

COMMUNICATION

Identification of a Minimal Binding Element within the T7 RNA Polymerase Promoter

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The T7 RNA polymerase promoter has been proposed to contain two domains: the binding region upstream of position –5 is recognized through apparently traditional duplex contacts, while the catalytic domain downstream of position –5 is bound in a melted configuration. This model is tested by following polymerase binding to a series of synthetic oligonucleotides representing truncations of the consensus promoter sequence. The increase in the fluorescence anisotropy of a rhodamine dye linked to the upstream end of the promoter provides a very sensitive measure of enzyme binding in simple thermodynamic titrations, and allows the determination of both increases and decreases in the dissociation constant. The best fit value of $K_d = 4.0$ nM for the native promoter is in good agreement with previous fluorescence and steady state measurements. Deletion of the downstream DNA up to position –1 or to position –5 leads to a fivefold increase in binding, while further sequential single-base deletions upstream result in 20 and 500-fold decreases in binding. These results indicate that the (duplex) region of the promoter upstream of and including position –5 is both necessary and sufficient for tight binding, and represents the core binding element of the promoter. We propose a model in which part of the upstream binding energy is used by T7 RNA polymerase to melt the downstream initiation region of the promoter. We also show that the presence of magnesium is necessary for optimal binding, but not for specific enzyme-promoter complex formation, and we propose that magnesium is not required for melting of the promoter.

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In the initiation of transcription, an RNA polymerase (or appropriate complex of RNA polymerase plus activators) must bind to a specific sequence in the DNA (the promoter) and then must facilitate melting of the duplex DNA near the start site, directing the template strand into the enzyme active site, and initiating phosphodiester bond formation. Although the mechanistic details of this process may vary among RNA polymerases (and indeed, among promoters for the same RNA polymerase), the fundamental requirements for initiation of transcription are the same. The relatively unregulated single subunit RNA polymerase from bacteriophage T7 requires no protein cofac-

tors, yet initiates transcription with high specificity and fidelity. It is perhaps the simplest RNA polymerase (and the only RNA polymerase for which a high resolution structure is known; Sousa *et al.*, 1993) and so presents an ideal model system in which to study the fundamental structural and mechanistic features of this process.

The highly conserved consensus promoter sequence has been studied by a variety of approaches and early studies suggested that the promoter can be loosely divided into an upstream binding domain and a downstream domain required for catalysis (Chapman & Burgess, 1987). Recent *in vitro* measurements of transcription from oligonucleotide-based promoters have identified specific base functional groups involved in direct read-out of the sequence from position –11 to position –5, through contacts in the major groove (Li

Abbreviations used: DTT, dithiothreitol; KGlu, potassium glutamate.

et al., 1996). Interactions with this upstream binding domain appear then to be typical of many duplex DNA binding proteins, and it is in this region that the primary sequence specificity which distinguishes T7, T3, and SP6 RNA polymerases resides (Diaz *et al.*, 1993; Joho *et al.*, 1990; Raskin *et al.*, 1992,1993). The fact that different species-distinguishing sequences are allowed in this region suggests that this region of the DNA is not directly involved in RNA polymerization and may serve simply as a binding tether for downstream elements. In fact, a recent study has shown that the downstream elements need only be loosely tethered to the upstream domain in order to provide precise and efficient initiation of transcription (Weston *et al.*, 1997).

In the binary complex, the non-template strand near the start site has been shown to be susceptible to endonucleolytic cleavage, suggesting that this region of the DNA is melted in the static enzyme-DNA complex (Muller *et al.*, 1989; Strothkamp *et al.*, 1980). Steady state kinetic studies of artificially melted promoter constructs have provided evidence that melting of the promoter DNA presents a small energetic barrier to initiation (Maslak & Martin, 1993). Indeed, kinetic and thermodynamic measurements of binary complex formation (in the absence of turnover) show that melting of the TATA region immediately adjacent to the transcription start site occurs very rapidly, perhaps coincident with binding (Jia *et al.*, 1996; Újvári & Martin, 1996).

