Cross-linking of Promoter DNA to T7 RNA Polymerase Does Not Prevent Formation of a Stable Elongation Complex*

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T7 RNA polymerase recognizes a small promoter, binds DNA, and begins the process of transcription by synthesizing short RNA products without releasing promoter contacts. To determine whether the promoter contact must be released to make longer RNA products and at what position the promoter must be released, a mutant RNA polymerase was designed that allows cross-linking to a modified promoter via a covalent disulfide bond. The modifications individually have no measurable effect on transcription. Under oxidizing conditions that produce the protein-DNA cross-link, the complex is able to synthesize short RNA products, strongly supporting a model in which promoter contacts are not lost on translocation through at least position +6. However, cross-linked complexes are impaired in promoter escape in that only about one in four can escape to make full-length RNA. The remainder release 12- and 13-mer RNA transcripts, suggesting an increased energetic barrier in the transition from an initial transcribing complex to a fully competent elongation complex. The results are discussed in the context of a model in which promoter release helps drive initial collapse of the upstream edge of the bubble, which, in turn, drives initial displacement of the 5’-end of the RNA.

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runoff products, cross-linking of the promoter to the enzyme presents a new (or increased) energetic barrier leading to premature release of RNA at positions +12 and +13.

MATERIALS AND METHODS

Mutant Construction—An expression vector coding for the mutant polymerase (His-tagged A94C) was prepared by utilizing the Strategene QuikChange™ site-directed mutagenesis kit. The parental plasmid was isolated from cells (pBH161/BL21) generously provided by W. T. McAllister. The oligonucleotide primers (5'-GACTTTGTTAGGGAAGTGAATGACCCGGCAGGCAGGCGCGG-3' and its complement) directing the single amino acid mutation were purchased from Integrated DNA Technologies (Coralville, IA). The underlined region encodes the alanine (GCT) to cysteine (TGT) mutation. Candidate plasmids were sequenced to confirm the mutation and then transformed into BL21 cells. Mutant polymerase activity, which was the same as wild type, was assayed using methods described below.

Protein Expression and Purification—His-tagged wild type and mutant T7 RNA polymerase were overexpressed in Escherichia coli strain BL21 and purified using Qiagen nickel-nitrilotriacetic acid as described (22). Protein purity (>95%) was determined by SDS-PAGE analysis. The purified protein was dialyzed against storage buffer (20 mM potassium glutamate, pH 7.8, 100 mM NaCl, 50% glycerol, and 1 mM freshly prepared DTT (absent in oxidized samples). Protein purity was determined by SDS-PAGE analysis.

Transcription Assays—Transcription reactions were performed in a total volume of 20 μl at 37 °C for 10 min and quenched with an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.01% bromphenol blue, and 0.01% xylene cyanol). Equimolar concentrations of double strand DNA and enzyme were used at final concentrations of 0.2 μM in a reaction buffer containing 30 mM HEPES, pH 7.8, 25 mM potassium glutamate, 15 mM magnesium acetate, 0.25 mM EDTA, 0.5% glycerol, 50 mM NaCl, and 1 mM freshly prepared DTT (absent in oxidized samples). Reactions were initiated by the addition of nucleoside triphosphates to a final concentration of 400 μM each and were labeled with 1 μCi of [α-32P]GTP.

Transcription assays with samples on beads or samples run through the G-25 Sephadex spin columns were performed at 37 °C for 10 min and then quenched with an equal volume of stop solution. RNA products were separated on a 7 M urea, 20% polyacrylamide Tris-borate gel and quantified using a Storm 840 PhosphorImager as described previously (25).

Oxidation by Glutathione or Diamide—In some experiments, oxidized glutathione and 1,1′-azobis(N,N-dimethylformamide) (diamide) were utilized to ensure that any non-cross-linked complex was driven toward complete oxidation (26, 27). To demonstrate that the disulfide cross-link was not reversibly exchanging, a 1-μl volume of 10 mM oxidized glutathione, 50 mM oxidized glutathione, and 10 mM diamide or diamide deoxidized H2O (as a control) was added to 9 μl of bead-isolated, cross-linked complexes to increase the oxidizing strength of the buffer solution. Each sample was then incubated for 1 h at 4 °C before use in transcription assays as described above.

RESULTS

Recent crystal structures of elongation complex models strongly support a mechanism for transcription in which enzyme-promoter contacts are completely lost on progression to an elongation complex (2, 10, 28, 29). To determine whether initial promoter contacts must be released when the enzyme switches to a more stable elongation complex, a mutant polymerase was designed that allows covalent cross-linking of the promoter DNA to the promoter binding region of the enzyme in the initially bound complex. There are three regions of the enzyme that make up the promoter binding domain as revealed by contacts seen in the DNA-bound crystal structures (3, 21).

