fluorescence anisotropy and 2-AP fluorescence, and stopped-flow association and dissociation kinetics were used to obtain the on and off rates for $K_d$ determination. At the same time, the transcription activity of these promoter fragments was investigated by pre-steady-state assays. The results indicated an essential role for the upstream AT-rich region that was further investigated for physiological significance and for sequence-specific effects by studying of a natural...
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class II promoter φ1.1B and a randomly chosen AT/GC-mixed sequence from the −18 to −22 position.

MATERIALS AND METHODS

T7 RNA Polymerase and Buffer—T7 RNAP was purified as described previously (17, 18). The enzyme concentration was calculated from its absorbance at 280 nm in 8 mM urea using the molar extinction coefficient of 1.4 × 10⁵ M⁻¹·cm⁻¹ (19). The reaction buffer 50 mM Tris-acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 2 mM dithiothreitol was used throughout the study.

Oligodeoxynucleotides and Dye Coupling—Oligodeoxynucleotide strands were custom-synthesized by Integrated DNA Technologies (Coralville, IA). 2-AP was directly incorporated into the template strand during oligodeoxynucleotide synthesis. To make dye-labeled oligodeoxynucleotides, a terminal aminomethyl phosphorothioate linker was incorporated at the 5′-end. The oligodeoxynucleotide was labeled with a succinimidyl ester of tetramethylrhodamine carboxylic acid (TAMRA) (Molecular Probes, Portland, OR). The dye-labeling reaction was performed in 100 mM fresh sodium carbonate buffer (pH 8.0) in the dark for 12 h at room temperature by mixing the oligodeoxynucleotide strand with a 10× excess of the dye that was freshly resuspended in dimethyl sulfoxide. The large excess of the unreacted dye was removed by gel-filtration (Bio-gel P-6, Bio-Rad). The labeled strands were then purified using 14–16% polyacrylamide (acrylamide:bis-acrylamide = 19:1)/5 m urea gel electrophoresis (PAGE) in 1× TBE buffer (18 cm wide × 32 cm long, constant voltage (400 V) for 7–8 h). Fluorophore-labeled strand migrated slower as a visibly discrete band from the unlabeled strand that moves faster and has to be visualized by UV shadowing. >95% pure oligodeoxynucleotide was recovered by electroelution followed by ethanol precipitation and storage in 10 mM Tris-HCl buffer (pH 7.5) plus 1 mM EDTA in −20°C.

The labeling ratio was determined by comparing the absorbance at 260 and 560 nm for DNA and TAMRA, respectively. Contributions from the dye to the observed absorbance at 260 nm were corrected using the equation: 

\[
A_{\text{real}} = A_{\text{obs}} - (A_{\text{max}} \times CF),
\]

where \( A_{\text{obs}} \) is the absorbance at 260 nm, \( A_{\text{max}} \) and \( CF \) are the peak absorbance of TAMRA at 560 nm, and CF is the correction factor (0.38 for TAMRA). The coefficient used to calculate the concentrations of the dye is 91,000 M⁻¹·cm⁻¹. For each oligodeoxynucleotide strand, the coupling ratio of dye to DNA was determined to be very close to 1:1.

Unlabeled oligodeoxynucleotide strands were purified similarly. Double-stranded DNA was made by mixing two complementary strands in equimolar ratio for unlabeled double-stranded DNA or with a 5% excess of the non-fluorescent strand (the non-template strand in this study) for a singly labeled double-stranded DNA, heating to 70–80°C for 5 min followed by slow cooling to room temperature over 2 h.

