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Tests of a Model for Promoter Recognition by T7 RNA Polymerase: Thymine Methyl Group Contacts†

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ABSTRACT: The DNA-dependent RNA polymerase from bacteriophage T7 is highly specific for a 17 base promoter sequence. Interactions between T7 RNA polymerase and its promoter DNA have been probed using modified oligonucleotides and a steady-state kinetic assay. The incorporation of deoxyuridine in place of thymidine at individual sites in the promoter sequence results in the replacement of an exocyclic methyl group by hydrogen (effectively removing the thymine methyl). This substitution has been placed individually at each of the thymines in the T7 consensus promoter. Many of these substitutions do not affect binding or catalysis; however, the thymine methyl group at position −6 is critical to recognition. The kinetic parameter K_m increases approximately 10-fold while k_{cat} is only slightly affected, suggesting that this thymine methyl is critical to binding specificity, but not to the kinetics of initiation. Two methyl groups near the start site on the template strand (at positions −1 and −3) also contribute to promoter specificity, while nearby methyl groups on the nontemplate strand do not. The implications of these results are discussed with respect to recent models for promoter binding.

T7 RNA polymerase is a single-subunit DNA-dependent RNA polymerase with high specificity for a well-defined promoter sequence (Chamberlin & Ryan, 1982; Dunn & Studier, 1981). As such, it offers an ideal model system in which to study promoter recognition and the initiation of transcription. Comparison of the phage promoter sequences first predicted a minimal consensus sequence from positions −17 to +5 (Oakley & Coleman, 1977; Dunn & Studier, 1983), and kinetic studies have demonstrated that a 17 base pair consensus promoter sequence is sufficient for optimal promoter recognition and precise initiation of transcription (Martin & Coleman, 1987). The small size of the T7 promoter allows the simple chemical synthesis of oligonucleotide promoters containing modified base analogs. The ability to introduce small changes into the DNA, combined with sensitive functional assays, allows a detailed study of DNA functional groups involved in promoter recognition.

The relatively small size of the T7 promoter has also been confirmed by a variety of footprinting studies (Basu & Maitra, 1986; Ikeda & Richardson, 1986; Gunderson et al., 1987), including studies using Fe(II)EDTA·H_2O_2 (Muller et al., 1989). This footprinting agent presumably acts by generating hydroxyl radicals in solution, which then react with unprotected ribose rings, to provide a high-resolution footprint (Tullius & Dombroski, 1986; Tullius et al., 1987). The hydroxyl radical footprint of the T7 RNA polymerase–promoter complex is summarized in Figure 1 and suggests that the polymerase binds to one face of the duplex spanning one or two major grooves and two flanking minor grooves. Indeed, recognition of major groove contacts in the central region has been supported by recent studies targeting individual base functional groups (Jorgensen et al., 1991; Schick & Martin, 1993). Hence the T7 promoter might be analogous to the *Escherichia coli* RNA polymerase promoter "recognition domain" (spanning positions −14 to −55) in which the RNA polymerase is thought to bind primarily to one face of the duplex DNA (Metzger et al., 1989; Schickor et al., 1990; Mecsas et al., 1991).

The above model for promoter recognition makes specific predictions regarding the involvement of specific functional groups in the T7 promoter. Assuming initially a direct read-out of base functional groups, contacts should appear in the predicted central major groove, the two flanking minor grooves, and possibly in the upstream major groove. As an initial probe, we have used the specific substitution of thymidine by deoxyuridine (T → dU) to simply remove a methyl group from the major groove of duplex DNA at precise locations along the promoter sequence. This substitution should have a minimal effect on the extended DNA structure (Fliess et al., 1988; Delort et al., 1985). A similar study of the RNA polymerase from *E. coli* has identified, via directed substitution of thymidine by deoxyuridine, critical thymine methyl group contacts at positions −34, −35, and −7 of the λ P_R promoter (Dubendorff et al., 1987).

