The Stability of Abortively Cycling T7 RNA Polymerase Complexes Depends upon Template Conformation†

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ABSTRACT: We have developed a promoter competition assay to determine whether T7 RNA polymerase dissociates from its template during abortive cycling. We find that the stability of the initiation complex (IC) depends upon the conformation of the promoter, and that the degree to which the template is unwound contributes importantly to the stability of the IC. On linear DNA or a relaxed plasmid template, the stability of the IC is very low ($t_{1/2}$ < 1 min). However, on a supercoiled template, the IC has a stability that is comparable to that of a paused elongation complex ($t_{1/2}$ = 14 min). At a synthetic promoter that is single stranded in the initiation region (from −5 and downstream), the polymerase forms a highly stable complex ($t_{1/2}$ > 30 min) even in the absence of RNA synthesis. These findings are important to our understanding of the transition from the IC to an EC.

Bacteriophage T7 RNA polymerase (RNAP) consists of a single polypeptide of 883 amino acids (98 kDa) (Moffatt et al., 1984) which can accurately initiate, elongate, and terminate transcription *in vitro* in the absence of auxiliary factors (Chamberlin & Ryan, 1983; McAllister, 1993). The relative simplicity of the phage RNAP, as compared to the multisubunit bacterial and eukaryotic RNAPs, makes this enzyme an excellent model for studies of RNAP structure and function. This work addresses the nature of the interaction between the phage RNAP and its promoter early in the transcription cycle.

Promoter binding and transcript initiation by T7 RNAP are thought to proceed along pathways similar to those described for *Escherichia coli* RNAP. During, or shortly after, promoter recognition, the phage RNAP melts open the DNA helix from −6 to +2 within the 23 base pair (bp) promoter consensus sequence, rendering the nontemplate (NT) strand susceptible to cleavage by single-strand-specific endonucleases while protecting the template (T) strand in this region (Strothkamp et al., 1980; Osterman & Coleman, 1981). The DNA sequence in the initiation domain of the promoter may have evolved to facilitate rapid melting upon T7 RNAP binding, as suggested by the inability to detect closed complexes in gel retardation assays (Muller et al., 1988), and by the absence of a detectable lag time for transcription initiation (Ikeda et al., 1992). Further support for this notion comes from the finding that the rate of initiation (k_{cat}) at synthetic promoters in which the NT strand in the initiation domain is absent is the same as at promoters that are double stranded over their entire length, suggesting that melting of the DNA poses little or no thermodynamic barrier to initiation (Maslak & Martin, 1993).

Initiation of transcription by T7 RNAP proceeds in two distinct phases. In the first phase, the initiation complex (IC) engages in repeated cycles of abortive initiation events, producing oligomeric transcripts that range from 2 to 12 nucleotides in length (Martin et al., 1988; Ling et al., 1989). It had not been determined whether the entire ternary complex dissociates and the RNAP needs to bind a promoter anew during this process, or whether the RNAP maintains a sufficiently tight binary association with the promoter to release the nascent RNA and re-form an IC without dissociating from the promoter, as is believed to be the case with *E. coli* RNAP (Carpousis & Gralla, 1980).

After the synthesis of a transcript of a promoter-specific critical length, the IC undergoes a transition to form a stable elongation complex (EC) (Martin et al., 1988; Ling et al., 1989). Current models propose that interaction of the nascent RNA with a product binding site in the RNAP is responsible for this stabilization, either by anchoring the template to the RNAP (Muller et al., 1988) or by inducing a conformation change in the protein which prevents dissociation from the template (Sousa et al., 1992; Macdonald et al., 1993). Once formed, the EC is capable of transcribing long stretches of template *in vitro* [>15 × 10^3 bp (Golomb & Chamberlin, 1977)] at an average rate of 200−260 nt/s (Bonner et al., 1994b). Polymerization by the RNAP proceeds until the EC encounters a termination signal or the complex runs off the end of the template.

In this study, we have used a promoter competition assay to characterize the stability of T7 RNAP initiation complexes under a variety of conditions. We have found that the conformation of the template contributes importantly to the stability of abortively cycling complexes, and that a highly stable binary complex may be formed at synthetic promoters in which the NT strand in the initiation region has been deleted. These findings are important to our understanding of the transition from an IC to an EC.