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As shown in Fig. 1, these regions include the AT-rich recognition loop centered on Arg-96, the specificity loop (residues 745–759), and an intercalating loop centered on Val-237.

Residue Ala-94, in the first of these regions, was chosen for mutation based on its close proximity to the 3'-hydroxyl of the adenosine at position −17 of the template strand DNA, its lack of contact with the DNA or the rest of the protein, its lack of conservation among the closely related phages T3, SP6, and K11 (18–20), and biochemical evidence that minor changes in this region of the promoter DNA are reasonably tolerated (30). Template strand DNA was synthesized with a 3'-thiol modification so that it could be directly cross-linked via formation of a disulfide bond with Cys-94. We expected that cross-linking of the promoter DNA would allow normal initiation and initial translocation, whereas full run transcription would be eliminated because the enzyme would not be able to escape the promoter.

Modifications Have No Effect on Transcription in the Absence of Cross-linking—To ensure that modification of the protein alone has no direct effect on transcription, we compared transcription assays with mutant A94C and wild type T7 RNA polymerase. In each case we used double-stranded DNA containing an upstream consensus promoter and a downstream sequence encoding a 20-mer runoff transcript. Results presented in lanes 3 and 4 of Fig. 2 show that, under reducing conditions, transcription from the mutant polymerase is essentially identical to that of wild type polymerase.

Similarly, to ensure that adding a thiol group to the 3'-end of

The abbreviation used is: DTT, dithiothreitol.

the template strand DNA has no detrimental effect on binding or on the ability of the enzyme to transcribe, we analyzed transcription by wild type T7 RNA polymerase on an identical DNA sequence containing a 3'-thiol on the template strand (see Fig. 2). As shown in lane 2 of Fig. 2, the addition of the 3'-thiol to the DNA has no effect on transcription. Finally, comparison of lanes 1 and 4 in Fig. 2 shows that under the normal reducing conditions of our assays, which should prevent formation of the disulfide, the combination of both A94C and the addition of a 3'-thiol on the DNA has no effect on transcription. In all cases, there is no significant difference in the amount of transcribed product using either enzyme, nor is there a difference in the product profile.

**Covalent Cross-linking Does Not Alter Short Product Synthesis**—Because T7 RNA polymerase retains promoter contacts from binding through initial transcription (at least through position +6), it is expected that a cross-linked complex should initiate as well as or better than an uncross-linked complex and that translocation to at least position +6 should be unimpeded. To test this hypothesis, DTT was removed from solutions containing DNA and enzyme (as described under “Materials and Methods”) to drive the formation of a disulfide bond between the protein and the DNA. In a transcription assay with only GTP as the substrate on a promoter encoding GGGA at the start site, T7 RNA polymerase produces a range of poly(G) products up to 14 bases in length (8, 25). Because forward translocation is minimal in this system, it is a simple measure of initiation. As shown in Fig. 3, cross-linking the promoter to the enzyme has no effect on the enzyme's ability to synthesize poly(G) products. Similarly, in the presence of GTP and ATP on a promoter encoding the substrate-limited six-base product GGGAGA, there is no difference in the product profile (intensity differences between lanes are attributed to differences in concentrations after processing). With no change in the product profile (relative amounts of 3-mer or 4-mer to 6-mer) evident, we conclude that initiation and early transcription are not adversely affected by the covalent cross-linking of the promoter DNA to the enzyme.

**Cross-linked Complex Can Make Full Run Product**—Based on earlier observations from both footprinting and fluorescence experiments, we predicted that restricting promoter release would allow the synthesis of only a 9-mer to 11-mer product. Footprinting experiments have shown that restricting promoter release occurs at some time after the synthesis of a 6-mer product and before the synthesis of a 15-mer (2, 10). Similarly, we have shown that the initially melted bubble collapses after synthesis of a 8- to 9-base pair product, presumably concurrent with promoter release (11). Thus, we hypothesized that locking the promoter onto the enzyme by covalently cross-linking the upstream promoter DNA to the AT-rich recognition loop (a sub-
domain of the promoter binding region) would limit the length of the final product to an ~10-mer RNA. The results presented in Fig. 4, however, indicate that the cross-linked complex is indeed able to synthesize a full-length runoff (20-mer) product. Although a full-length transcript is produced by the cross-linked complex, there is a substantial increase in the amount of 12- and 13-mer falloff products relative to the 20-mer runoff product.