The substitution T → dU is expected to introduce a very small perturbation both in the DNA and in the interaction between T7 RNA polymerase and its promoter. A quantitative in vitro steady-state kinetic assay of transcription initiation can be used to measure such relatively small changes in recognition (Martin & Coleman, 1987). In this assay, the steady-state velocity of synthesis of a short five base message is measured as a function of both enzyme and DNA concentrations. Since the average elongation rate for T7 RNA polymerase is approximately 230 s^{-1} (Golomb & Chamberlin, 1974) and initiation rates are 0.5 s^{-1}, the rate of production of a five base message should approximately equal the rate of initiation. The concentration dependence of the velocity of production of the five base transcript can be fit to the exact steady-state solution of the kinetic scheme described by eq 1.

To the extent that k_{-1} is substantially larger than k_{cat}, K_m approximates the dissociation constant for the complex of enzyme and promoter. The rate constant k_{cat} reflects the rate-limiting step or steps in the complex process leading up to nucleotide triphosphate binding and catalysis of phos-

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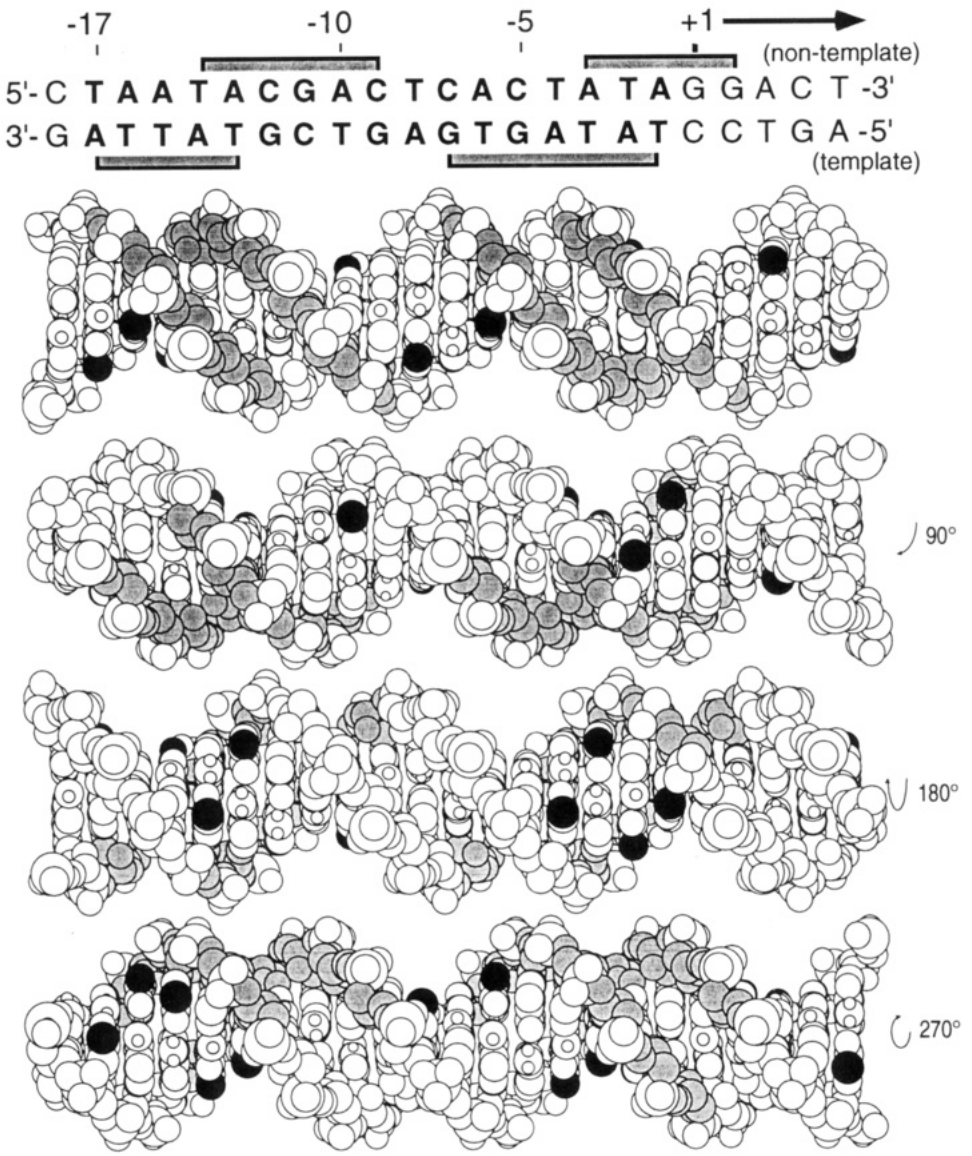