The introduction of the 2-aminopurine fluorescent base analog into the DNA has provided thermodynamically valid measurements of static enzyme-DNA complex formation (Jia *et al.*, 1996; Újvári & Martin, 1996). The relatively low fluorescence intensity of 2-aminopurine, however, places a lower limit on the concentration of promoter which can be assayed, which limits the accuracy of the dissociation constants determined for tight binding constructs. Also, since most of the structural changes in the DNA which accompany binding (for example, melting of the DNA) occur near the start site, limitations are placed on exactly what types of modifications can be introduced into the promoter DNA. Here, we follow polymerase binding by measuring fluorescence anisotropy of a rhodamine fluorophore attached to the upstream end of the DNA (Dunkak *et al.*, 1996; Heyduk & Lee, 1990; LeTilly & Royer, 1993; Perez-Howard *et al.*, 1995). This technique is based on the increase in the rotational correlation time and, thus in the fluorescence anisotropy of the labeled promoter DNA, as the very large (100 kDa) RNA polymerase binds to the free oligonucleotide. The increased sensitivity afforded by this probe allows the determination of changes which increase, as well as decrease, binding affinity. The placement of the probe at the upstream end of the promoter allows the construction of promoters truncated in one or both strands at the downstream end, providing a direct test of the proposed domain structure for

the T7 RNA polymerase promoter. Finally, this approach allows us to compare the effects of solution conditions on promoter binding by the entire promoter and by the various truncated constructs.

In the current study, rhodamine was covalently attached to the upstream end of the T7 RNA polymerase promoter *via* the 5' or 3' end of the non-template or template strands, respectively, through a three carbon linker. Previous studies have demonstrated that a promoter truncated upstream at position -17 is sufficient for maximal steady state kinetics (Martin & Coleman, 1987), and footprinting data suggest that protein-DNA contacts extend at least to position -17 (Basu & Maitra, 1986; Chapman *et al.*, 1988; Gunderson *et al.*, 1987; Ikeda & Richardson, 1986; Muller *et al.*, 1989). We have attached rhodamine to one construct ending upstream at position -17 and, to avoid any possible protein-fluorophore interactions, to another construct extended with an arbitrary sequence to position -22, as shown below:

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-22      -17      -10      -5      +1      +5
GAAATTAATACGACTCACTATAGGGAA non-template
CTTTAATTATGCTGAGTGATATCCCTT  template
                                     ↓

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The rhodamine-labeled, double-stranded oligonucleotides were used in equilibrium titrations in which a fixed concentration of DNA was titrated with increasing amounts of T7 RNA polymerase. Throughout the titrations, no significant change in the total fluorescence intensity of the rhodamine was observed. The increase in the fluorescence anisotropy as a function of the enzyme concentration was fit to the exact solution of a simple two-state binding equilibrium (with corrections for dilution) to obtain the apparent dissociation constant as shown in Figure 1.

Previous fluorescence measurements based on the incorporation of 2-aminopurine within the TATA region of the promoter measured a K_d of 4.8 nM for binding of the enzyme to a consensus promoter construct truncated upstream at position -17 (Újvári & Martin, 1996). The measured K_d value of 27 nM for the construct truncated and labeled with rhodamine at position -17 suggests that attachment of the fluorophore this close to the promoter interferes with optimal binding of RNA polymerase. In contrast, the obtained K_d value of 4.0 nM for constructs truncated and labeled at position -22 suggest that the placement of the label five base-pairs upstream of position -17 is sufficient to avoid interactions with the fluorophore. Similar results were obtained with a six-carbon linker (data not shown) consistent with the model in which rhodamine stacks onto the end of the duplex, independent of the tethering linkage. Consequently, promoters labeled upstream at position -22 through a three-carbon linker are used in the following experiments.