Fluorescence Titrations with 2-AP DNA—Experiments were carried out at 25°C as reported previously (20). 2-AP was excited at 314 nm, and the emission fluorescence was collected at 376 nm. The blank-corrected fluorescence \( F \) was plotted as a function of total DNA concentration, \([D]_t\). The linear increase in the observed fluorescence at high \([D]_t\) corresponding to free DNA fluorescence was then subtracted from \( F \) at each \([D]_t\), and the data were replotted and fit to Equations 1 and 2 to obtain the \( K_d \) values,

\[
F_{\text{corr}} = C + ([D]_t - [ED]) f_D + [ED] f_{ED} \tag{Eq. 1}
\]

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

\[
[ED] = \frac{(K_d + [E] + [D]_t) - \sqrt{(K_d + [E] + [D]_t)^2 - 4[E][D]}}{2} \tag{Eq. 2}
\]

where \([E] \) is the total concentration of the polymerase.

Fluorescence Anisotropy—Equilibrium titrations using fluorescence anisotropy were performed at 25°C on a PTI QM-3 spectrofluorimeter (Photon Technology International) mounted with Glen-Thomson calculated prism polarizers in an L-format scheme. The titration was conducted by adding small aliquots of concentrated T7 RNAP to the solution of dye-labeled promoter DNA (20–50 nM). Fluorescence anisotropy of TAMRA was calculated from the vertical \( F_{vh} \) and horizontal \( F_{vh} \) polarized fluorescence intensities with a vertical excitation polarizer according to \( r_{obs} = \frac{F_{vh} - GF_{vh}}{F_{vh} + 2GF_{vh}} \). The grating correction factor \( G \) is measured from the ratio of the vertical \( F_{vh} \) and horizontal \( F_{vh} \) intensities from a horizontally polarized excitation. The mixed solution of DNA and enzyme was incubated in a 3-mL cuvette for ~4 min before taking 10 measurements of fluorescence anisotropy to obtain an averaged value of fluorescence anisotropy \( r_{obs} \). The observed anisotropy \( r_{obs} \) is contributed both by free DNA (with anisotropy \( r_f \) ) and from protein-bound DNA (with anisotropy \( r_p \) ),

\[
r_{obs} = r_f f_b + r_p (1 - f_b) \tag{Eq. 3}
\]

where \( f_b \) is the fraction of DNA in the form of T7 RNAP complex, \( f_b = \frac{[ED]}{[D]_t} \). The increase in fluorescence anisotropy as a function of increasing T7 RNAP concentration was fit by the least-squares method to the quadratic Equation 2 to obtain the dissociation constant, \( K_d \). The starting anisotropy of attached TAMRA prior to binding was slightly below 0.18 and constant for all promoter fragments.

Stopped-flow Fluorescence Measurements—The apparent rates of association were measured from the time-dependent increase in 2-AP fluorescence after rapidly mixing the 2-AP promoter fragments (final concentration of 100 nM) with excess T7 RNAP (final concentration of 200, 300, 400, 500, and 600 nM) at 25°C using a KinTek 2000 stopped-flow setup as described previously (17, 20). The observed rates were plotted as a function of T7 RNAP concentration, and the association rate constant \( k_{on} \) was determined from the slopes. In the displacement assay, 2 μM (final concentration) non-fluorescent bubble DNA was mixed with 100 nM 1:1 preformed complex of 2-AP DNA and T7 RNAP at 25°C using the same KinTek 2000 stopped-flow setup. The dissociation of the complex was monitored by following the time-dependent decrease of 2-AP fluorescent. The 2-AP signals in both measurements were collected using a 360-nm cut-off filter with excitation at 315 nm. The averaged time courses were fit to a single or double exponential Equation 4,

\[
F_{obs} = \text{Constant} + \sum_{i=1}^{n=1 or 2} \alpha_i \exp(-k_f t) \tag{Eq. 4}
\]

where \( \alpha_i \) is the amplitude and \( k_f \) is the rate constant (s⁻¹) of each component.