FIGURE 1: Potential protein contacts in the T7 RNA polymerase consensus promoter sequence. The lower figures depict a B-form helical representation of the promoter. The uppermost of these is oriented as the enzyme would face the DNA in the closed duplex binding model. Subsequent views are rotated by 90°. Sugars protected from attack by hydroxyl radical are shown in light gray. Thymine methyl groups are shown in darker gray.

$$\text{Enz} + \text{DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Enz–DNA} \xrightarrow[\text{NTP's}]{k_{cat}} \text{Enz} + \text{DNA} + \text{RNA} \qquad K_m = \frac{k_{-1} + k_{cat}}{k_1} \quad (1)$$

phodiester bond formation. The combination of chemical synthesis of oligonucleotides containing simple base modifications with this kinetic assay allows one to determine which functional groups are involved in transcription initiation and may help identify whether the effect is at the level of polymerase binding (K_m) and/or catalysis (k_{cat}).

MATERIALS AND METHODS

T7 RNA Polymerase. T7 RNA polymerase was prepared from *E. coli* strain BL21 carrying the overproducing plasmid pAR1219, which has the polymerase gene cloned under inducible control of the *lac* UV5 promoter (Davanloo et al., 1984). T7 RNA polymerase was purified as previously described (King et al., 1986). A molar extinction of $\epsilon_{280} = 1.4 \times 10^{-5}$ M^{-1} was used to determine enzyme concentrations (King et al., 1986). The purity of the enzyme was checked periodically by SDS–polyacrylamide gel electrophoresis.

Oligonucleotides. Oligonucleotides were synthesized and purified as described in Schick et al. (1993). Double-stranded oligonucleotides were prepared by annealing a 1:1 mixture of complementary single strands in 40 mM HEPES, pH 7.8, and 1 mM EDTA.[1] The single-strand extinction coefficients were added to approximate the double-strand extinction coefficient and compared with the double-strand extinction coefficient obtained from a method described by Newman et al. (1990), which takes into account hyperchromic effects in short oligonucleotides. The hyperchromic effect for double-stranded native promoter oligonucleotide is 1.44.

The incorporation of dU modifications at specific positions in the promoter sequence was verified using the uracil-specific reagent uracil-*N*-glycosylase (Life Technologies), as described in Schick and Martin (1993). This procedure was able to unequivocally identify incorporation of dU at all sites except

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

those near the ends of the oligonucleotides (positions −17 and −16).