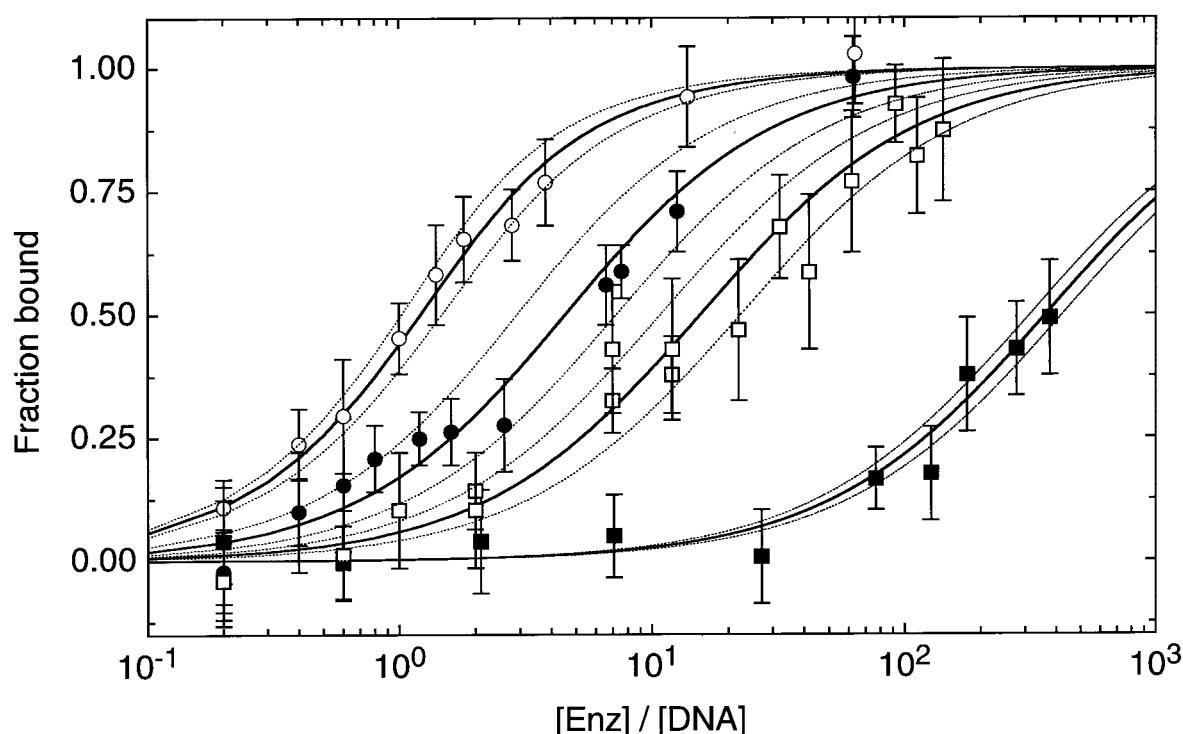


Figure 1. Equilibrium titrations at 1 nM DNA concentration for some of the promoters shown in Figure 2. Titrations were carried out in a buffer containing 30 mM Hepes (pH 7.8), 15 mM magnesium acetate, 100 mM potassium glutamate and 0.05 % Tween-20 (Calbiochem, protein grade). All DNA constructs started upstream at position -22 . Open circles; promoter ending downstream at position -5 ($K_d = 0.7$ nM); filled circles; promoter ending at position $+5$ ($K_d = 4.0$ nM); open squares promoter ending at position -6 on the non-template strand and at position -5 on the template strand ($K_d = 15$ nM); filled squares; promoter ending at position -7 on the non-template strand and at position -5 on the template strand ($K_d = 364$ nM). Continuous lines represent curves corresponding to the fitted K_d values, dotted lines show calculated curves based on the 67 % error ranges of the best fit dissociation constants, and emphasize the significance of the differences in the K_d values determined for the different promoter constructs. Note that the final anisotropy value for the weakest construct (truncated at position -7 and -5 on the non-template and template strands, respectively) could not be measured experimentally due to the weak binding of the enzyme to this DNA. In the fitting process for this construct, we arbitrarily fixed the final anisotropy value based on numbers from the titrations with the other promoter constructs. Consequently the reported error range appears smaller than expected and we consider the fitted value for K_d of ≈ 360 nM to be a lower limit. Methods: T7 RNA polymerase was purified as described by King *et al.* (1986). Oligonucleotides were synthesized by the phosphoramidite method on a Milligen/Bioscience Cyclone Plus DNA synthesizer. Rhodamine was attached (post synthesis) to the 5' or 3' end of oligonucleotides *via* a 5' or 3'-amino-modifier C3 linker (Glen Research, Sterling, VA). 5'-Amino modifiers and the spacer analog 9-O-dimethoxytrityl-triethyleneglycol,1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (Prime Synthesis) were coupled off machine as described previously (Schick & Martin, 1993). Amino labeled oligonucleotides were coupled to Rhodamine-X isothiocyanate (Molecular Probes, Eugene, OR) as described by Dunkak *et al.* (1996) and Perez-Howard *et al.* (1995). Labeled single strands were then purified by denaturing gel electrophoresis. Fluorescence experiments were performed using a QM-1 Photon Technology International T-format fluorimeter with emission monochromator set to 580 nm with a 3 to 5 nm slit width and excitation monochromators set to 602 nm with slit width of 7 to 17 nm. All measurements were carried out at 25°C. The increase in fluorescence anisotropy was fit as a function of the amount of added enzyme by varying K_d , and the initial and final anisotropy values simultaneously. The precise value of the initial and final anisotropies, $0.19(\pm 0.02)$ and $0.27(\pm 0.02)$, respectively, varied between constructs and reflected changes in the length of the DNA (and possibly changes in the size of the polymerase-promoter complex).

