The T7 RNA Polymerase Intercalating Hairpin Is Important for Promoter Opening during Initiation but Not for RNA Displacement or Transcription Bubble Stability during Elongation†

Luis G. Brieba and Rui Sousa*

Department of Biochemistry, University of Texas Health Sciences Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7760

Received November 28, 2000; Revised Manuscript Received January 26, 2001

ABSTRACT: The recently described crystal structures of a T7RNAP−promoter complex and an initial transcription complex reveal a β-hairpin which inserts between the template and nontemplate strands of the promoter [Cheetham, G. M., et al. (1999) *Nature 399*, 80; Cheetham, G. M., et al. (1999) *Science 286*, 2305]. A stacking interaction between the exposed DNA bases and a valine at the tip of this hairpin may be especially important for stabilizing the opened promoter during initiation. It has been suggested that this hairpin may also be important for holding the transcription bubble open during transcript elongation, and a proposed model for how the RNA exits the transcription complex implies that this hairpin may also help displace the RNA from the template strand. To test these hypotheses, we have characterized both point and deletion mutants of this element. We find that these mutants exhibit reduced activity on linear, double-stranded templates but not on supercoiled or partially single-stranded templates. Probing of promoter−polymerase complexes, initial transcription complexes, and elongation complexes with $KMnO_4$ and a single-strand specific endonuclease reveals that the mutants have greatly reduced promoter unwinding activity during initiation. However, the structure and stability of the transcription bubble during elongation are not altered in the mutant enzymes, and RNA displacement activity is also normal. Thus, the T7RNAP intercalating hairpin is important, though not essential, for stabilizing the opened promoter during initiation, but is not important for RNA displacement or for transcription bubble structure or stability during elongation.

Following promoter binding, RNA polymerases must open the DNA to expose the template bases for pairing with NTPs. This essential step in transcription initiation is a critical point of transcriptional regulation, and the regulatory and kinetic aspects of promoter opening have consequently been the focus of intense study. The structural mechanisms of this process are, however, less well understood. Structure−function studies have led to proposals that aromatic side chains from the polymerase could stack on exposed DNA bases to stabilize the opened DNA (*1*−*4*). The recently described crystal structures of Taq core RNAP[1] (*5*) and of T7RNAP−promoter (*6*) and initial transcription complexes (ITCs) (*7*) reveal β-hairpins which appear to be positioned to insert between the template (T) and nontemplate (NT) strands of the DNA and/or between the RNA and template (*8*), to separate paired strands. The T7RNAP−promoter complex also reveals direct interactions between the side chains of this intercalating β-hairpin and DNA bases. In particular, a stacking interaction between Val237 at the tip of this hairpin and the −5 base pair has been proposed to be important for stabilizing the melted DNA (*6*). Thus, both wedging open of the DNA by insertion of a β-hairpin between the T and NT strands and stacking interactions between a hydrophobic side chain and exposed bases may play a role in T7 promoter opening. The structure of the T7RNAP ITC also led to a proposal that the RNA exits the transcription complex (TC) through a cleft between the thumb subdomain of the RNAP and this intercalating β-hairpin (*7*), suggesting that the intercalating hairpin could also play a role in holding apart the RNA and T strand immediately upstream of the RNA−DNA hybrid (Figure 1).

To evaluate the role of the T7RNAP intercalating hairpin in template unwinding and RNA displacement, we constructed point and deletion mutants in this structure. Our results reveal that the intercalating hairpin is important, though not essential, for promoter opening during initiation, but that it is not important for RNA displacement or maintenance of the transcription bubble during elongation.

MATERIALS AND METHODS

Mutant Construction and Protein Purification. V237A, V237G, and the Δ236−238 deletion were constructed with the Quick Change Site-Directed Mutagenesis Kit (Stratagene) using pDT7 (*9*) as a template. The Δ231−241 deletion was constructed using the PCR−SOEING method as described previously (*10*). Mutant G235D DNA was provided by F. W. Studier and X. Zhang (*11*). Proteolytically nicked

† Supported by NIH Grant GM 52522 (to R.S.) and a Fulbright-CONACYT fellowship (to L.G.B.).

* To whom correspondence should be addressed. Phone: (210) 567-8782. Fax: (210) 567-8778. E-mail: sousa@biochem.uthscsa.edu.

[1] Abbreviations: RNAP, RNA polymerase; EC, elongation complex; ITC, initial transcription complex; TC, transcription complex; T strand, template strand; NT strand, nontemplate strand; ds, double-stranded; pss, partially single-stranded.

10.1021/bi002716c CCC: $20.00
Published on Web 03/03/2001

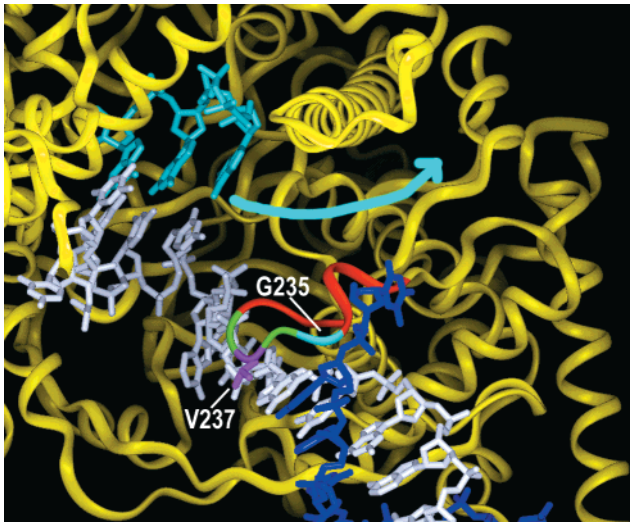

FIGURE 1: Structure of a T7RNAP ITC[7] (PDB entry QLN1). The template and NT strands are white and blue, respectively. A three-base RNA molecule is cyan, and a previously proposed exit path for the RNA[7] is represented by the cyan arrow. The mutagenized side chains V237 and G235 are magenta and cyan, respectively. The two deletions (Δ236−238 and Δ231−241) are indicated with green for residues 236 and 238, and with red for residues 231−234 and 239−241.

polymerase was prepared as described previously (*12*). Wild type and mutant RNAPs were purified as described previously (*9*).

Template Preparation for Activity Assays. A 46-base oligonucleotide (5′-GTTAATTATGCTGAGTGATAT^{+1}CCCTC-TGGCCTTAAGCTCGAGCGGG) was synthesized on an Applied Biosystems DNA synthesizer. This oligonucleotide was annealed to either a complementary 46-base oligonucleotide to generate a double-stranded (ds) promoter template or a 17-base oligonucleotide corresponding to the segment of the T7 promoter from position −21 to −5, to generate a partially single-stranded (pss) template. Plasmid DNAs were purified with the Qiagen Maxiprep kit according to the manufacturer's instructions.