Kinetic Assay of Transcription Initiation. Kinetic assays of transcription (Martin & Coleman, 1987) were carried out in a total volume of 20 μL containing 0.02–0.20 μM DNA in 30 mM HEPES, pH 7.8, 15 mM magnesium acetate, 100 mM potassium glutamate, 0.25 mM EDTA, 1 mM DTT, 0.1 mg/mL *N,N*-dimethylated casein (Sigma), 0.05% TWEEN-20 (Calbiochem, 10% protein grade), 0.8 mM GTP, and 0.4 mM ATP. [α-^{32}P]GTP at approximately 100 μCi/mL was present to label products. A 10.0-μL aliquot of a 2× mix [20 mM HEPES, pH 7.8, 30 mM magnesium acetate, 1.6 mM GTP (ultrapure, Pharmacia), 0.8 mM ATP (ultrapure, Pharmacia), and [α-^{32}P]GTP] on ice was premixed with 5.0 μL of 4× oligonucleotide in 40 mM HEPES, pH 7.8 and 1 mM EDTA and incubated in 0.5-mL plastic tubes for 3 min at 37 °C. To start the reaction, 5.0 μL of 4× enzyme (freshly diluted to 0.08–0.32 μM in 40 mM HEPES, pH 7.8, 400 mM potassium glutamate, 0.4 mg/mL *N,N*-dimethylated casein, 4 mM DTT, and 0.2% TWEEN-20) on ice was added and the sample returned to 37 °C. Aliquots of 5.0 μL were withdrawn at 3, 6, and 9 min and spotted onto individual lanes of Whatman 3 MM filter paper, prespotted with 10 μL of 200 mM EDTA. Small RNA products were separated from unincorporated nucleotide by ascending paper chromatography in 60% saturated ammonium sulfate, pH 8.0 (Mulligan et al., 1985). Periodically, autoradiograms of the chromatograms were taken to verify complete separation of product from unincorporated nucleotides. For each sample, the band corresponding to the product was cut out and counted with ScintiVerse E (Fisher) liquid scintillant.

For each template, reaction velocities were measured at various enzyme and DNA concentrations. The error in each velocity was approximated as the higher of 0.1 μM/min or the *t* distribution 80% confidence interval of the fitted slope for the three time points. Velocity data were then fit as previously described (Martin & Coleman, 1987) to the exact solution to the steady-state equation, using a weighted nonlinear least-squares minimization algorithm based on the Gauss–Newton method (Johnson et al., 1981). Ranges in the values represent a 65% joint confidence interval of the fitted parameters. As a direct result of the nonlinear nature of the velocity equation, the confidence intervals are not symmetric. In particular, given the enzyme and promoter concentration ranges of the current study, increases in K_m (weakening of binding) have more confidence than do decreases in K_m. Representative data and best fits to the data are compared in Figure 2. For a given promoter construct, the predicted velocity curves presented for the various enzyme concentrations all correspond to the single pair of best fit values to K_m and k_{cat}.

Product Analysis. Transcription products from each promoter were qualitatively analyzed by denaturing polyacrylamide (18–20%) gel electrophoresis (data not shown), and in all cases the correct five base RNA was the predominant product. In some cases, four base RNA products were also produced in low amounts and were included as total product formed when analyzed in the kinetic study.

RESULTS

The model for promoter recognition by T7 RNA polymerase derived from hydroxyl radical footprinting studies (Muller et al., 1989) makes simple first-order predictions regarding the relative importance of base functional groups at each position and on each strand of the promoter DNA. In particular,