Identification of the tight binding core within the promoter

We have previously shown that the introduction of different spacers into the TATA region of the template strand (positions -4 to -1) does not effect the kinetics of transcription initiation significantly (Weston *et al.*, 1997). With the use of the rhodamine anisotropy assay, we now demonstrate

that binding of T7 RNA polymerase to its promoter is similarly not disturbed. Figure 2 shows that the introduction of a very long polyethylene glycol spacer at position -1 of the template strand does not significantly weaken formation of the enzyme-DNA complex, consistent with the above model, in which the TATA region lies outside of the binding domain of the promoter.

An oligonucleotide-based steady state kinetic assay was used by Maslak and Martin to show that removal of (only) the non-template strand downstream of and including position -4 results in no decrease in polymerase binding and that specific initiation continues at a high rate. In the kinetic assay the steady state parameter, K_m was used as an approximation of the dissociation constant. We have recently suggested (Újvári & Martin, 1996) that in the expression of $K_m = (k_{-1} + k_{cat})/k_1$, the values of k_{cat} and k_{-1} are comparable, and that consequently K_m is relatively insensitive to decreases in k_{-1} (tighter binding) as observed for the partially single-stranded constructs, but is sensitive to increases in k_{-1} (weaker binding). Consequently the steady state kinetic assay will be insensitive to changes which lead to increased binding affinity.

It has previously been observed that promoter constructs which are partially single-stranded in the downstream domain show stronger binding than the full length duplex construct (Diaz *et al.*, 1996; Jia *et al.*, 1996; Újvári & Martin, 1996). Consistent with these expectations, current binding measurements on a partially single-stranded construct (non-template strand deleted downstream of position $+1$) show fourfold tighter binding ($K_d = 1.1$ nM) relative to the fully duplex construct ($K_d = 4.0$ nM; see Figure 2). This suggests that some of the binding energy derived from strong upstream promoter interactions may be used to drive formation of the downstream, presumably melted, structure. In order to test this model and to determine whether the upstream region is sufficient for tight binding, we prepared constructs truncated on both strands at either position -1 or position -5 . Binding measurements presented in Figures 1 and 2 demonstrate that these constructs bind 4 to 5 times more tightly than the full length promoter.