MATERIALS AND METHODS

DNA Preparation. Synthetic DNAs were prepared by the solid phase phosphoramidite method and purified by gel electrophoresis (Sambrook et al., 1989). Following precipitation in ethanol, oligomer DNA was resuspended in TE (0.01 M Tris-HCl, pH 7.4, 1.0 mM EDTA-Na_4), and the

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concentration was determined from the UV absorbance, assuming a molar extinction coefficient of $\epsilon_{260} = 8.4 \times 10^3$ per base. Complementary oligomers were annealed at a concentration of 50 μM (each strand) in 200 mM NaCl, and then diluted to a final concentration of 2−10 μM in 50 mM NaCl and stored at −20 °C.

pLM45 was constructed by subcloning a 366 bp *Bgl*II−*Hin*dIII fragment containing the Φ10 promoter from pT7−7 (Tabor & Richardson, 1985) into the *Bam*HI−*Hin*dIII sites of pUC 19 (Macdonald, 1993). Plasmid DNA was purified by banding in CsCl gradients (Macdonald, 1993). To prepare relaxed circular DNA, supercoiled plasmid (20 μg) was treated with 100 ng of vaccinia virus topoisomerase I (courtesy of Dr. Stewart Shuman) for 30 min at 37 °C in 50 mM Tris-HCl, pH 8, 100 mM NaCl, and 2.5 mM EDTA. Relaxation of the template was confirmed by electrophoresis of samples in 0.8% agarose gels.

Transcription Assays. Transcription reactions were carried out at 37 °C in 20 mM Tris-HCl, pH 7.9, 8 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.05% Tween-20. The test promoter (2.5 pmol) and T7 RNAP (0.5 pmol) were mixed in a volume of 40 μL of reaction buffer and equilibrated at 37 °C for 60 s. Polymerization was initiated by the addition of GTP and ATP (5 mM each in a volume of 5 μL) and was allowed to proceed for 60 s, whereupon radioactive labeling was initiated by the addition of 25 μCi of [α-^{32}P]ATP (specific activity of 800 Ci/mmol) in 5 μL. The final reaction volume was 50 μL. Synthetic promoter (125 pmol) was introduced either prior to the initial 37 °C equilibration or together with the radiolabel, as noted. At intervals after the addition of radiolabel, 5 μL aliquots were quenched with 5 μL of stop buffer (5 M urea, 50 mM EDTA, 0.01% xylene cyanole FF, and 0.01% bromphenol blue).

Samples were heated at 90 °C for 2 min and resolved by electrophoresis at 70 W in 20% polyacrylamide gels (31 × 39 × 0.04 cm) containing 6 M urea, until the bromophenol blue dye front had run 25 cm. The products were visualized and quantified by exposure of the gel to a Molecular Dynamics PhosphorImager screen for 30 min. All PhosphorImager data were within the linear range of screen response.

Calculation of Stabilities of RNAP−Promoter Complexes. The rate of radioactive product accumulation is described by eq 1:

$$dP/dt = k_{cat}[ED] \quad (1)$$

where P is the concentration of radioactive product, k_{cat} is a first-order rate constant, and [ED] is the concentration of the RNAP−promoter complex. In the absence of a sink promoter, the rate of accumulation is constant, as expected for a steady-state reaction ([ED] = $[ED]_o$). Upon the addition of a large excess of sink promoter, [ED] is expected to decay with a first-order rate constant, k_{off}:

$$dP/dt = k_{cat}[ED]_o e^{-k_{off}t} \quad (2)$$

Integration of eq 2 yields eq 3:

$$P = \frac{k_{cat}[ED]_o}{k_{off}}(1 - e^{-k_{off}t}) \quad (3)$$

The value of $k_{cat}[ED]_o$ is taken as the rate of radioactive product accumulation in the absence of sink promoter.

The value of k_{off} is estimated from the observed data by a least-squares fit of the product accumulation *vs* time to curves predicted by eq 3. Half-lives of the complexes are related to k_{off} by the relationship:

$$t_{1/2} = \ln(2)/k_{off} \quad (4)$$

Gel Retardation Assays. DNA oligomers were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Each 20 μL binding reaction contained 3000 cpm of DNA (*ca*. 0.5 nM) in 10 mM $NaPO_4$, pH 7.8, 1 mM EDTA, 20 mM NaCl, and 4% glycerol (Muller et al., 1988). Denatured salmon sperm DNA (25 μg/mL) was present to eliminate nonspecific binding. After incubation at 30 °C for 10 min, samples were loaded onto a preelectrophoresed 8% polyacrylamide gel and resolved by electrophoresis at room temperature at 3.8 V/cm for 2 h in 0.045 M Tris−borate, 1 mM EDTA.