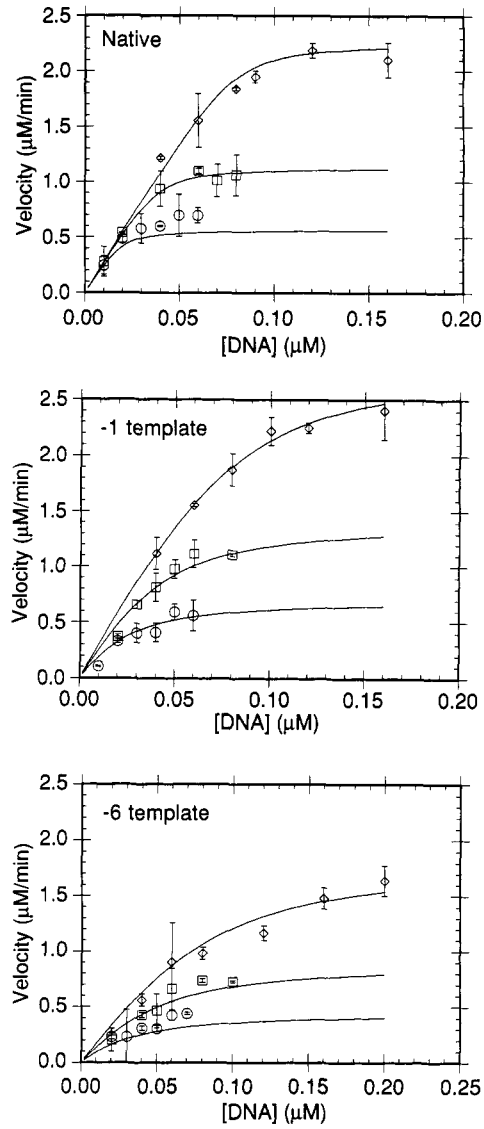

FIGURE 2: Comparison of representative steady-state kinetic fits for initiation of transcription from the native promoter (K_m = 2.0 nM; k_{cat} = 28.3 min^{-1}), the promoter containing dU at position −1 on the template strand (K_m = 12.5 nM; k_{cat} = 35.2 min^{-1}), and the promoter containing dU at position −6 on the template strand (K_m = 23 nM; k_{cat} = 22.6 min^{-1}). The enzyme concentrations are (○) 0.02, (□) 0.04, and (◇) 0.08 μM. Other details of the experiment are described in the text.

thymine exocyclic methyl groups are predicted to protrude into the major groove on both the front and back faces of the helix. Methyl groups at positions −16 and −6 are predicted to lie in upstream and central major grooves, respectively, facing the polymerase. In contrast, methyl groups at positions −14, −13, −10, −4, −3, and −1 are predicted to lie on the face of the helix directly opposite the enzyme.

Incorporation of dU in place of T at single sites within the promoter sequence allows direct tests of this model. This substitution should have no first-order effect on Watson–Crick hydrogen bonding within the DNA, and there is evidence that it produces minimal perturbation of the overall structural features of duplex DNA (Fliess et al., 1988; Delort et al., 1985). For each thymine methyl in the promoter, a double-stranded promoter was constructed containing a single T → dU substitution. The illustration in Figure 1 compares the relative positions of thymine methyls predicted by the simple duplex DNA binding model. The best-fit values for K_m and

Table I: Comparison of Kinetic Parameters for the Native Promoter Sequence and Promoter Sequences Lacking Individual Thymine Methyl Groups

position of T → dU substitution	K_m (nM)	k_{cat} (min^{-1})
native	2.0 (0.8–4.2)	28.3 (26.7–29.6)
−1 template	12.5 (8.5–18.4)	35.2 (32.6–37.8)
−2 nontemplate	1.0 (0.4–2.0)	26.1 (25.1–26.9)
−3 template	3.7 (1.6–8.1)	15.9 (14.7–17.1)
−4 nontemplate	0.5 (0.2–1.2)	23.1 (22.6–23.6)
−6 template	23 (13–40)	22.6 (19.9–25.4)
−8 nontemplate	2.9 (1.6–5.0)	39.1 (37.2–40.9)
−10 template	3.1 (1.6–6.1)	25.9 (24.3–27.3)
−13 template	2.4 (1.1–4.7)	27.2 (25.8–28.5)
−14 nontemplate	0.3 (<0.1–1.1)	28.0 (26.8–29.0)
−15 template	1.4 (0.6–3.2)	31.4 (29.9–35.9)
−16 template	4.5 (2.9–6.8)	29.6 (28.3–30.9)
−17 nontemplate	1.0 (0.2–3.2)	25.8 (24.1–27.1)

k_{cat} associated with each modified promoter are presented in Table I.