Li *et al.* (1996) have identified specific base functional group contacts on the template strand at position -5 (the 2-amino group of guanine) and at position -6 (the 5-methyl group of thymine). To further assess the minimal tight binding promoter, we have incrementally removed the non-template strand bases at positions -5 and -6 (leaving the potential template strand contacts). These deletions result in 20 and 500-fold increases in the dissociation constant (relative to the tightest binding construct), respectively, indicating that although the primary contacts in this region may lie on the template strand, their recognition appears to require a duplex structure. Furthermore, the DNA from position -5 upstream must represent the tight binding core of the promoter, consistent with the simple two-domain model (Chapman & Burgess, 1987) and with direct read-out across the major groove from position -6 through position -11 (Li *et al.*, 1996). The observation that the upstream binding core binds polymerase more tightly than the full length double-stranded DNA supports our model

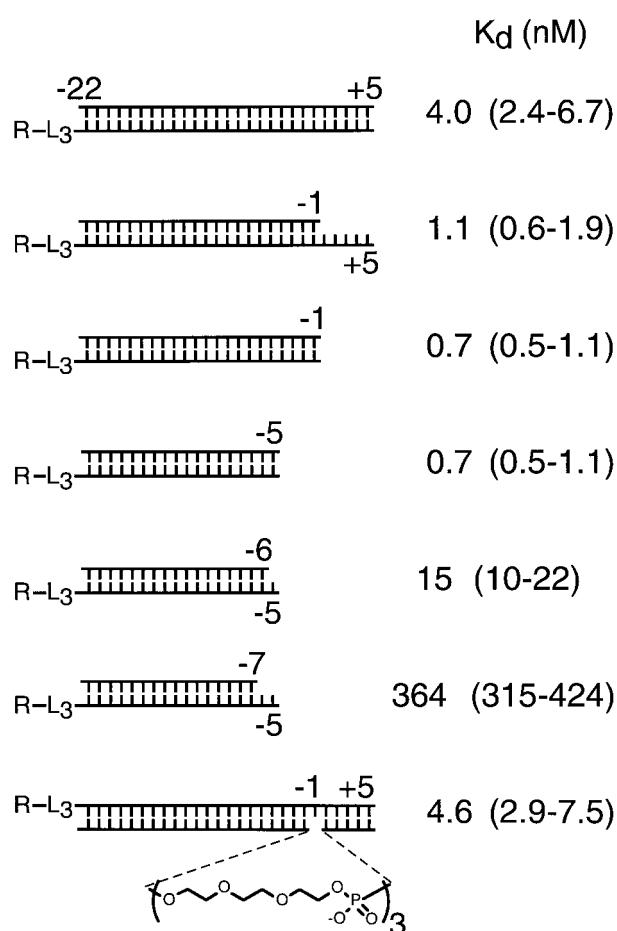


Figure 2. Identification of a minimal upstream promoter element for optimal binding to T7 RNA polymerase. Promoters were gradually truncated from the downstream end as shown in the cartoons. For the first six constructs, titrations were carried out at 1 nM DNA concentration, as described in the legend to Figure 1. The last construct shown in this Figure contains a polyethylene-glycol spacer at position -1 on the template strand. The dissociation constant for this construct was determined from a titration at 10 nM DNA.

in which energy derived from the upstream contacts is utilized by the enzyme to melt the region of the promoter near the start site.

The duplex sequence TATA is known to present a low energetic barrier for DNA melting (Breslauer *et al.*, 1986; SantaLucia *et al.*, 1996; Sugimoto *et al.*, 1996) and it has been suggested that the consensus promoter sequence has evolved to facilitate promoter melting (Maslak & Martin, 1993). Since removal of either the non-template strand or both strands downstream of position -1 decreases the dissociation constant fourfold, but subsequent removal of both strands upstream to position -5 results in no further decrease in K_d , we propose that the GGGAA duplex (positions $+1$ to $+5$) within the initially transcribed region presents the primary barrier to promoter melting. It is likely that the sequence of