Activity Assays. Measurement of the rates of runoff transcript synthesis was carried out by incubating RNAPs at a final concentration of 0.5 μM in reaction mixes containing either pss or ds promoter (0.1 μM) for 5, 10, or 20 min at 37 °C in transcription buffer (*13*) with NTP concentrations of 0.5 mM and 0.1 mCi/μL of 800 mCi/mM [α-^{32}P]GTP. Abortive initiation assays were carried out similarly, but with only GTP and ATP present at concentrations of 20 and 200 μM, respectively, to limit transcript synthesis to a 6mer. Measurement of the rate of transcript synthesis on supercoiled and linear templates was carried out as described previously (*14*), using either supercoiled or *Pvu*II-linearized pT75 (*15*), as templates. Reactions were stopped by addition of equal volumes of 95% formamide, 25 mM EDTA, and 0.1% xylene cyanol, resolved by PAGE in 20% polyacrylamide, 1% bisacrylamide, 7 M urea gels in 1× TBE, and analyzed with a Molecular Dynamics phosphorimager.

Template Preparation and Binding Experiments. To generate a promoter labeled on the NT strand, a *Pvu*II−*Hin*dIII fragment from pT7-5 (*15*) was labeled with [α-^{32}P]-dCTP using Klenow fragment (Promega). T strand-labeled promoter was prepared from *Hin*dIII-linearized pT7-5 using [γ-^{32}P]ATP and T4 kinase, followed by cutting with *Pvu*II and gel purification. Labeling of the 5′-end of the T strand of a pss promoter was carried out with [γ-^{32}P]ATP and T4 kinase, after which the labeled T strand was annealed to the NT strand. Unincorporated NTPs were removed with the Qiagen nucleotide removal kit. Promoter−RNAP complexes were formed in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 4 mM $MgCl_2$, and 8% glycerol (*16*). Binding reactions were carried out with the labeled promoter at 2×10^{-8} M and polymerase at 6×10^{-7} M, in the presence and absence of 1 mM 3′-dGTP (Trilink Biotechnologies). After 10 min at 37 °C, 0.25 unit of *Ase*I (NEB) was added. After an additional 10 min, digestion was stopped by addition of an equal volume of 95% formamide, 25 mM EDTA, and 0.1% xylene cyanol. Reaction mixtures were resolved by PAGE in 6% polyacrylamide, 0.3% bisacrylamide, 8 M urea gels in 1× TBE and analyzed by phosphorimaging.

Promoter Opening Assessed by Endonuclease or $KMnO_4$. Promoter opening was assessed under the same conditions used in the binding experiments. After formation of the promoter−polymerase complexes, T7 endonuclease (NEB) was added to a final concentration of 1.2 units/μL. Reactions were stopped after either 15 or 45 min and analyzed as described for the binding reactions. Permanganate treatment was carried out by forming promoter−polymerase complexes in binding buffer with the labeled promoter at 2×10^{-8} M and RNAPs at 6×10^{-8} M. Complexes were formed in the presence of different mixtures of NTPs (as indicated in the figure legends). $KMnO_4$ was added to a concentration of 1.5 mM and allowed to react for 1 min, before being quenched by addition of $^1/_{10}$ reaction volume of 50% β-mercaptoethanol. Cleavage of modified nucleotides was carried out by addition of $^1/_{10}$ volume of piperidine and incubation at 90 °C for 15 min.

Elongation Complex (EC) Stability. Halted ECs were formed with either *Bgl*II-linearized or supercoiled pKP10 (*17*) and RNAPs at 0.1 mM in the presence of GTP, ATP, and 3′-dUTP at 0.5 mM, and 0.1 mCi/μL of 800 mCi/mM [α-^{32}P]CTP in transcription buffer. After 5 min at room temperature, heparin was added to a final concentration of 0.2 mg/mL to prevent further initiation. Reaction mixtures (30 μL) were incubated for an additional 2 h at room temperature, and then applied to Centricon ultrafiltration units (Amicon) with a 100 kDa molecular mass cutoff. Centrifugation was carried out at 14 000 rpm for 5 min at room temperature, and the filtrates and retentates were resolved by PAGE in 20% acrylamide, 1% bisacrylamide, 7 M urea gels in 1× TBE and analyzed by phosphorimaging.

RNA Displacement Assessed by RNAse H Sensitivity. Rnase H at a final concentration of 0.5 unit/μL was added to halted ECs formed as described for the EC stability experiments. Reaction aliquots were taken at 1, 2, 4, and 8 min, and resolved and analyzed as described for the EC stability experiments.

RESULTS

Mutagenesis of the T7RNAP Intercalating Hairpin. Figure 1 shows the crystal structure of a T7RNAP ITC (*7*). The promoter is unwound downstream of position −5, and the NT strand is disordered downstream of position −4. A stacking interaction between Val237 of the intercalating

hairpin and the −5 base pair was observed in this structure. This interaction was proposed to be especially important in stabilizing the opened DNA (*6*). We therefore generated V237A and V237G mutations to assess the effects of progressively reducing the hydrophobic surface of the side chain at this position. Initial characterization revealed that neither of these point mutations fully disrupted template unwinding, so to more completely disrupt the function of the intercalating hairpin, we constructed two deletions which removed either 3 (Δ236−238) or 11 (Δ231−241) amino acids from this element. We also characterized a point mutation (G235D) which had been identified in a screen for T7RNAP mutants which were hypersensitive to the inhibitory effects of T7 lysozyme (*11*).

The Mutations Reduce Activity on Double-Stranded, but Not Single-Stranded, Templates. Figure 2 shows the activity of the mutant enzymes in multiple-round runoff transcription reactions. In this experiment, and those which follow, we also characterize the activity of an enzyme ("nicked") which has been proteolytically cleaved between residues 172 and 173, but in which the N- and C-terminal polypeptides remain noncovalently associated (*12*). This nicked enzyme has been found to be partially deficient in both promoter opening (*18*) and RNA displacement (*19*), and therefore provides a valuable comparison with the intercalating hairpin mutants. The templates that have been used either are fully double-stranded (ds) (Figure 2A, lanes 8−14) or lack the NT strand downstream of position −5 (Figure 2A, lanes 1−7). On the partially single-stranded (pss) template, the nicked enzyme and Δ231−241 are 2-fold less active than the wild type enzyme, while the other intercalating hairpin mutants exhibit wild type levels of activity. However, the intercalating hairpin mutants, with the exception of V237A, exhibit greatly reduced activity on the ds template (Figure 2A, lanes 8−14). Figure 2B plots the ratios of the activity of the different RNAPs on ds versus pss templates. G235D shows the largest specific reduction in activity on the ds template and, relative to the wild type or nicked enzymes, is ∼12-fold less active on ds than on pss templates. V237G and the two deletion mutants are 5−9-fold less active, relative to the wild type or nicked enzymes, on the ds than on the pss templates.