RESULTS

Promoter Competition Assay: Use of a "Sink" Promoter To Sequester Free RNAP. The consensus T7 Φ10 promoter in pLM45 (the "test" promoter) initiates RNA synthesis with the sequence GGGAGAC... (Dunn & Studier, 1983) (see Table 1). In the presence of only GTP and ATP as substrates, repeated initiation and release of abortive products at this promoter lead to the accumulation of the 6 nucleotide (nt) product 5′-GGGAGA-3′ (Ling et al., 1989; Gross et al., 1992). To determine whether these repeated cycles of initiation are accompanied by dissociation of RNAP from the template, we utilized a promoter competition assay in which a second promoter was used as a "sink" for free polymerase. The sink promoter [the promoter in the plasmid Bluescript II SK+ (Stratagene)] initiates RNA synthesis with the sequence GGGCGA... (Table 1). Transcription from this promoter in reaction mixtures that contain only GTP and ATP as substrates results in the synthesis of an extended ladder of poly(G) products as a result of "idling" by the RNAP at the run of three C's on the template strand in the initiation region (Martin et al., 1988; Gross et al., 1992). Thus, products made from the test promoter are labeled by the incorporation of [α-^{32}P]ATP (Figure 1, lane 2), whereas products made from the sink promoter are not (Figure 1, lane 1); products from the latter promoter may be visualized if [α-^{32}P]GTP is the labeled substrate (data not shown).

To determine whether abortive initiation involves rapid dissociation of the RNAP from the promoter, a 10-fold molar excess of the sink promoter was added to reactions either before or after abortive cycling had been initiated at the test promoter, and the production of the 6 nt product was monitored during a subsequent 2 min incubation period. As seen in Figure 1 (lane 5), the addition of the sink promoter prior to the addition of the substrate inhibited the synthesis of abortive products at the test promoter. In contrast, if the sink promoter was added after abortive cycling had been initiated, continued accumulation of the 6 nt product was observed during the subsequent 2 min incubation at nearly control levels (i.e., the level observed in the absence of sink; compare Figure 1, lanes 2 and 6). The presence of equivalent amounts of nonspecific DNA that does not contain a T7 promoter had little effect on the production of abortive products when added prior to substrate (not shown). These

Table 1: Sequences of Promoter and Test DNA[a]

Plasmids:	
	-20 -10 +1 10 20
	. . . .
pLM45:	5′.....TCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCT.....3′
	3′.....AGCTTTAATTATGCTGAGTGATATCCCTCTGGTGTTGCCAAAGGGAGA.....5′
Bluescript II SK+:	5′.....GAGCGCGCGTAATACGACTCACTATAGGGCGAATTTGGGTACCGGCCC.....3′
	3′.....CTCGCGCGCATTATGCTGAGTGATATCCCGCTTAAACCCATGGCCGGG.....5′
Synthetic DNA:	
ds test promoter:	5′ TCGAAATTAATACGACTCACTATAGGGAGACCACAACCGCGCGTTCTG 3′
	3′ AGCTTTAATTATGCTGAGTGATATCCCTCTGGTGTTGGCGCGCAAGAC 5′
partially ss test promoter	5′ TCGAAATTAATACGACTCA 3′
	3′ AGCTTTAATTATGCTGAGTGATATCCCTCTGGTGTTGGCGCGCAAGAC 5′
ds sink promoter:	5′ ATTAATACGACTCACTATAGGGT 3′
	3′ TAATTATGCTGAGTGATATCCCA 5′
partially ss sink promoter	5′ ATTAATACGACTCA 3′
	3′ TAATTATCGTGAGTGATATCCCA 5′
ds non-promoter:	5′ CTATGTATTCTGTAACTAGATTGC 3′
	3′ GATACATAAGACATTGATCTAACG 5′

[a] The promoter in pLM45 is the consensus T7 promoter, Φ10 {314}; initiation is at +1. The ds test promoter has the same sequence as Φ10 from −24 to +13. The ds and ss sink promoters have the same sequence as the T7 promoter in Bluescript. The ds nonpromoter DNA has the same base composition as the consensus T7 promoter (Φ10), but in a scrambled order.