Contacts in the Upstream Region −17 to −13. In the predicted upstream major groove, methyl groups at positions −13 and −14 lie on the back face of the helix, and, as expected, removal of each of these results in little deviation from the native values for K_m (2.0 nM) and k_{cat} (28.3 min^{-1}). The methyl groups at positions −17 and −15 are predicted to lie near the edge of the recognition face, and their removal also has no apparent effect on the initiation kinetics. Although the thymine methyl at position −16 is predicted by the footprint to directly face the enzyme, removal of this methyl group has only a slight, if any, effect on K_m (4.5 nM) and no significant effect on k_{cat} (29.6 min^{-1}). This result preliminarily suggests that the upstream major groove is not a primary determinant of specificity.

Contacts in the Central Region −10 to −6. The thymine methyl at position −10 appears to lie far from the protected regions, and, consistent with this, its removal results in no change in the steady-state kinetics. The methyl at position −8 is predicted to lie near the edge of the protected region but nevertheless does not seem to contribute significantly to binding (K_m = 2.9 nM and k_{cat} = 39.1 min^{-1}). At position −6, the thymine methyl on the template strand is predicted to lie very close to the center of the recognition domain, directly facing the protein. Its removal has a very substantial effect on binding (K_m = 23 nM), with a slight effect on catalysis (k_{cat} = 23 min^{-1}). This 10-fold increase in K_m identifies the thymine methyl at position −6 as critical to binding between the DNA and T7 RNA polymerase.

Contacts in the Region near the Start Site −4 to −1. Closer to the start site of transcription (within the "TATA" region of the promoter), the thymine methyl groups at position −1, −2, −3, and −4 are predicted to lie near the back face of the helix. Consequently, the simple model predicts that they are not involved directly in recognition. As expected, removal of the methyl groups at positions −2 and −4 has little effect on measured kinetic parameters. In contrast, removal of the thymine methyl at position −1 increases K_m (12.5 nM) while removal of the methyl at position −3 reduces k_{cat} (15.9 min^{-1}) slightly. The results at positions −1 and −3 are not predicted by the simple model for promoter recognition involving fully duplex DNA. Closer examination of these results reveals that the thymine methyls at positions −2 and −4 lie on the nontemplate strand of the promoter, while the thymines at positions −1 and −3 lie on the template strand.

DISCUSSION

The combination of the chemical synthesis of oligonucleotides to introduce simple functional group modifications into promoter DNA and a steady-state kinetic assay to quantitatively measure the effects of these changes presents a powerful approach to begin a detailed study of protein–DNA interactions in transcription by T7 RNA polymerase. In particular, specific predictions of a recent model for promoter binding can now be directly tested (Muller et al., 1989).

To the extent that equilibration of the enzyme–DNA complex is fast relative to the rate-limiting step in catalysis ($k_{-1} \gg k_{cat}$), the Michaelis constant K_m approximates an apparent dissociation constant, K_d, and provides a functional measure of promoter binding. Indeed, native K_m values we have measured under various conditions (Maslak and Martin, unpublished results) generally agree with the association constant measured by quantitative footprinting (Gunderson et al., 1987). The kinetic parameter k_{cat} is likely to reflect the rate-limiting step(s) in a more complex series of events leading to the initiation of transcription, including a small contribution from promoter melting (Maslak & Martin, 1993). In a number of modified promoters (Schick & Martin, 1993; Maslak & Martin, 1993) K_m and k_{cat} have been observed to vary independently, providing further support for the idea that K_m and k_{cat} reflect separate changes in binding and initiation kinetics, respectively.

Although modifications resulting in large disruptions in promoter binding lend valuable insight into the details of the interaction, changes in the promoter which produce no significant changes in K_m or k_{cat} provide equally important insight into recognition. In our current assay, increases in K_m of more than about a factor of 3 are estimated to be significant. Decreases in K_m from native values are less reliably determined under current concentration ranges of enzyme and promoter. Assuming that K_m approximates K_d, a 3-fold increase in K_m corresponds to a minimal change in binding energy of approximately 0.7 kcal/mol. Full base pair modifications to the T7 RNA promoter sequence have been quantitatively studied in the past (Schneider & Stormo, 1989; Chapman & Burgess, 1987; Jorgensen et al., 1991). The current kinetic assay is sensitive enough to measure small changes in binding and catalysis associated with much simpler modifications to the DNA.