To determine if the reduced activities of the mutant enzymes on ds templates were due to a reduction in the initiation rate, we carried out multiple-round abortive transcription assays with ds and pss templates using NTP limitation to restrict transcript synthesis to six nucleotides. In these reaction mixtures, the concentrations of the initiating NTPs were kept low so that transcript synthesis would be limited by the initiation rate, since at saturating NTP levels the rate of short transcript synthesis by T7RNAP is limited by the rate of transcript dissociation (*18*, *20*). The relative rate of initiation on the ds versus pss templates is plotted in Figure 2C, and reveals that the intercalating hairpin mutations specifically reduce the rates of initiation on ds templates, and that G235D displays the largest reduction in activity.

We also compared the activity of the mutant enzymes on supercoiled and linear templates (Figure 3). On a supercoiled plasmid template (Figure 3A), all of the enzymes, with the exception of the nicked enzyme, are similarly active (within a factor of ∼2). However, on the same template linearized by restriction digestion (Figure 3B), the intercalating hairpin

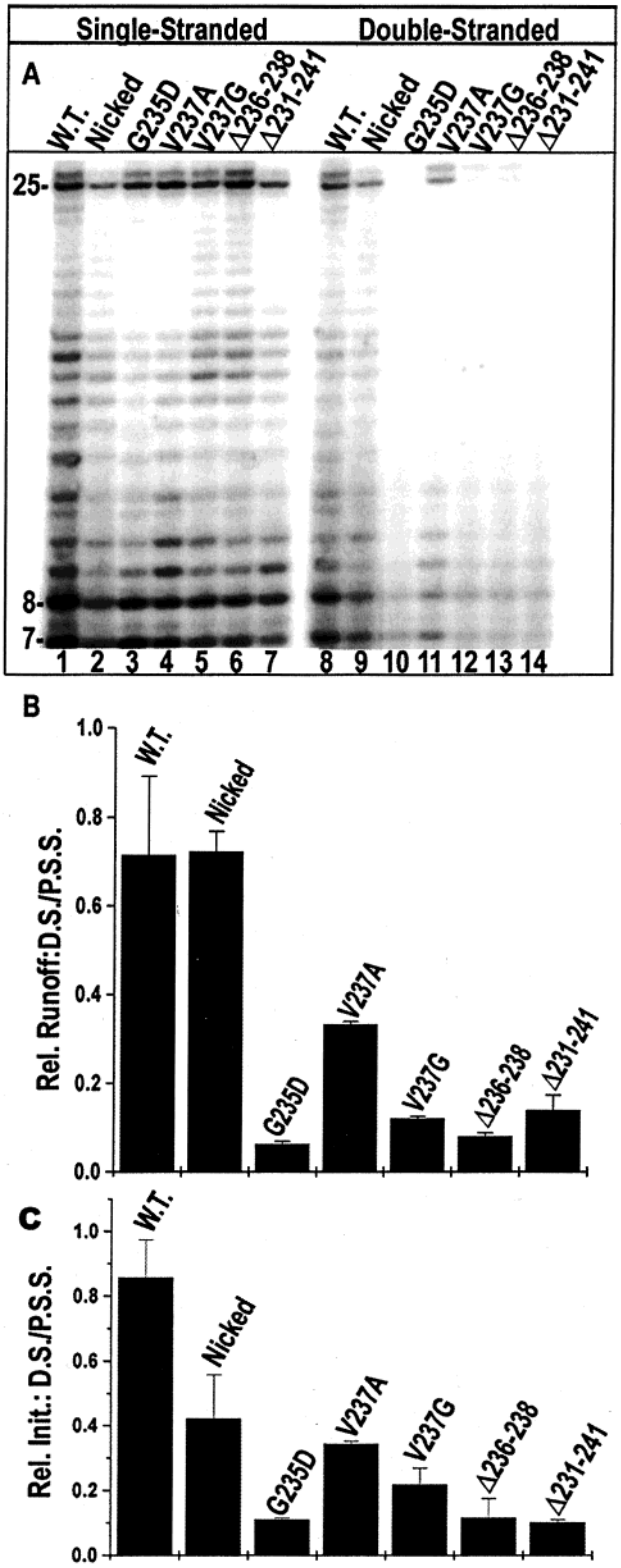

FIGURE 2: Activity of intercalating hairpin mutants on double- and single-stranded templates. (A) Transcription reactions carried out with the indicated mutants on either pss (lanes 1−7) or fully ds templates (lanes 8−14) of identical sequence. The product of runoff transcription is 25 bases in length. (B) Plot of the ratio of runoff transcription by the indicated enzymes on ds vs pss templates. Error bars show the range from three experiments. (C) Plot of the ratio of initiation rates of the enzymes in abortive initiation assays on ds vs pss templates. Error bars show the range from two experiments.

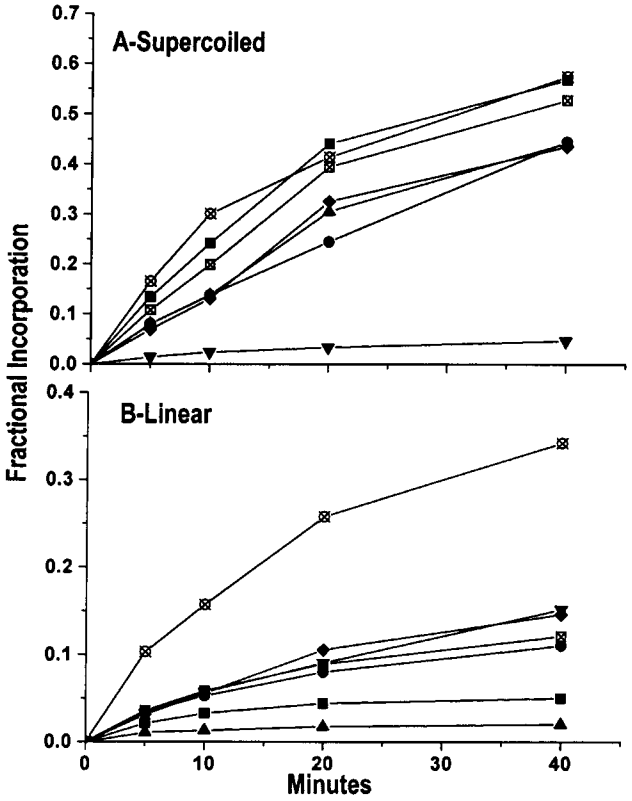

FIGURE 3: Activity of intercalating hairpin mutants on supercoiled and linear templates. (A) Fractional incorporation as a function of time using a supercoiled plasmid template and the following RNAPs: (■) Δ236−238, (●) Δ231−241, (▲) G235D, (▼) nicked, (◆) V237A, (⊞) V237G, and (⊕) wild type. The template and RNAP concentrations are 0.5×10^{-8} M. (B) As described for panel A but using a linearized plasmid template.