Pre-steady-state Transcription Assay—The pre-steady-state kinetics of RNA synthesis were measured at 25°C using a rapid chemical quench-flow instrument fitted with a temperature-controlled water bath (KinTek Corp., Austin, TX). T7 RNAP (15 μM final) and promoter fragment (10 μM final) preincubated in high salt buffer (50 mM Tris acetate, 100 mM sodium acetate, 10 mM magnesium acetate, 5 mM dithi-
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Equilibrium DNA binding titrations can be carried out in two ways. One can titrate a fixed amount of promoter DNA with increasing concentrations of T7 RNAP (forward titration) or titrate a fixed concentration of T7 RNAP with increasing concentrations of the promoter DNA (reverse titration). Titrations with 2-AP-modified promoters have been carried out in both ways (17, 20, 21). In practice, however, when using 2-AP DNA, the forward titration provides a low signal change. This is primarily due to the high fluorescence background from free T7 RNAP, which masks the increase in 2-AP fluorescence as a result of complex formation. In contrast, the reverse titration provides a sensitive measurement of 2-AP DNA binding because the fluorescence of 2-AP in the unbound DNA is much weaker than the 2-AP fluorescence increase that results upon complex formation. Therefore, equilibrium titrations with the 2-AP promoter fragments were carried out by titrating T7 RNAP (100 nM) with increasing concentrations of 2-AP DNA fragments (20) as shown in Fig. 1a.

Equilibrium DNA binding assays by the anisotropy method were carried out using forward titrations. The probe TAMRA was attached on the 5′-end of the template strand via a C6 amino linker at position +19, which is believed to be far away from specifically bound RNAP on the promoter upstream. To minimize quenching effect due to a neighboring guanine base, the downstream terminal GC bp in all the fragments was modified to TA. We observed similar binding of P-22 irrespective of whether TAMRA was present at the 5′-end of the template strand or the 5′-end of the non-template strand, that is, at the downstream or the upstream end of the duplex DNA. Similarly, the same binding affinity was reported previously when TAMRA was incorporated at the 3′-end of the template strand at position −22 via a C3 amino linker (22). The extrinsic label at the −22 position therefore does not appear to affect the binding affinity of the complex. Each DNA duplex contained only a single attached dye as determined by UV-visible absorbance measurements. In all titrations, the mixture of T7 RNAP and DNA was incubated for at least 4–6 min prior to data acquisition. After correction of blank, experimental data were least-squares fitted to the quadratic equation to determine the values of $K_d$. A typical plot of TAMRA anisotropy measurement is shown in Fig. 1b.

TABLE TWO summarizes the $K_d$ values of the promoter fragments obtained from the two types of titrations. The results show that the extended promoter fragments with a longer AT-rich sequence bind to T7 RNAP with a greater affinity relative to the minimal P-17 promoter fragment. This trend of stabilization becomes more obvious when the $K_d$ values are plotted versus the length of upstream extension (Fig. 2). T7 RNAP binds the P-17 promoter, which has a half-truncated AT-rich region, with a $K_d$ of ~60 nM, which is generally in agreement with previous estimates of a weak binding affinity for promoter DNA fragments truncated at −17 based on DNase I footprinting protection and gel-shift assays (20–55 nM) (5, 6, 25) but is significantly larger than ~4 nM obtained from the forward titration of 2-AP DNA (21). Recovery of the full AT-rich region helps to stabilize the complex of T7 RNAP such as with P-21. Although the 2-AP fluorescence assay showed a decreased $K_d$ of ~39 nM for P-21, the stabilization was more substantial when measured by the anisotropy assay, resulting in a $K_d$ of ~16 nM for P-21. Both binding assays showed that the T7 RNAP-DNA complex is more stable when the promoter region is extended to −22 (~17 nM from the 2-AP assay and ~4 nM from the TAMRA anisotropy assay). Similar stabilization was observed whether the −22 bp was GC or AT, suggesting no sequence-specific interaction with T7 RNAP at this position. Further extension from −22 to −27 had little effect on the $K_d$ values as

\[2-\text{AP} \]