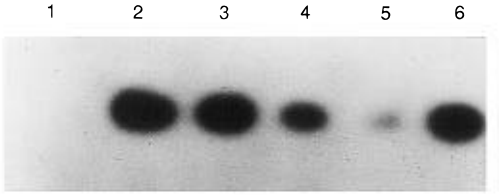

FIGURE 1: Excess plasmid containing a T7 promoter effectively prevents abortive transcription from pLM45. Sequences of promoters used as test and sink promoters are given in Table 1. Transcription reactions were assembled as described under Materials and Methods, with the following variations. Lane 1: 0.5 pmol of Bluescript as test promoter (no sink DNA). Lane 2: 0.5 pmol of pLM45 as test promoter (no sink DNA). Lanes 3−5: 0.5 pmol of pLM45 as test promoter, mixed with 0, 2.5, or 5 pmol of Bluescript, respectively, prior to addition of RNAP. Lane 6: 0.5 pmol of pLM45 as test promoter, 5 pmol of Bluescript as sink promoter, added at the same time as radiolabel. Transcription reactions proceeded for 2 min, and the products were analyzed by PAGE.

results demonstrate the utility of a sink promoter in preventing synthesis of labeled product by free RNAP, and suggest that most of the abortively cycling RNAP remains in association with the test promoter during the course of the reaction.

Abortive Cycling by T7 RNAP on a Supercoiled Template Is Nondissociative. To confirm that abortive cycling by T7 RNAP is nondissociative, and to establish the kinetic parameters of the process, time-course studies were carried out. To extend the flexibility of the system, and in order to allow the use of higher molar concentrations of sink promoter, the plasmid-based promoter used as a sink in the previous experiments was replaced with a synthetic promoter (see Table 1, ds sink promoter).

Figure 2A displays representative data from time-course experiments in which reaction mixtures containing pLM45 and RNAP were incubated with a 50-fold molar excess of synthetic sink either prior to or subsequent to the addition of substrate. As expected, the addition of sink promoter prior to the addition of substrate effectively inhibited synthesis of the 6 nt product. Addition of sink DNA to reactions that were already engaged in abortive cycling resulted in continued production of the abortive products over a 60 min period, but at steadily decreasing rates. The shape of the curve indicates that the RNAP and the test promoter maintain an association over repeated cycles of abortive initiation events, but that these complexes decay during the course of the reaction.

The stabilities of RNAP complexes with promoters may be derived from the data shown in Figure 2A using the kinetic model described under Materials and Methods. The results of these analyses from promoter complexes in different structural contexts are summarized in Table 2.

The half-life of initiation complexes with a promoter in supercoiled plasmid template is about 14 min. Elongation complexes paused at the site of a missing nucleotide, or blocked by a cross-link between the two DNA strands, have been found to have a similar stability (Shi et al., 1988; Sastry & Hearst, 1991) (Karasavas and McAllister, unpublished observation). The rate of 6 nt product accumulation in the absence of sink under these conditions is 0.3 mol^{-1} s (mole of enzyme)$^{-1}$ (Diaz, 1995). Given a half-life of the IC of 24 min, this means that, on average, each IC will carry out *ca*. 430 cycles before dissociating.

The Stability of Abortively Cycling T7 RNAP−DNA Complexes Depends upon Template Conformation. The test promoter used in the experiments above was in a supercoiled plasmid, and it was of interest to know whether the stability of the abortive cycling complexes might be affected by the conformation of the template. To address this question, the assays were repeated using pLM45 as a template in two additional conformations—linear and relaxed circular. Linearizing the template essentially abolished the resistance of the abortive initiation complexes to excess sink promoter, as did relaxing the plasmid with vaccinia virus topoisomerase (Figure 2B,C, Table 2). From these experiments, we may conclude that template superhelicity contributes importantly to the stability of the IC. A potential explanation for this finding is that the greater tendency of linear or relaxed