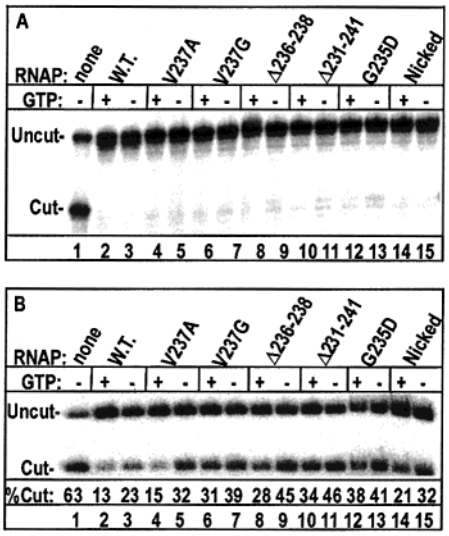

FIGURE 4: T7 promoter occupancy by intercalating hairpin mutants as measured by the level of protection from restriction digestion. (A) *Ase*I-treated complexes formed with the indicated RNAPs and a partially single-stranded template (see Materials and Methods for the sequence) either with (even-numbered lanes) or without (odd-numbered lanes) 1 mM 3′-dGTP present. (B) As described for panel A, but using a fully ds template, the percent template cut in this experiment is given below the gel lanes.

mutants are markedly less active, with G235D again displaying the largest reduction in activity.

Since it is conceivable that the activity of any structurally altered enzyme might be enhanced on supercoiled or pss templates relative to ds templates, we compared the activity of a number of active site mutants (Y639M, Y639L, D552S, H784A, H784Q, L637A, F644A, and G645A) and a mutant with a thumb subdomain deletion (Δ359−381), on ds and pss templates. We found that the activity of such mutants was similarly reduced on both templates (not shown). The specific reductions in activity of the intercalating hairpin mutants on the ds versus pss templates are not, therefore, a general feature of any polymerase with reduced activity or altered structure.

The Intercalating Hairpin Mutants Are Deficient in Promoter Unwinding As Assessed by Sensitivity to $KMnO_4$ or Single-Strand Specific Endonucleases. The specific reductions in the activity of the intercalating hairpin mutants on linear, ds templates versus pss or supercoiled templates suggest that these mutants are specifically deficient in promoter opening. To test this directly, we used $KMnO_4$, which reacts with unpaired thymidines, and single-strand specific T7 endonuclease, which has been previously shown to cleave the NT strand in an open promoter complex at position −6 (*21*). It was first essential to determine whether the mutant enzymes could form complexes with a T7 promoter, since it would otherwise be impossible to distinguish between a deficiency in promoter opening and loss of promoter binding. To assess promoter−polymerase complex formation, we used protection from *Ase*I, which cuts the T7 promoter between positions −17 and −18 on the NT strand, and between positions −15 and −16 on the T strand. Binding of T7RNAP should block digestion of the promoter by *Ase*I. Figure 4A shows an experiment in which a pss promoter was treated with *Ase*I either alone (lane 1) or in the presence of the different RNAPs (lanes 2−15). One set of reaction mixtures (even numbered lanes) also contained 1 mM 3′-dGTP. In the absence of any RNAP, 60−70% of the promoter is digested by a 10 min treatment with 0.25 unit of *Ase*I (lane 1), but addition of the polymerases completely blocks digestion (lanes 2−15). Figure 4B shows the results of a similar experiment carried out with a fully ds promoter. Protection from *Ase*I digestion by RNAP is less effective with the ds promoter than with the pss promoter, consistent with studies showing that T7RNAP binds to ds promoters with lower affinity than to pss promoters (*22*). It is also seen that the mutant enzymes bind somewhat more poorly than the wild type enzyme. However, in all cases the addition of RNAP leads to protection from *Ase*I digestion with the percent occupancy of the promoter by RNAP, as calculated from the decrease in the exponential rate of decay of the intact DNA, ranging from 74% for the wild type enzyme to 39% for the Δ231−241 mutant (in the absence of 3′-dGTP). The calculated occupancy for the wild type enzyme corresponds to an apparent dissociation constant of 2×10^{-7} M, which is consistent with previously measured values (*16*, *22*, *23*). Addition of 1 mM 3′-dGTP (even-numbered lanes) increases the level of protection of the promoter, in agreement with previous observations that addition of the initiating NTPs stabilizes the RNAP−promoter complex (*23*).

To determine whether the ds promoters are unwound in these complexes, they were treated with T7 endonuclease (Figure 5). In reactions with the wild type or nicked enzymes and in the presence of 1 mM 3′-dGTP (lanes 2 and 14, respectively), ∼60% of the NT strand is cut at position −6 by a 15 min treatment with 2.5 units of endonuclease (the

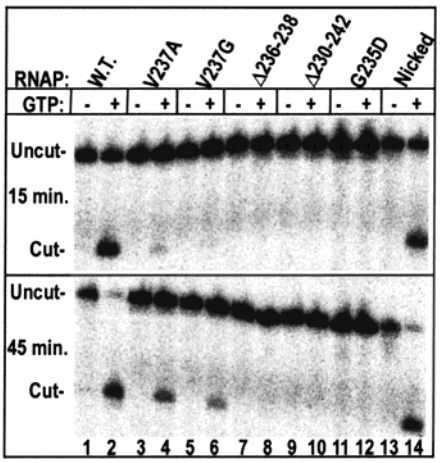

FIGURE 5: Promoter opening in mutant RNAP−promoter complexes as measured by sensitivity to single-strand specific endonuclease. RNAP−promoter complexes formed with a ds template as described for Figure 4B and treated for either 15 or 45 min, as indicated, with T7 endonuclease.

position of the cut site was confirmed by comparison to a Maxam−Gilbert G+A sequencing ladder; not shown). In the absence of 3′-dGTP, no cutting is detected after 15 min; in a 45 min digestion, a small amount of cleaved product is detected with the wild type and nicked RNAPs even without 3′-dGTP, while in the reactions with 3′-dGTP, ~90% of the promoter is cut after 45 min. The observation that addition of 3′-dGTP greatly increases the sensitivity of the promoter to endonuclease is consistent with observations that the DNA in a T7RNAP−promoter complex exists predominately in a closed conformation and that the binding of the initiating NTP is required to stabilize the unwound form of the promoter (*18*, *24*). The intercalating hairpin mutants all display decreased levels of promoter opening, as revealed by the greatly decreased sensitivity to endonuclease. In the presence of 1 mM 3′-dGTP, only 6 or 20% of the promoter is cut after 15 or 45 min, respectively, in the reaction with V237A; with V237G no cutting was detected after 15 min, and only 8% was cut after 45 min. With the other intercalating hairpin mutants, cutting was undetectable even after 45 min with 3′-dGTP present.