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**TABLE ONE**

| Promoter DNA sequences | DNA sequence of synthetic non-template strand (5′ → 3′) |
|------------------------|--------------------------------------------------------|
| P-27                   | CCTGGGTATTACGACTCATATAGGGAGACCTCATCTTTGAA |
| P-24                   | GGGATAATTACGACTCATATAGGGAGACCTCATCTTTGAA |
| P-22                   | GAATTATACGACTCATATAGGGAGACCTCATCTTTGAA |
| P-21                   | ATTTATACGACTCATATAGGGAGACCTCATCTTTGAA |
| P-17                   | TAATACGACTCATATAGGGAGACCTCATCTTTGAA |
| mP-22                  | CAGTCTAATACGACTCATATAGGGAGACCTCATCTTTGAA |
| mP1.1B-22              | CGGTTAATACGACTCATATAGGGAGACCTCATCTTTGAA |
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FIGURE 1. Representative equilibrium titrations to measure the binding affinity of T7 RNAP and promoter DNA at 25 °C. a, normalized fluorescence changes of 2-AP during the formation of T7 RNAP-DNA complexes. 100 nM T7 RNAP was separately titrated by concentrated 2-AP-incorporated P-17 (circles), P-21 (triangles), and P-22 (squares). Experimental data of 2-AP fluorescence were fitted according to Equations 1 and 2 to obtain the equilibrium dissociation constants \( K_d \) for the complex of T7 RNAP with promoter DNA (see “Materials and Methods” for details). b, normalized changes of the fluorescence anisotropy of tethered TAMRA during the formation of T7 RNAP-DNA complexes. Changes in the fluorescence anisotropy of 20 nM TAMRA labeled on P-17 (circles), P-21 (triangles), or P-22 (squares) monitors the titration with increasing T7 RNAP concentrations. The equilibrium dissociation constants \( K_d \) were obtained by fitting the anisotropy values according to Equations 2 and 3 (see “Materials and Methods” for details).

TABLE TWO

| DNA   | \( K_d \) (2-AP) \( nM \) | \( K_d \) (anisotropy) \( nM \) | On-rate \( k_{on} \) \( \mu M s^{-1} \) | Off-rate \( k_{off} \) \( s^{-1} \) | \( k_{off}/k_{on} \) |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|
| P-17  | 61 ± 6          | 63 ± 12         | 95 ± 6          | 3.9 ± 0.2       | 41 ± 3          |
| P-21  | 39 ± 6          | 16 ± 4          | 85 ± 5          | 0.86 ± 0.02     | 10 ± 1          |
| P-22  | 17 ± 1          | 4 ± 2           | 144 ± 18        | 0.73 ± 0.04     | 5.1 ± 0.7       |
| P-24  | 12 ± 2          | 2 ± 1.3         | 153 ± 2         | 0.62 ± 0.01     | 4.0 ± 0.5       |
| P-27  | 3.3 ± 1.1       | 3.1 ± 0.9       | 144 ± 14        | 0.56 ± 0.01     | 3.9 ± 0.4       |
| mP-22 | ND              | 45 ± 12         | 80 ± 3          | 1.4 ± 0.1       | 18 ± 2          |
| mP1.1B-22 | ND         | 53 ± 15         | 78 ± 9          | 1.7 ± 0.5       | 22 ± 7          |

* Based on equilibrium titrations of a fixed amount of T7 RNAP with increasing 2-AP DNA.
* \( K_d \) was obtained from the initial slope of the dependence of observed rates \( k_{on} \) on T7 RNAP concentrations as measured by 2-AP fluorescence changes. 2-AP signals in all other DNA constructs except P-17 displayed a single exponential increase. The result of P-17 comes from the main and fast phase of a biexponential increase.
* Shown here are observed rates of the fast and main phase (≈80%) whereas the observed rates of the slow and minor phase are essentially irreversible (within 0.06–0.1 s^-1 ). Fitting deviations are included for both on- and off-rates.
* Calculated on the assumption of a simple kinetic process of T7 RNAP complex formation, \( E + P \leftrightarrow EP_{(final)} \). However, a multiple-step pathway was proposed at least for P-21 (see Ref. 23).