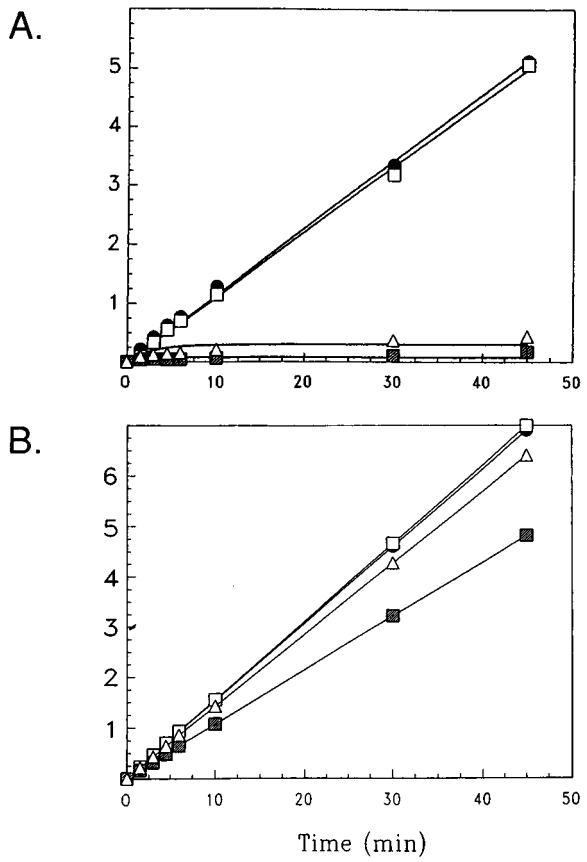

FIGURE 2: Kinetics of decay of abortively cycling complex on plasmid templates. Transcription reactions were performed as described under Materials and Methods. The test promoter was the Φ10 promoter in pLM45; the sink promoters are described in Table 1. Accumulation of hexameric transcript (in PhosphorImager units) is plotted as a function of reaction time following the addition of [α-^{32}P]ATP. Sink DNA was added as follows: (●) no sink DNA added; (□) ds nonpromoter sink; (■) ds sink promoter mixed with the template prior to the addition of radiolabel; (△) ds sink promoter added to ongoing reaction together with [α-^{32}P]ATP. The test promoter was either in supercoiled plasmid (panel A), linearized plasmid (panel B), or plasmid relaxed by vaccinia virus topoisomerase (panel C).

Table 2: Half-Lives of T7 RNAP Initiation Complexes at Various Promoters[a]

template	$t_{1/2}$ (min)[a]
supercoiled plasmid	14
linearized plasmid	<1
relaxed plasmid	<1
ds oligomer	<1
partially ds oligomer	>30

[a] Half-lives were determined from experiments such as that described in Figure 2 using the kinetic model described in the text.

templates to reanneal results in the displacement of the RNAP, the RNA, or both from the template, and that the relative underwinding of superhelical plasmid DNA contributes importantly to the stability of the IC. As the promoter is melted open during initiation, and as it has been shown that the NT strand in the melted region is not required for initiation, a prediction of this model is that a partially single-stranded promoter that lacks the NT strand should behave like a superhelical template in an abortive initiation assay (i.e., such a promoter is permanently "melted").

To test this, we constructed synthetic promoters that either were completely double stranded (ds) or were single stranded

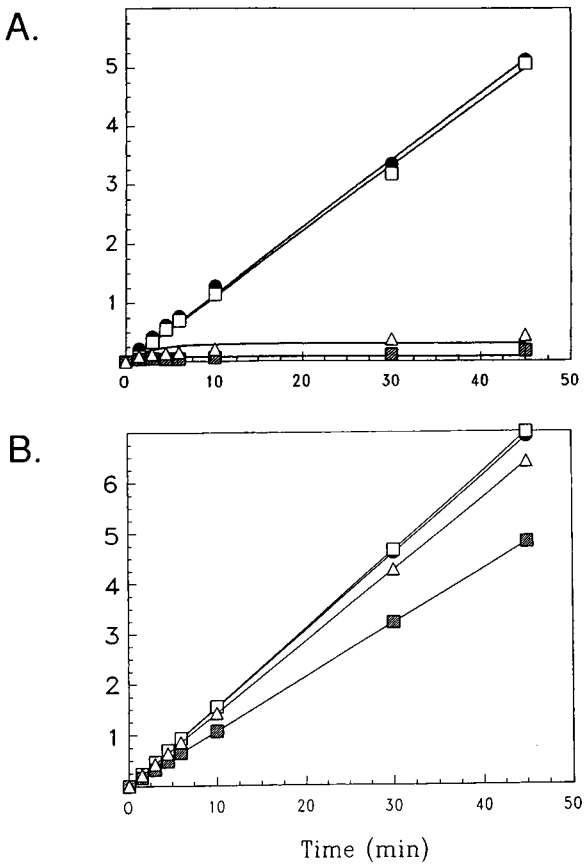

FIGURE 3: Kinetics of decay of abortively cycling complex on synthetic templates. Assays were performed as in Figure 2. The test promoter was a completely double-stranded oligomer (panel A) or partially single-stranded promoter (panel B; see Table 1). Sink DNA was added as follows: (●) no sink DNA added; (□) ds nonpromoter sink; (■) ds sink promoter mixed with the template prior to the addition of radiolabel; (△) ds sink promoter added to ongoing reaction together with [α-^{32}P]ATP.