The effects of the mutations on promoter opening and transcription bubble structure were also assessed by $KMnO_4$ reactivity. It has been previously demonstrated that the promoter in a T7RNAP−promoter complex formed with a linear template is unreactive with $KMnO_4$, indicating that in such a complex the promoter exists primarily in a closed form. It was also shown that $KMnO_4$ reactivity could be detected in a binary complex formed with a supercoiled template, or in a complex formed with a linear template upon addition of GTP and ATP, indicating that either supercoiling or addition of the first nucleotides stabilizes the open complex (*25*). Our results with the wild type enzyme and linear templates agree with these observations. In the presence of RNAP and 3′-dGTP (lane 5 in panels A and B of Figure 6), reactivity with $KMnO_4$ was weak and similar to that seen in the absence of RNAP (lane 4 in panels A and B). Upon addition of both GTP and ATP, we detected strong $KMnO_4$ reactivity at positions −1 and −3 of the template (T) strand (lane 6 in panel A), as well as at position −2 of the nontemplate (NT) strand (lane 6 in panel B). Weak reactivity at position −4 of the NT strand was also detected,

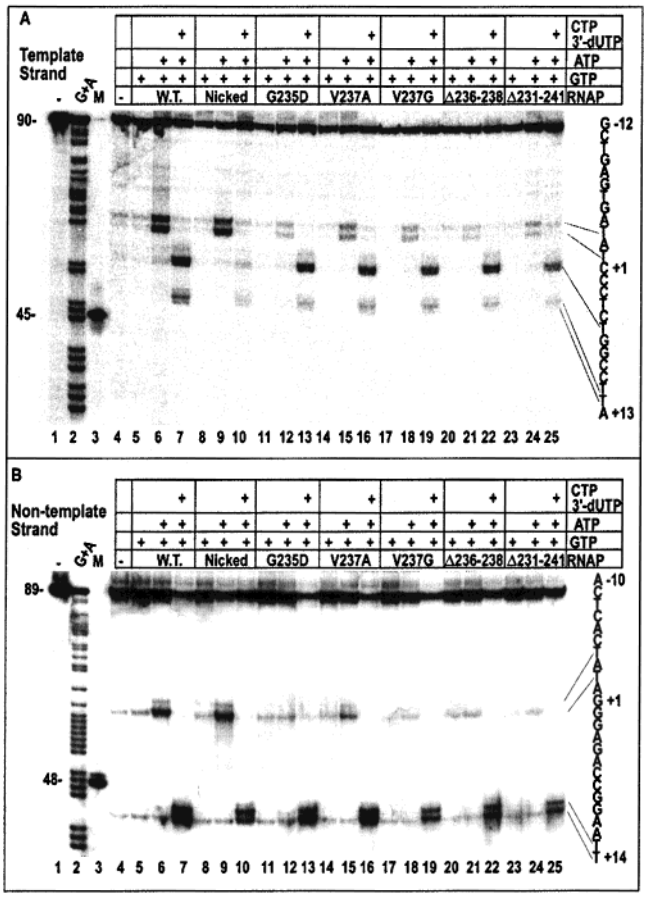

FIGURE 6: Promoter opening and transcription bubble structure in mutant RNAP−DNA complexes as measured by $KMnO_4$ sensitivity. Lanes 5−11 contained RNAP−DNA complexes formed with the indicated NTPs and linearized pT75 end-labeled on either the template (A) or nontemplate (B) strand (lanes 5−11), treated with $KMnO_4$, and subsequently cleaved with piperidine: lane 1, unreacted DNA; lane 2, Maxam−Gilbert (G+A) sequencing reaction; lane 3, DNA cut with *Eco*RI to generate markers of the indicated lengths; and lane 4, DNA alone reacted with $KMnO_4$ and cleaved with piperidine.

indicating that the −4 base pair in this initiation complex is only partially open. Since the addition of GTP and ATP allows extension of the RNA to position 6, and since it has been shown that the DNA in a T7RNAP elongation complex (EC) is closed almost immediately downstream of the RNA 3′-end (*13*), these observations imply that the bubble in this ITC extends from approximately position 6 to approximately position −3/−4. A bubble size of 9−10 bases would be consistent with the size of the bubble in an EC, as previously measured by $KMnO_4$ reactivity, or in an ITC as measured by topoisomerase relaxation (*13*, *18*). The $KMnO_4$ sensitivity of the nicked ITC (lane 9 in panels A and B) is similar to that of the wild type enzyme. However, the mutant ITCs all exhibit lower levels of reactivity with permanganate (lanes 12, 15, 18, 21, and 24). V237A displays a degree of reactivity intermediate between those of the wild type enzyme and the other mutants. These observations agree with those from the endonuclease sensitivity studies in showing that the mutant enzymes are deficient in holding the promoter open during initiation, with V237A displaying the least defective phenotype.

To determine whether the intercalating hairpin is also important for maintaining the structure of the transcription

bubble during elongation, we formed ECs by adding GTP, ATP, CTP, and 3′-dUTP to these reaction mixtures so that the RNA was extended to position 13. Upon addition of these NTPs to the reaction mixture with the wild type enzyme, the enhanced $KMnO_4$ reactivity between positions −1 and −4 disappears and strong reactivity is observed at positions 6 and 13/14 on the T and NT strands, respectively (lane 7 in panels A and B of Figure 6). This indicates that the transcription bubble in the EC halted at position 13 is 9−10 bases in length, and that the RNA−DNA hybrid may be 7 base pairs long. These values are consistent with the previous characterization of halted ECs (*13*). Interestingly, while the T strand bases at position 11/12 are expected to be paired with RNA, we detected weak $KMnO_4$ reactivity at these positions (lane 7 in Figure 6A). Similar observations were made by Kainz and Roberts (*26*) and Lee and Landick (*27*), who found that T strand segments expected to be paired with RNA in an *Escherichia coli* RNAP EC were reactive with $KMnO_4$ or DEPC, though the reactivity was much lower than at the margins of the transcription bubble. It may be that the 3′-end of the hybrid is fraying or that its structure diverges sufficiently from the canonical A- or B-form duplex to be weakly reactive with permanganate. The mutant ECs show patterns of $KMnO_4$ reactivity which are very similar to that of the wild type enzyme (lanes 13, 16, 19, and 25). Thus, at the resolution level of $KMnO_4$ footprinting, the intercalating hairpin mutants do not alter the presence or structure of the transcription bubble in an EC. Interestingly, the nicked EC displays reduced reactivity at position 6 on the T strand, though reactivity at positions 11/12 (T) and 13/14 (NT) is similar to that of the wild type EC (lane 10 in panels A and B of Figure 6). This could mean that the transcription bubble in the nicked EC is smaller than in the wild type EC. However, it is more likely that the reduced reactivity at position 6 in the nicked EC reflects formation of an extended RNA−DNA hybrid, since the nicked enzyme is defective in RNA displacement (*19*).