TABLE ONE

| DNA   | \( K_d \) | \( K_d \) (anisotropy) |
|-------|--------|-----------------|
| P-17  | 61     | 63              |
| P-21  | 39     | 16              |
| P-22  | 17     | 4               |
| P-24  | 12     | 2               |
| P-27  | 3.3    | 3.1             |
| mP-22 | ND     | 45              |
| mP1.1B-22 | ND | 53             |

We noted that the forward titrations done with TAMRA anisotropy provided consistently a lower \( K_d \) than the reverse titrations done by 2-AP fluorescence except for the P-27 promoter. For example, the anisotropy method provided a P-21 promoter \( K_d \) of ~16 nM, which is consistent with a previous measurement from a forward titration using 2-AP DNA (\( K_d \) of 15–20 nM for P-21 DNA binding by T7 RNAP under comparable conditions (17)). However, this \( K_d \) value of P-21 DNA is several times lower than the previously reported value measured by reverse titrations using 2-AP fluorescence (20). Thus far, we do not have a clear explanation for the discrepancy. Based on the observation that promoter fragments truncated equally at −22 but at different positions downstream (+19, +9, and +5) show similar \( K_d \) values (22), we propose that nonspecific binding of T7 RNAP to the non-coding sequence is not the reason for the observed discrepancy. We suspect, however, that protein-protein interactions within T7 RNAP could be a cause for the discrepancy in the measured \( K_d \) values measured by forward versus reverse titrations. In the reverse titrations, a large excess of

evaluated from the TAMRA anisotropy assays. These studies indicated that P-22 is the minimal promoter fragment that shows optimal binding interactions with T7 RNAP. To investigate whether the observed stabilization due to extension beyond −17 was AT sequence-specific, we replaced the −18 to −22 with a randomly chosen sequence (60% GC content, modified P-22 or mP-22). The mP-22 DNA bound to T7 RNAP with a significantly decreased affinity relative to P-22, and in fact, its affinity was close to that of P-17. Therefore, the stabilization effect of the extended promoters observed above appeared to be due to the AT-rich sequence. To investigate the physiological significance of this result, we chose the upstream sequence of a natural class II promoter ph1.1B that contains a relatively GC-rich upstream sequence. The synthesized promoter was identical to the consensus class III promoters from −17 to +2 for comparison but contained the 1.1B-specific sequence in the −22 to +2 region (TABLE ONE, mP1.1B-22). The binding affinity of mP1.1B-22 DNA was measured by anisotropy titrations, and these experiments showed that mP1.1B-22 binding is significantly weakened to the level of mP-22 and P-17 DNAs. These results strongly supported a role for the upstream AT-rich region in modulating T7 promoter strength.

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free T7 RNAP was present at the beginning stages. If multiple T7 RNAP molecules bound to the DNA fragment under these conditions, this phenomenon was likely to affect the fitting and thus the $K_d$ value measured by the reverse titration. During forward titration, free RNAP concentration became higher than the DNA binding sites only at the later stages, and thus, this phenomenon was less likely to affect the fitting and the measured $K_d$ value. In support of this hypothesis, we did observe the beginnings of a second phase in the anisotropy assay during P-17 and P-21 binding at very high protein/DNA ratios, which was not used in our fitting for $K_d$ determination but likely caused by the binding of additional RNAP molecules.

**Kinetics of Promoter Binding and Dissociation**—The interactions between T7 RNAP and the promoter fragments were characterized additionally by measurements of the kinetics of T7 RNAP-promoter DNA association and dissociation. Previously, we have characterized a minimal two-step mechanism for the formation of T7 RNAP-promoter complexes during initiation (17, 26). To measure the rate of association ($k_{on}$), the 2-AP signal was collected after rapidly mixing 2-AP DNA fragments with T7 RNAP in a stopped-flow setup. The real-time formation of T7 RNAP-promoter complex was measured from the time course of 2-AP fluorescence emission by about 1.5-fold. On the other hand, the pre-steady-state 2-AP signals showed a biexponential increase containing a fast major phase and a slow minor phase. The fast phase of association of mP-22 had a reduced pre-exponential amplitude relative to P-17 (~80% in mP-22 versus 90% in P-17). For the mP1.1B-22, the fast phase of association accounted for 76% of the total amplitude at T7 RNAP concentration below 400 nM and accounted for ~90% at higher enzyme concentrations. Thus, mP-22, mP1.1B-22, and P-17 appear to have equivalent rates of complex association ($k_{on}$) (TABLE TWO). These results indicate that the AT-rich region upstream of −17 affects the kinetics of T7 RNAP complex association in a sequence-specific manner.