(ss) in the initiation region (−5 to +6; see Table 1). As was the situation for linear DNA or a relaxed circular plasmid, product accumulation from the ds synthetic promoter is sensitive to challenge by the sink promoter (Figure 3A). In contrast, synthesis of abortive products from a partially ss promoter is highly resistant to challenge by the sink promoter (Figure 3 B).

Surprisingly, the addition of a 50-fold molar excess of ds sink DNA prior to the addition of substrate failed to inhibit the subsequent accumulation of abortive transcripts from the partially ss promoter (Figure 3B; $t_{1/2}$ > 30 min). These results are reproducible using different preparations of template, and with up to a 250-fold excess of sink DNA (not shown). In these experiments, the RNAP and the test promoter were mixed together before the addition of the sink promoter. The resistance of these complexes to the subsequent addition of sink promoter suggests that the RNAP may bind more readily to, or form an unusually stable binary complex with, the partially ss template.

Partially Single-Stranded Promoter Templates Form Highly Stable Binary Complexes with RNAP. To examine these questions, experiments were performed in which the order of addition of the reaction components was varied. In the first set of reactions, RNAP was allowed to form a binary complex with the test promoter prior to addition of the sink promoter (Figure 4, lanes 1−6). In the second set of reactions, the test promoter and the sink promoter were mixed

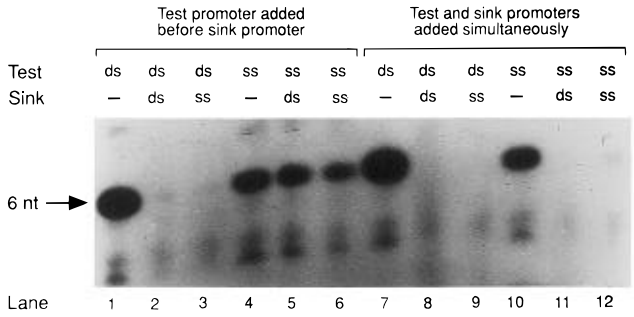

FIGURE 4: Effect of order of addition of components. Transcription reactions were performed at 37 °C as described under Materials and Methods. In lanes 1−6, RNAP was mixed with the test promoter, and sink promoter was added 1 min later. In lanes 7−12, test promoter and sink promoter were mixed together prior to addition of RNAP. After 1 min, GTP and [α-^{32}P]ATP were added, and the reaction was allowed to proceed for a further 5 min. The synthetic test and sink promoters were either completely double-stranded (ds) or partially single-stranded (ss), as noted (see Table 1). The position of the 6 nt product made from the test promoter is indicated.

together before the addition of RNAP (Figure 4, lanes 7−12). Both sets of reactions were allowed to equilibrate for 1 min prior to the initiation of transcription.

When the test promoter is incubated with RNAP prior to the addition of sink promoter, the ds test promoter (but not the partially ss test promoter) is quenched by excess ds or partially ss sink DNA (Figure 4, lanes 1−6). These results are consistent with those of the time-course assays described above, in which the reaction components were added in the same order (see Figures 2 and 3). In contrast, when the test promoter and the sink promoter are mixed prior to the addition of RNAP, the partially ss promoter is inhibited by both ds and partially ss sink promoter (Figure 4, lanes 7−12).

Tight Binding of T7 RNAP to Partially Single-Stranded Promoters Revealed by Gel Permeation Assays. The binding parameters of the T7 RNAP for ds or partially ss promoters were determined directly by means of a gel retardation assay. Synthetic DNA oligomers that form a base pair structure having a fully duplexed promoter region (oligo A, Figure 5A) or a partially ss promoter region (oligo B, Figure 5A) were labeled with ^{32}P and incubated with varying concentrations of T7 RNAP. The relative amounts of oligomer bound to RNAP or remaining free were then determined by electrophoresis in nondenaturing polyacrylamide gels (Figure 5B). The binding constant (K_b) for each oligomer was determined as the slope of a plot of the ratio of bound *vs* free oligomer as a function of enzyme concentration (Figure 5C) (Ikeda & Richardson, 1986). The results demonstrate that the apparent affinity of T7 RNAP for the partially ss promoter is at least 5 times greater than for the fully ds promoter. Under these conditions, the RNAP did not exhibit significant binding to oligomers having a random sequence but a similar structure, indicating that this effect is promoter-specific.