The Intercalating Hairpin Mutants Form Stable ECs. While $KMnO_4$ probing reveals the presence of a transcription bubble of apparently normal structure in mutant ECs, this observation does not rule out the possibility that the intercalating hairpin is important for transcription bubble stability. In particular, if mutations in the hairpin increase the tendency of the transcription bubble to collapse and displace the RNA, then they should increase the rate at which RNA is released from the EC. To assess this, we formed halted ECs on either linear or supercoiled templates and measured the amount of RNA released into solution (as assessed by ultrafiltration) after a 2 h room temperature incubation. On linear templates, all of the ECs, with the exception of that formed with the nicked RNAP, were very stable with only 3−7% of the RNA released into solution (Figure 7A). Consistent with previous results, the nicked EC on the linear template was less stable and released more than 40% of the RNA (*17, 19*). The wild type EC on the supercoiled template was less stable than the wild type EC on the linear template (Figure 7B). The decreased stability of the wild type T7RNAP EC on supercoiled templates has been observed previously and has been attributed to the increased level of formation of extended RNA−DNA hybrids on such templates (*17, 19*). Similar observations and interpretations have recently been obtained for RNAP II (*28*).

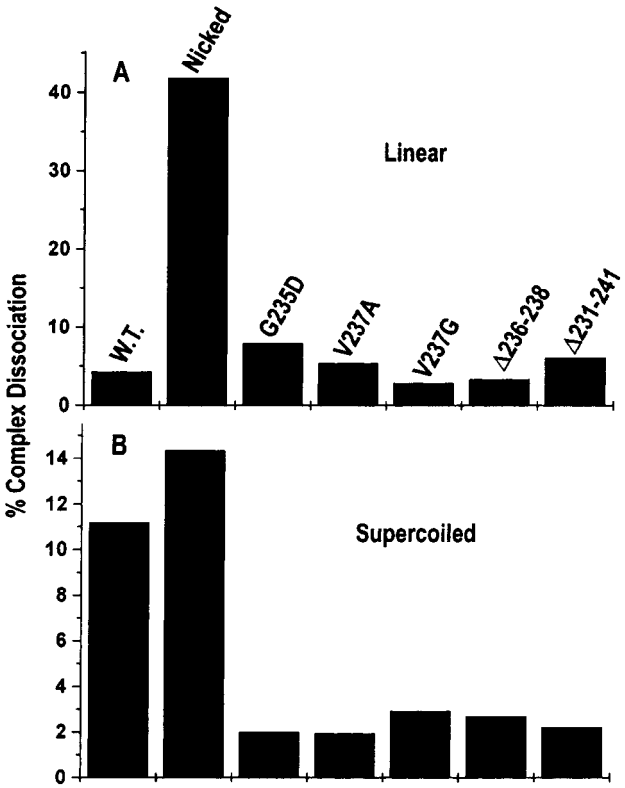

FIGURE 7: EC stability of mutant RNAPs. Halted ECs containing 16mer RNAs were formed with the indicated RNAPs (100 nM) on either linearized (A) or supercoiled (B) pPK10 (100 nM) in a 5 min reaction at room temperature. Heparin was then added to a concentration of 0.2 mg/mL to prevent reinitiation, and complexes were incubated for 2 h at room temperature, after which the reaction mixtures were filtered through membranes with 100 kDa molecular mass cutoffs. The graphs plot the percentage of the RNAs present in the filtrate.

Interestingly, the ECs formed with mutant RNAPs are not destabilized by template supercoiling and, consequently, form complexes on supercoiled templates which are more stable than the wild type EC (Figure 7B).

The Intercalating Hairpin Mutants Are Not Defective in RNA Displacement. The experimental results depicted in Figure 3 show that the nicked enzyme is poorly active on a supercoiled template. This may seem surprising in light of the previous statement that this enzyme is partially deficient in promoter opening. However, it has been shown that the nicked enzyme has poor RNA displacement activity, and this results in extensive formation of extended RNA−DNA hybrids, especially on supercoiled templates (*19*). Formation of such hybrids inhibits the enzyme's activity (*19*). The observation that the intercalating hairpin mutants are active on supercoiled templates therefore suggests that these mutants are not deficient in RNA displacement. To test this directly, we formed halted ECs on both linear and supercoiled templates and treated the complexes with RNAse H. Previous observations have shown that the RNA in a T7RNAP EC is in a dynamic equilibrium between properly displaced and extended hybrid conformations (*19*). If the RNA in the EC is properly displaced from the template, it is RNAse H resistant, but if it forms an extended hybrid, it becomes RNAse H sensitive (*19*). As shown in Figure 8A, RNAse H treatment of the nicked EC on a linear template rapidly degrades the RNA, while the RNA in the wild type EC is relatively resistant to RNAse H, as is the RNA in the EC

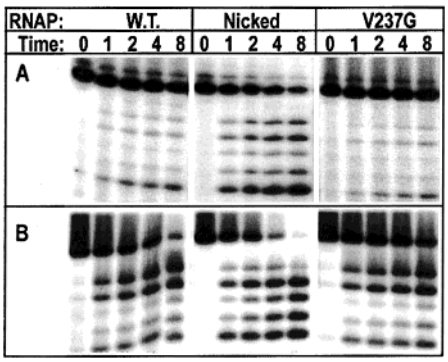

FIGURE 8: RNA displacement activity of mutant RNAPs as assessed by RNAse H treatment. Halted ECs containing 16mer RNAs formed with the indicated RNAPs (100 nM) on 100 nM linearized (A) or supercoiled (B) pPK10 and treated with 0.5 unit/μL RNAse H for the indicated times at room temperature.

Table 1: Rates[a] (min^{-1}) of RNAse H-Mediated RNA Degradation in Halted ECs

enzyme	linear	supercoiled	enzyme	linear	supercoiled
wild type	0.083	0.43	V237G	0.056	0.16
nicked	0.45	0.77	Δ236−238	0.042	0.19
G235D	0.025	0.10	Δ231−241	0.029	0.11
V237A	0.063	0.20			

[a] Incubations were at room temperature with an RNAse H concentration of 0.5 unit/μL and an EC concentration of 10^{-7} M.

formed with V237G. The extent of formation of extended hybrids is increased on supercoiled templates, resulting, as seen in Figure 8B, in increased RNAse H sensitivity of ECs on supercoiled templates. The rates of RNAse H digestion for all of the enzymes, on both supercoiled and linear templates, are compiled in Table 1. Two conclusions may be drawn from these data. First, the mutant ECs do not exhibit the increased RNAse H sensitivity which is seen with the nicked EC, indicating that the mutants are not defective in RNA displacement. Second, the mutant ECs actually show less RNAse H sensitivity than the wild type EC, indicating that the level of formation of extended hybrids is reduced in the mutant ECs. Since formation of extended hybrids has been shown to destabilize ECs (*17*, *19*, *28*), this could explain why the mutant ECs are more stable than the wild type EC, especially on supercoiled templates (Figure 7).