The rate of T7 RNAP-promoter complex dissociation ($k_{off}$) was measured by a displacement assay. A non-fluorescent promoter DNA (40-bp fragment from −21 to +19) with multiple mismatches in the TATA melting region of non-template strand (bubble DNA) was used as a trap. The affinity of T7 RNAP for the bubble DNA is much stronger than that of the native duplex DNA (20, 22). In the displacement assay, 2 μM (final concentration) trap DNA was mixed with 100 nM 1:1 pre-formed complex of 2-AP DNA and T7 RNAP in a stopped-flow setup. Dissociation of the RNAP-DNA complex was monitored by following the decrease in 2-AP fluorescence that results from base restacking in the enzyme-dissociated DNA. A biexponential decrease of 2-AP fluorescence was observed in all tested promoter fragments despite a single exponential increase observed in the association kinetics of the complex of P-21 to P-27. About 80% of the signal occurred in the fast phase, which was assigned as the dissociation rate of T7 RNAP-DNA complex. This rate decreased from 3.9 s^{-1} in P-17 to ~0.9 s^{-1} in P-21 (Fig. 3b). The rate of P-17 dissociation that we observed here was significantly faster (~20-fold) than the one reported in a previous study (21). It is not clear whether the short sequence from −17 to +5 used in the previous study is responsible for this difference. Extension upstream from −21 to −27 had a smaller effect on the fast phase rate that decreased only slightly (TABLE TWO). On the other hand, the rate of minor phase remained largely identical among these promoter fragments (~0.08–0.11 s^{-1}). Thus, in contrast to the association rate constant that increased by about 1.5-fold, the AT-extended upstream region to −22 and beyond led to a decrease in the dissociation rate by about 7-fold. The dissociation rates of mP-22 and mP1.1B-22 were slower than that of P-17 (1.4 and 1.7, respectively, versus 3.9 s^{-1}) but faster than those of the AT-extended promoters (TABLE TWO). Thus, both the association and the dissociation kinetics were affected by the upstream DNA region from −18, and this effect was AT sequence-specific.

A direct comparison of $k_{on}$ and $k_{off}$ values reveal the stability ($K_d = k_{off}/k_{on}$), which increased as the upstream AT-rich sequence was extended from −17 to −27 (TABLE TWO). For the weaker promoter P-17 and mP-22, the $K_d$ values derived from $k_{off}/k_{on}$ were smaller than those determined from equilibrium titrations. For other promoters, they were very close to those obtained from the TAMRA anisotropy assay. Thus, both the kinetic and the equilibrium methods demonstrated the trend of stabilizing T7 RNAP complexes by extending the AT-region sequence from −17 to −27. Furthermore, the kinetic parameters revealed that the stability of the AT-region extended promoter fragments relative to P-17 is primarily due to the slow rate of DNA dissociation. This is distinct from observations made with *Escherichia coli* RNAP AP8 promoter, in which DNA sequences upstream of −47 were found to affect the initial steps of transcription bubble formation in the open complex by stimulating the rate of association rather than decreasing the rate of complex dissociation (27).