DISCUSSION

The formation of an unstable initiation complex that carries out multiple cycles of abortive initiation before making the transition to a stable elongation complex appears to be characteristic of all RNAPs studied (Carpousis & Gralla, 1980; Ackerman et al., 1983; Luse & Jacob, 1987; Martin et al., 1988; Ling et al., 1989). However, the mechanisms by which the transition to a stable complex occurs and stability is conferred are not understood. In view of the information that is available concerning the structure of T7 RNAP, a better understanding of the process of abortive cycling by this enzyme is of general interest.

A number of factors that might be expected to contribute to the production of abortive transcripts by T7 RNAP have been studied previously. Removal of the nontemplate strand in the transcribed region does not suppress abortive cycling [(Martin et al., 1988) and this work], indicating that the tendency of the DNA strands in the open complex to reanneal is not solely responsible for abortive cycling. Similarly, mutations in the binding region of the promoter which diminish RNAP−promoter affinity do not suppress abortive

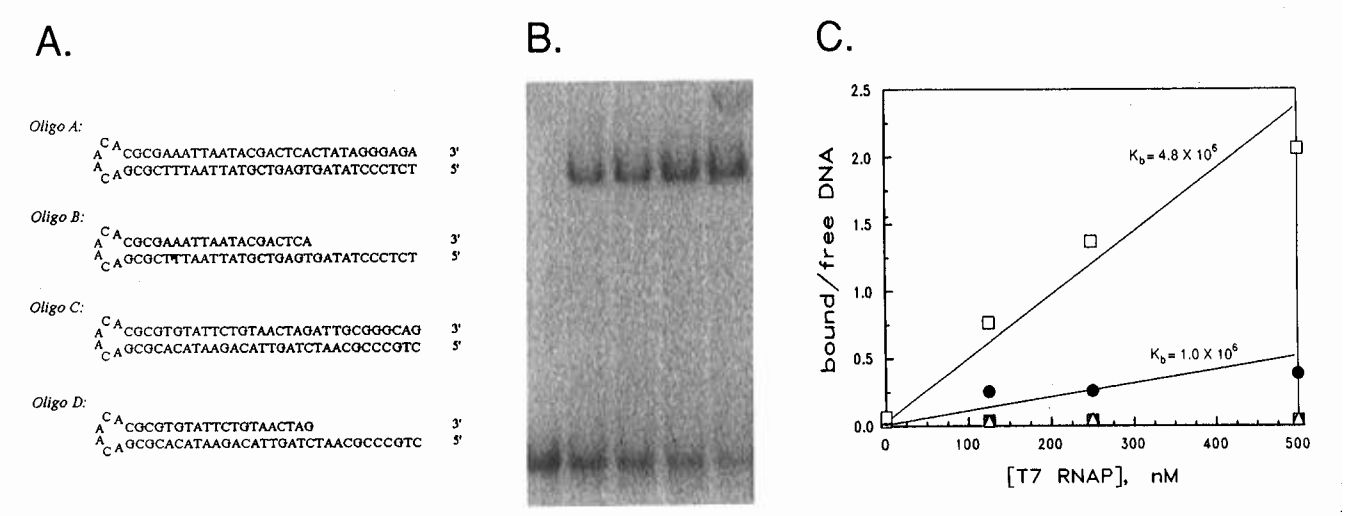

FIGURE 5: Gel retardation assay of RNAP−DNA complexes. Panel A: Oligomeric DNAs were synthesized to include either a promoter (A, B) or a sequence with the same base composition but in randomized order (C, D). The oligomers were designed to self-hybridize in a hairpin configuration (in order to ensure complete annealing of template and nontemplate strands) with either fully duplexed termini (A, C) or a recessed 3′ end resulting in a single-stranded region of 12 nucleotides on the "template" strand (B, D). Panel B: Promoter-containing DNA bound by RNAP was separated from unbound DNA by electrophoresis on nondenaturing PAGE. Results shown are those involving oligo B; RNAP was present at 0, 125, 250, 500, or 1000 ng (lanes 1−5). Panel C: The ratio of bound to unbound DNA is plotted as a function of RNAP concentration. The slope of the line corresponds to the binding constant (K_b) between the RNAP and DNA. (●) Oligo A; (□) oligo B; (■) oligo C; (△) oligo D.

cycling, indicating that the energetic cost of breaking promoter interactions is also not the primary cause of abortive cycling (Martin et al., 1988). Factors that do influence the production of abortive transcripts by the enzyme include the concentration of substrates, the initially transcribed sequence of the promoter, temperature, and ionic strength (Martin et al., 1988; Ling et al., 1989). These observations suggest that the production of abortive products is the result of a complex interplay between enzyme, template, and transcript.