	[Mg ²⁺] (mM)	K _d (nM)
	15	4.2 (2.0-8.9)
	1.0	12 (8-18)
	0.4	29 (17-55)
	0.0	56 (33-92)
	15	1.6 (0.7-3.5)
	0.0	17 (13-25)

Figure 3. Magnesium dependence of the dissociation constants for the full length double-stranded and for the partially single-stranded (non-template strand missing from position +1 to +5) promoters. Titrations were carried out as described in the legend to Figure 1, but with 10 nM DNA, and with concentrations of magnesium acetate as indicated.

this initially transcribed region has evolved subject to additional constraints (for example, the energetics of the initially formed heteroduplex), while the immediately adjacent TATA sequence has evolved to facilitate local melting.

Mg²⁺ is required for optimal binding

For the interaction between *Escherichia coli* RNA polymerase and the λP_R promoter, Suh *et al.* (1992) proposed the existence of two closed and two open complexes. They showed that magnesium is a non-specific competitor for the negatively charged phosphate groups of the promoter, during formation of the closed complex and up to the formation of the first open complex. They proposed that a specific uptake of magnesium ions converts the first open complex to the second open complex.

T7 RNA polymerase is a member of a class of structurally related polymerases (Delarue *et al.*, 1990; McAllister & Raskin, 1993; Sousa *et al.*, 1993) and the catalytically essential and conserved Asp537 and Asp812 residues are known to coordinate active site Mg²⁺ (Osumi-Davis *et al.*, 1992, 1994; Patra *et al.*, 1992). Woody *et al.* (1996) have recently used Mn²⁺ electron paramagnetic resonance spectroscopy to determine that there are approximately two binding sites for Mn²⁺ cations on the polymerase. A model was proposed wherein Mg²⁺ or Mn²⁺ are coordinated to the above aspartate residues with a dissociation constant of approximately 2 mM. Previous studies of T7 RNA polymerase have also indicated a requirement for Mg²⁺ in promoter binding (Gunderson *et al.*, 1987) and steady state kinetic data show a dependence of K_m on the concentration of Mg²⁺ in solution (Maslak & Martin, 1994).

In the results summarized in Figure 3, we find tight polymerase-promoter binding ($K_d = 56$ nM) in the complete absence of Mg²⁺. However, addition of 15 mM Mg²⁺ leads to a 13-fold increase in binding ($K_d = 4.2$ nM), consistent with a role for magnesium in the formation of the final complex. If this increased binding results from an interaction

with Mg²⁺ which specifically facilitates melting, then we would expect promoter binding to show such a dependence on Mg²⁺ concentration, but would expect the dissociation constant for the partially single-stranded promoter (non-template strand removed from position +5 to +1) to be independent of (or less dependent on) the magnesium concentration. In contrast, we measure a similar 11-fold increase in the dissociation constant for the partially single-stranded construct. Preliminary studies monitoring the fluorescence of 2-aminopurine placed at position -2 in the TATA region show that the polymerase-dependent increase in fluorescence does not require Mg²⁺, further suggesting that open complex formation does not require magnesium (unpublished results).

In the current study, labeling of the upstream end of the promoter with rhodamine allows the study of modifications on both strands of the promoter near the transcription start site. In future studies, labeling of the downstream end of the promoter will allow complementary studies of modifications at the upstream end of the promoter. Rhodamine-labeled DNA will also serve as an ideal probe in stopped flow kinetic experiments, to follow the binding of T7 RNA polymerase to its promoter, independent of conformational changes in either the DNA or protein.

Conclusion

We have identified the region upstream of position -5 as the region of the promoter both necessary and sufficient for tight binding by T7 RNA polymerase. This result supports a two domain model for the promoter in which the upstream part is responsible only for binding and the downstream part is required for optimal catalysis (Chapman & Burgess, 1987). We further propose a model in which a part of the upstream binding energy is used by the enzyme to introduce a conformational change in the downstream DNA, presumably melting of the initiation region of the promoter.

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