**Effect on RNA Synthesis**—How does the DNA sequence upstream of −17 position affect transcription? An earlier study reported an isolated 10-fold increase in the efficiency of transcription of −500-nt-long transcript upon extension of upstream duplex sequence by just one GC pair at
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FIGURE 3. Stopped-flow kinetics of the formation and dissociation of T7 RNAP-promoter complexes. Fluorescence intensity >370 nm from 2-AP incorporated duplex DNA was detected with excitation at 315 nm. Representative data of P-17 and P-21 binding are shown, and data for other DNA promoters were similar to those of P-21. a, association kinetics. Averaged traces from 8 to 10 shots for 100 nM promoter DNA (black, truncated upstream on −17, P-17; gray, extended upstream on −21 with a full AT-rich region, P-21) upon rapidly mixing with 200 nM T7 RNAP (final concentrations) at 25 °C are shown. The curve for P-17 is best-fitted with a biexponential function ($k_1 = 25.8 ± 1.3 s^{-1}$ and $k_2 = 0.25 ± 0.11 s^{-1}$), whereas the curve for P-21 is fitted with a single exponential function ($k = 21.8 ± 0.7 s^{-1}$). b, dissociation kinetics. 100 nM preformed complex of T7 RNAP and 2-AP DNA promoter (equimolar) was rapidly mixed with 2 μM non-fluorescent bubble DNA (final concentrations), and the decrease of 2-AP signal upon displacement was monitored. Both averaged curves (black, P-17; gray, P-21) were best-fitted with a biexponential function with $k_1 = 3.9 ± 0.22 s^{-1}$ and $k_2 = 0.10 ± 0.01 s^{-1}$ for P-17 and $k_1 = 0.86 ± 0.02 s^{-1}$ and $k_2 = 0.090 ± 0.004 s^{-1}$ for P-21.

FIGURE 4. Effect of upstream promoter extension on transcription activity. A time course of transcription reaction was carried out at 25 °C using a rapid chemical quench-flow instrument. T7 RNAP (15 μM, final) and promoter DNA (10 μM) were preincubated, and all four NTPs (500 μM each), spiked with [$\gamma$-32P]GTP, were added to initiate transcription reaction. After time intervals ranging from 0.05 to 25 s, the reaction was quenched with EDTA (200 mM). The RNA products (2–19 nt) from each reaction were subsequently resolved on a 23% polyacrylamide, 4M urea sequencing gel, as shown for P-17 promoter (29, 30). We have measured the kinetics of 2–19-nt RNA synthesis on P-17, P-21, P-22, P-27, mP-22, and mP1.1B-22 promoter fragments. A typical time course of RNA synthesis ranging from 50-ms to 25-s reaction times on P-17 and P-21 promoters is shown in Fig. 4, a and b. As seen from the figure, the kinetics of RNA formation was fast, and the full-length 19-nt RNA was synthesized in 0.7–1 s. The absence of RNA products 14–18 nt long indicated that processive RNA synthesis occurred upon promoter clearance after 13-nt RNA synthesis.

The use of [$\gamma$-32P]GTP allowed incorporation of one radiolabel per RNA product initiated from +1G. The amounts of various RNA products from 2 to 19 nt were quantified for each promoter DNA for all reaction times studied. Fig. 4c shows the fractions of short (2–8 nt) and long (9–13 nt) abortive products formed. All promoter fragments from P-17 to P-27 as well as mP-22 and mP1.1B-22 showed similar amounts of short abortive products. This is in agreement with equal amounts of 5-nt RNA (full-length) produced from −19 and −17 truncated promoters, as reported earlier (30). Interestingly, the amounts of long abortive products from P-17 to P-27 promoter fragments exhibited a direct dependence on the specific upstream promoter length. The amount of 9–13-nt RNA increased with increasing upstream length. The dependence was purely AT-rich sequence-specific because the natural class II promoter mP1.1B-22, and the modified mP22 promoter with nonspe-
fic sequence between −18 and −22, produced nearly three times fewer amounts of long abortive products than the consensus P-22 counterpart. The amounts of long abortive products synthesized by mP1.1B-22 and mP-22 promoters were very similar to that of the P-17 promoter. These results suggested that retaining the strong RNAP-promoter interactions by extending specifically the AT-rich upstream region results in longer abortive products, 9–13 nt in length. It has been reported that promoter clearance in T7 RNAP is facilitated by general weakening of the T7RNAP-promoter interactions (31), which is also the case in E. coli RNAP (32). The P-17 promoter fragment bound weakly to T7 RNAP and made reduced amounts of long abortive products, thus exhibiting early promoter clearance. The mP1.1B-22 and mP-22 promoters behaved more like P-17 in this regard, showing similar amounts of long abortive products and early promoter clearance despite the presence of a longer upstream region devoid of AT-rich sequence that does not enhance its stability or activity.