DISCUSSION

The Intercalating Hairpin Is Important for Promoter Opening. Our results confirm the proposal, based on X-ray studies (*6*), that a β-hairpin formed by T7RNAP residues 226−245 is important for promoter opening. Deletion mutants in this structure specifically reduce the activity of the polymerase on ds templates without markedly reducing activity on pss or supercoiled templates, and they also reduce the level of promoter opening during initiation as assessed by sensitivity to $KMnO_4$ or a single-strand specific endonuclease. The proposal that a stacking interaction between the −5 template base and V237 (*6*) is important for stabilizing the open complex was supported by the observation that V237A and V237G exhibit reduced activity on ds templates and decrease the level of open complex formation as assessed by $KMnO_4$ or endonuclease sensitivity. Significantly, the V237G substitution is more disruptive than the V237A substitution. The latter mutant would be expected to retain at least part of the stacking interaction normally made between V237 and the −5 base.

In light of its functional importance, it is surprising that the sequence of the intercalating hairpin is poorly conserved among the family of T7-like RNAPs. The highly homologous T7 and T3 RNAPs, for example, are 82% identical over their entire sequences, but only 45% identical within residues 226−245 (*29*). Even functionally important residues within this sequence are poorly conserved. Thus, while G235 is conserved in the T3 and T7 RNAPs, and V237 is conservatively replaced with an alanine in T3 RNAP, in the closely related K11 RNAP, G235 and V237 are replaced with alanine and glycine, respectively (*30*, *39*). With the more divergent members of this family, it becomes impossible to generate a reliable alignment of this region. In fact, the intercalating hairpin is one of the most poorly conserved sequence elements in this polymerase family. If all these RNAPs use a similar mechanism for promoter opening, then this poor sequence conservation implies that all that needs to be conserved to maintain such a mechanism is a β-hairpin which can serve as a wedge to open the DNA, while individual side chain−DNA interactions may vary. This conclusion is reinforced by studies of T7 promoter mutants, which have shown that the effects of mutations in the −6 to −1 region of the promoter can be suppressed by negative supercoiling (*31*). This is in contrast to the effects of mutations in the upstream "recognition half" of the promoter, which cannot be suppressed by supercoiling (*32*). In fact, the NT strand downstream of position −6 can be deleted without markedly affecting initiation, and the polymerase will specifically bind a promoter from which both the NT and T strands downstream of position −6 have been deleted (*33*, *34*). Sequence conservation in the −6 to −1 segment of the T7 promoter therefore appears to reflect a requirement for a sequence which lowers the thermodynamic barrier to promoter opening, rather than the presence of base-specific interactions with polymerase.

Our results also reveal that, while the β-hairpin is important for promoter opening, it is not essential. Even a deletion which removes almost this entire element (Δ231−241) fails to completely eliminate polymerase activity on ds templates, and the effects of the deletion can be almost fully overcome by using a supercoiled template. This suggests that other interactions, most probably between residues of the template binding cleft and the unwound T strand (*6*, *7*) and between the initiating NTPs and the template, provide enough stabilization to allow transient promoter melting and transcription initiation, albeit at a reduced rate. It has also been demonstrated that T7RNAP binding induces a bend in the promoter (*35*). Bending might contribute to stabilization of an unwound region in the promoter.

An unexpected observation was that a G235D substitution is more disruptive of activity on ds templates than are deletions of 3 or 11 amino acids which encompass G235. The G235D mutation was identified in a screen for mutants which increase the sensitivity of T7RNAP to the negative regulator T7 lysozyme (*11*). It was exceptional inasmuch as the majority of such hypersensitivity mutants map to the C-terminal region of the polymerase. Examination of a space-filling model of the promoter−polymerase complex (Figure 9) reveals that the α-carbon of G235 approaches the

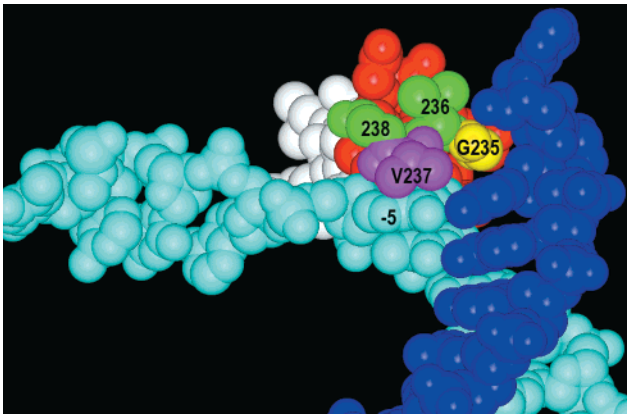

FIGURE 9: Space-filling model of the intercalating hairpin (residues 226−246) and the template (cyan) and nontemplate (blue) strands as seen in a T7RNAP−promoter co-crystal (*6*). Valine 237 is magenta and stacks on the −5 template base. Glycine 235 in yellow closely approaches the −5 nontemplate nucleotide. Δ236−238 removes V237 as well as residues 236 and 238 (green). Δ231−241 removes residues 235−238 as well as the residues which are colored red.

nontemplate base at position −5. A larger side chain at this position would create a steric clash which could interfere with proper positioning of the promoter for initiation. The substitution of an aspartic acid at this position could exacerbate this by repelling the DNA phosphates. While this appears to provide a simple explanation for why a G235D substitution is more disruptive than a deletion of the entire intercalating hairpin, it is nevertheless surprising that the open complex cannot adjust to accommodate this steric clash. The intercalating hairpin itself is disordered in the absence of DNA (*36*, *37*), and the NT strand in the open promoter−polymerase complex is disordered downstream of position −4 (*6*), suggesting that both these elements are capable of some steric adjustment. The clash created by the G235D substitution could, for example, be relieved by extending the unwinding of the promoter by a single base pair at position −5. Apparently such an extension is strongly disfavored on a linear template, but may occur on supercoiled templates where G235D displays near wild type activity.

T7 promoter opening kinetics are similar to those of *E. coli* ribosomal promoters (*38*). Like *E. coli* rrn promoter−polymerase complexes, T7 promoter−polymerase complexes are unstable and sensitive to heparin until stabilized by addition of NTPs (*22*). In the absence of NTPs, open and closed forms of the complex are in rapid equilibrium, with the closed form being favored (*18*, *24*). Since T7 promoter melting is rapid [∼0.1 ms (*24*)], and does not limit the initiation rate (*20*), the effects of the intercalating loop mutations reported here must reflect destabilization of the open complex. Whether this is due to a decrease in the rate of opening, an increase in the rate of closing, or both would have to be addressed by kinetic experiments with time resolution on the order of 0.1 ms. Such experiments might also reveal if the intercalating hairpin simply stabilizes the opened DNA, or if it is also involved in nucleating formation of the open complex.