Confirmation of the Duplex Model—Upstream and Central Regions. Previous footprinting studies suggest that the methyl groups at positions −13, −14, and −15 lie on the back side of the recognition face (Figure 1) and do not contact the polymerase in the enzyme–DNA complex. The kinetic results from major groove T → dU substitutions at these positions appear to confirm this prediction. Neither K_m nor k_{cat} deviate from the kinetic parameters of the native promoter sequence in constructs which remove these thymine methyls.

Interestingly, the thymine methyl at position −16 is not critical to recognition, despite its predicted position along the face of the helix facing the enzyme. This is perhaps not surprising, given that the complete removal of nucleotide residues from positions −17 through −15 results in only a 10-fold increase in K_m (Martin & Coleman, 1987). The extreme upstream region of the promoter, although necessary for *maximal* promoter usage, does not appear as critical to recognition as the central and downstream regions of the promoter.

The thymine methyl group which appears to be most critical to recognition is that at position −6. Although its removal has no significant effect on k_{cat}, its replacement by a hydrogen

leads to a 10-fold increase in K_m (a change in K_d of this magnitude would correspond to $\Delta\Delta G \approx 1.4$ kcal mol^{-1}). This result can be compared with results from a study of the *Eco*RI endonuclease in which the simultaneous removal of *two* symmetry-related and noninteracting thymine methyls within a palindromic sequence produces a change in binding consistent with a total $\Delta\Delta G = 1.5$ kcal mol^{-1} (McLaughlin et al., 1987). Clearly the thymine methyl at position –6 of the template strand is involved in promoter specificity.

This result supports a model for recognition of major groove base functional groups in the central (duplex) region of the promoter. It is consistent with methylation interference studies which implicate involvement of adjacent guanine N7 functionalities at positions –7 and –5 along the same strand of the promoter (Jorgensen et al., 1991). It is, however, also consistent with recognition of template strand base substituents in a form of the promoter melted near the start site (Maslak & Martin, 1993).

Recognition near the Initiation Site. In the model summarized in Figure 1, all four thymine methyls within the sequence TATA from positions –4 to –1 are predicted to lie on the face of the helix opposite the protein. Although removal of thymine methyls at positions –4 and –2 has no effect on recognition, removal of those at positions –3 and –1 does disrupt transcription. The removal of the methyl group at position –1 significantly increases K_m with no effect on catalysis. Removal of the methyl group at position –3 reduces k_{cat} by approximately half with little or no apparent effect on binding (as reflected by K_m). Our new, revised model for recognition proposes that although the upstream half of the promoter remains duplex in recognition, the region near the initiation site is melted and the enzyme interacts in this region only with the template strand (Maslak & Martin, 1993).

In conclusion, these studies demonstrate that the kinetic parameters obtained from promoter constructs containing T → dU substitutions at positions –2, –4, –8, –10, –13, –14, –15, –16, and –17 do not significantly deviate from the kinetic parameters obtained for the native DNA sequence. This provides strong and unambiguous information identifying groups which are not required for recognition. However, incorporation of T → dU substitutions at positions –1 and –3 results in a small but significant change in kinetic parameters relative to the native promoter sequence, and removal of the thymine methyl at position –6 results in a large increase in K_m. The disruptions in recognition resulting from these changes suggest protein–DNA contacts and prompt further studies directed at elucidating the interactions between T7 RNA polymerase and its promoter sequence.

ACKNOWLEDGMENT

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SUPPLEMENTARY MATERIAL AVAILABLE

Steady-state kinetic fits for constructs listed in Table I but not included in Figure 2 (3 pages). Ordering information is given on any current masthead.

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