**DISCUSSION**

Equilibrium binding and kinetic studies demonstrated that the presence of the upstream DNA region from −18 to −22 is required in promoter fragments to form a tight T7 RNAP-promoter complex. The crystal structures of T7 RNAP with promoter fragments truncated at −17 shows that the AT-rich recognition loop (amino acids 93–101) interacts with the −13 to −16 region. These minor groove interactions result in a broader and shallower minor groove (2). Specifically, Lys-98 of the AT-rich interaction moves hydrogen bonds with T-14, Arg-96 with A-16 on the non-template strand, and Gly-97 with T-16 on the template strand (2). What is the role of the upstream DNA region from −18 to −22? Our studies showed that the so-called minimal promoter P-17 binds weakly to T7 RNAP. Tight interactions were restored when the upstream DNA was extended to −22 and the sequence was AT-rich. Thus, the minimal promoter fragment for forming a tight preinitiation complex with T7 RNAP required the extended AT-rich region to base pair −22.

One reason for observing tight binding of the longer promoter fragments might be that base pair fraying at the blunt end of the short promoter fragments such as P-17 interferes with the interactions of the DNA with the AT-rich recognition loop. Thus, extending the promoter to −22 simply restores the duplex DNA structure in the −13 to −16 region, thereby reconstituting the protein-DNA interactions. This possibility was tested by replacing the AT-rich sequence from −17 to −22 with a randomly chosen sequence that contained 60% GC. The results showed that the modified P-22 promoter fragment does not bind as tightly as the consensus P-22 DNA but acts more like P-17. Both the kinetics and the equilibrium binding assays supported this conclusion. The weakened affinity was also observed with a natural class II promoter sequence (31). Therefore, it appeared that the extended AT-rich region from −18 to −22 has a stabilization effect and provides additional contributions to the optimal interactions of the promoter with T7 RNAP.

Does the observation of the upstream AT sequence-specific effect mean that T7 RNAP interactions extend to −22? This is a possibility, and one can speculate that these additional interactions involve bending of the upstream DNA region as has been suggested for the E. coli RNAP and mammalian RNAP II initiation complexes (33–36). In T7 RNAP, residues Lys-95 and Lys-93 are close to the upstream region of the promoter, and these amino acids might contribute to additional interactions. Alternatively, it is not unprecedented that the formation of specific protein-DNA complexes is influenced by DNA sequences flanking the cognate site although no direct contacts are made with these flanking regions (37–39). For example, the replacement of TATA-flanking sequence with GC-rich blocks was found to stabilize the protein-DNA complex. It was suggested that this effect was caused by a unique DNA conformation at the edge of the TATA region, i.e. kinks or bends that help to stabilize TATA-binding protein once bound in the complex (37). Thus, an alternative and preferred explanation for the tighter affinity of extended promoter is that the AT-rich sequence affects the conformation of the DNA in the −13 to −16 region of the T7 promoter that favors the observed minor groove interactions with T7 RNAP. The AT-rich sequence upstream of base pair −17 is conserved among the five class III promoters, but it is variable among the class II promoters. Thus, the DNA sequence in the extended upstream AT-rich region can play a role in modulating the efficiency of transcription initiation by affecting the affinity of T7 RNAP for the promoter DNA and by affecting the efficiency of promoter clearance. Thus, in addition to the promoter binding region contributing to the differences in promoter strength among class II and between class II and class III promoters, the upstream AT-rich region also appears to be involved in promoter discrimination (15, 16).