The Intercalating Hairpin Is Not Important for Maintenance of the Transcription Bubble during Elongation. Though the intercalating hairpin clearly plays a role in promoter opening during initiation, we found that the $KMnO_4$ reactivity of halted ECs formed with the mutant enzymes was similar to that of the wild type EC. We also found that the stabilities of the ECs formed with the mutant enzymes were similar to, or greater than, that of the wild type EC. This suggests that while the intercalating hairpin helps hold the DNA open during initiation, once the RNA becomes long enough the RNA−DNA hybrid forms and serves to maintain the transcription bubble with no requirement for a contribution from the hairpin.

The Intercalating Hairpin Is Not Important for RNA Displacement. The proposal that the RNA in the T7RNAP TC passes between the thumb and the N-terminal domain (*7*) raises the possibility that the intercalating hairpin plays a role, not only in separating the T and NT strands but also in separating the RNA from the template (Figure 1). A similar proposal has been made for a structurally analogous hairpin formed by β' residues 315−324 in Taq RNAP (*5*). Our results, however, reveal that mutations in the T7RNAP intercalating hairpin do not lead to an increased level of formation of extended RNA−DNA hybrids, though an increased level of formation of such hybrids is readily detected with a polymerase cleaved within the N-terminal domain. This implies that the intercalating hairpin is not important for RNA displacement. Interestingly, the mutations actually decrease the level of formation of extended hybrids, as assessed by RNAse H treatment. It has been shown that reannealing of the T and NT strands is important for proper RNA displacement in a T7RNAP EC (*17*, *19*). It therefore seems simplest to conclude that disruption of the function of the intercalating hairpin enhances reannealing of the template and proper RNA displacement, without leading to complete transcription bubble collapse. This would also explain why, on supercoiled templates, the mutant ECs are more stable than the wild type EC, since it has been shown that extended hybrid formation destabilizes the EC (*17*, *19*, *28*).

ACKNOWLEDGMENT

We thank W. T. McAllister and P. Mentesanas for the gift of the pPK10 construct and F. W. Studier and X. Zhang for the strain expressing the G235D mutant.

REFERENCES

1. Fenton, M. S., Shun, J. L., and Gralla, J. D. (2000) *EMBO J. 19*, 1130−1137.
2. Panaghie, G., Aiyar, S. E., Bobb, K. L., Hayward, R. S., and de Haseth, P. L. (2000) *J. Mol. Biol. 299*, 1217−1230.
3. Juang, Y.-L., and Helmann, J. D. (1995) *J. Mol. Biol. 235*, 1470−1488.
4. Helmann, J. D., and Chamberlin, M. J. (1988) *Annu. Rev. Biochem. 57*, 839−872.
5. Zhang, G., Campbell, E. A., Minakhin, L., Richter, C., Severinov, K., and Darst, S. A. (1999) *Cell 98*, 6 811−824.
6. Cheetham, G. M., Jeruzalmi, D., and Steitz, T. A. (1999) *Nature 399*, 80−83.
7. Cheetham, G. M. T., and Steitz, T. A. (1999) *Science 286*, 2305−2308.
8. Naryshkin, N., Reyvakin, A., Kim, Y., Mekler, V., and Ebright, R. H. (2000) *Cell 101*, 601−611.
9. Bonner, G., Patra, D., Lafer, E. M., and Sousa, R. (1992) *EMBO J. 11*, 3767−3775.
10. Bonner, G., Lafer, E. M., and Sousa, R. (1994) *J. Biol. Chem. 42*, 25120−25128.
11. Zhang, X. (1995) Ph.D. Dissertation, State University of New York at Stony Brook, Stony Brook, NY.

12. Ikeda, R. A., and Richardson, C. C. (1987) *J. Biol. Chem. 262*, 3790−3799.
13. Huang, J., and Sousa, R. (2000) *J. Mol. Biol. 303*, 347−308.
14. Huang, Y., Eckstein, F., Padilla, R., and Sousa, R. (1997) *Biochemistry 36*, 8231−8242.
15. Tabor, S., and Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074−1078.
16. Gunderson, S. I., Chapman, K. A., and Burgess, R. R. (1987) *Biochemistry 26*, 1539−1546.
17. Mentesanas, P. E., Chin-Bow, S. T., Sousa, R., and McAllister, W. T. (2000) *J. Mol. Biol. 302*, 1049−1062.
18. Villemain, J., Guajardo, R., and Sousa, R. (1997) *J. Mol. Biol. 273*, 958−977.
19. Gopal, V., Brieba, L. G., Guajardo, R., McAllister, W. T., and Sousa, R. (1999) *J. Mol. Biol. 290*, 411−413.
20. Jia, Y., and Patel, S. S. (1997) *Biochemistry 36*, 4223−4232.
21. Strothkamp, R. E., Oakley, J. L., and Coleman, J. E. (1980) *Biochemistry 19*, 1074−1080.
22. Diaz, G. A., Rong, M., McAllister, W. T., and Durbin, R. K. (1996) *Biochemistry 35*, 10837−10843.
23. Ikeda, R. A., and Richardson, C. C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3614−3618.
24. Jia, Y., and Patel, S. S. (1997) *J. Biol. Chem. 272*, 30147−30153.
25. Place, C., Oddos, J., Buc, H., McAllister, W. T., and Buckle, M. (1999) *Biochemistry 38*, 4948−4957.
26. Kainz, M., and Roberts, J. (1992) *Science 255*, 838−841.
27. Lee, D. N., and Landick, R. (1992) *J. Mol. Biol. 228*, 759−777.
28. Kireeva, M. L., Komissarova, N., and Kashlev, M. (2000) *J. Mol. Biol. 299*, 325−335.
29. McGraw, N. J., Bailey, J. N., Cleaves, G. R., Dembinski, D. R., Gocke, C. R., Joliffe, L. K., MacWright, R. S., and McAllister, W. T. (1985) *Nucleic Acids Res. 13*, 6753−6766.
30. Rong, M., Castagna, R., and McAllister, W. T. (1999) *BioTechniques 27*, 690−692, 694.
31. Chapman, K. A., and Burgess, R. R. (1987) *Nucleic Acids Res. 15*, 5413−5432.
32. Imburgio, D., Rong, M., Ma, K., and McAllister, W. T. (2000) *Biochemistry 39*, 10419−10430.
33. Maslak, M., and Martin, C. T. (1993) *Biochemistry 32*, 4281−4285.
34. Ujvári, A., and Martin, C. T. (1997) *J. Mol. Biol. 273*, 775−881.
35. Ujvári, A., and Martin, C. T. (2000) *J. Mol. Biol. 295*, 1173−1184.
36. Sousa, R., Chung, Y. J., Rose, J. P., and Wang, B. C. (1993) *Nature 364*, 593−599.
37. Jeruzalmi, D., and Steitz, T. A. (1998) *EMBO J. 17*, 4101−4113.
38. Gaal, T., Bartlett, M., Ross, W., Turnbough, C. L., Jr., and Gourse, R. (1997) *Science 278*, 2092−2097.
39. Rong, M., Castagna, R., and McAllister, W. T. (1999) *BioTechniques 27*, 694.

BI002716C