The experiments described in this work demonstrate that abortive cycling by T7 RNAP is also influenced by the topology of the template. Abortive cycling at promoters in double-stranded linear or relaxed circular templates is a dissociative process, and the addition of excess sink promoter, either before or after transcription initiation, rapidly prevents significant transcript accumulation from these promoters (Figure 1). In contrast, initiation complexes formed on supercoiled and partially single-stranded templates continue to produce substantial amounts of transcript without leaving the template, and are resistant to the addition of excess sink (Figures 2 and 3). Unexpectedly, we have found that T7 RNAP forms a highly stable complex at partially single-stranded promoters even in the absence of RNA synthesis (i.e., in a binary complex; see Figure 3B).

The recent crystallization of T7 RNAP (Sousa et al., 1993) has suggested a physical basis for the switch from an unstable EC to a stable EC. The RNAP contains a prominent cleft defined by structural elements (fingers, palm, thumb) which are conserved in DNA polymerase and reverse transcriptase (Sousa et al., 1993; Joyce & Steitz, 1994). Isomerization to an elongation complex is proposed to result from movement of the conserved thumb subdomain, thereby closing off the binding cleft and preventing the dissociation of the RNAP from the template (Bonner et al., 1994a; Sousa et al., 1994).

Previous work with T7 RNAP has demonstrated that an amino-terminal RNA-binding domain is important for processive elongation (Muller et al., 1988; Martin et al., 1988) as well as for sequence-specific termination (Macdonald et al., 1993, 1994), and it has been suggested that an interaction of the nascent RNA with the RNA-binding domain triggers the conformation shift noted above (Sousa et al., 1992; Macdonald et al., 1993, 1994). In this model, continued interaction of the emerging RNA with the binding site is required to maintain a stable EC, and changes in the nature of this interaction are thought to trigger a reversal of the isomerization, resulting in termination (Sousa et al., 1992; Macdonald et al., 1993, 1994).

The studies presented here, however, demonstrate that changes in the conformation of the template can dramatically affect the stability of the initiation complex even in the absence of a stable RNA−RNAP interaction. Thus, for example, the abortively cycling complexes formed on a supercoiled promoter are nearly as stable as paused elongation complexes, even though the RNA product is continuously being synthesized and released. Perhaps more strikingly, T7 RNAP forms a highly stable complex with partially single-stranded promoters even in the absence of RNA synthesis (Figures 4 and 5).

A possible explanation for these findings is that the nature of a supercoiled template or partially single-stranded promoter allows the formation of a stable binary complex in the absence of a conformation change. For example, T7 RNAP is known to be able to bind nonspecifically to single-stranded DNA, and this may contribute to the stability of IC formed at single-stranded promoters or on supercoiled templates. Alternatively, the template may need to be melted in the initiation region prior to stabilization of the complex. In either case, a highly stable duplex DNA template would interfere with this isomerization.

In earlier work, Maslak and Martin measured the rate of accumulation of a short (5 nt) runoff product from promoter-containing oligomers as a function of DNA concentration, thus determining a K_m value for the promoter in this reaction (Maslak & Martin, 1993). This value was taken as an approximation to a dissociation constant (K_d) of the enzyme−promoter complex. There is an apparent discrepancy between the earlier results and those reported in this study, in that Maslak and Martin found little difference in the estimated K_d if the promoter was double-stranded or single-stranded in the initiation region, whereas our results imply that there is a large effect. This difference may reflect the nature of the assays involved. Indeed, using stopped-flow kinetics, the same group has recently found that T7 RNAP dissociates from a partially single-stranded promoter approximately 100 times more slowly than from a double-stranded promoter (C. Martin, personal communications).

The NT strand may also be involved in the displacement of the nascent RNA product. We have observed that the accumulation of product on partially single-stranded promoters is significantly reduced relative to that on double-stranded promoters, even though the stability of the RNAP−DNA complex is greater on the former (compare Figure 4, lanes 1 and 7 *vs* lanes 4 and 10).

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