Under our current conditions, the control (uncross-linked) wild type DNA and protein produce 12- and 13-mer products at ~8–10% each relative to the amount of 20-mer runoff product (Fig. 4, lane 3). In contrast, the cross-linked complex produces 12- and 13-mer products that are >200% the amount of the 20-mer product. The finding that these same short products are seen (at low levels) with the wild type enzyme and DNA suggests that they reflect an intrinsic barrier to escaping the promoter. Promoter release is likely to be that barrier. Covalent cross-linking of the DNA to the enzyme increases the probability that proper promoter release will not occur and, therefore, increases the amount of the short product generated.

In this experiment, the cross-link was allowed to form via simple air oxidation (4 days at 4 °C) following removal of DTT with a G-25 Sephadex spin column. As a result, differences in the overall amounts of transcripts between lanes might arise from a loss of protein during oxidation and/or gel filtration. In these experiments we can also not be sure that the complexes are 100% cross-linked, such that the observed full-length runoff product might arise from a population of uncross-linked complexes. The following experiments address this uncertainty.

Runoff Product Is Produced by Bona Fide Cross-linked Complexes—To better isolate cross-linked complexes, double-stranded DNA containing an upstream thiol group on the template strand and a downstream 3'-biotin moiety on the non-template strand was incubated with the mutant enzyme in the absence of DTT, as described above. The resulting cross-linked complexes were then captured using streptavidin-coated paramagnetic beads. The beads were then washed using buffer containing 1 mM NaCl to remove any non-covalently bound protein. After equilibrating the beads to transcription buffer, transcription assays performed directly from these beads showed ratios of 12- and 13-mer products relative to 20-mer products similar to those seen above (Fig. 5B). In a control experiment, wild type enzyme and DNA containing the downstream biotin but lacking the upstream 3'-thiol were incubated, captured, washed, and assayed as described above. No transcription was observed in this control, indicating efficient washing of the noncovalently bound enzyme from the DNA. The subsequent addition of free enzyme restored wild type levels of transcription, demonstrating that streptavidin-bound DNA was retained (data not shown).

Finally, to ensure that the observed 20-mer transcript does not arise from complexes in which the cross-link has reversed, we employed glutathione and diamide to maintain complete oxidation (26, 27). As shown in Fig. 5C, the addition of glutathione to the bead-isolated cross-linked complex (to a final concentration of 1 mM) does not alter the percentage of 12- and 13-mer products relative to 20-mer, demonstrating that the 12-, 13-, and 20-mer products are being synthesized by fully cross-linked complexes. The addition of glutathione to 5 mM (not shown) or the addition of diamide (a stronger oxidizer) to 1 mM (Fig. 5C, lane 2) significantly reduces the overall activity of the protein. A similar reduction in activity is seen for a parallel treatment of the wild type enzyme, suggesting that under these stronger oxidizing conditions the native cysteines are forming nonnative cross-links, thus reducing the protein activity (not shown).
Are All Complexes Coupled to Cys-94?—High salt washing of complexes containing the native alanine at position 94 and lacking a 3'-thiol on the DNA template led to a complete loss of transcription, demonstrating that noncovalently bound polymerase can be efficiently washed from the bead-DNA complex. This finding does not, however, preclude cross-linking of the modified DNA to any of the 12 native cysteine residues in T7 RNA polymerase.

Although the native cysteine nearest the 3'-thiol is 24 Å distant (C216), a secondary control was run to address the possibility that wild type polymerase could be cross-linked to the thiol-modified DNA through one of these native cysteines. In the previous transcription assay the wild type enzyme was not completely washed away, as there was residual activity resulting in a faint band corresponding to 20-mer RNA (10% of that seen in the A94C control) and very weak bands corresponding to 12- and 13-mer RNAs (data not shown). Cross-linking of promoter DNA to any of the native cysteine residues in T7 RNA polymerase would allow the RNA polymerase to survive the high salt challenge, but function with DNA would almost certainly happen in trans, as it should not allow correct positioning of the promoter on the protein to which it is cross-linked.

If one enzyme can utilize DNA tethered to a separately tethered enzyme, one would expect that complex to be susceptible to free, competing promoter DNA. In contrast, complexes with DNA bound at Cys-94 can be expected to be resistant to such competition. Therefore, the addition of a promoter sink that binds but will not support transcription can distinguish between complexes cross-linked properly via Cys-94 in the promoter binding domain and those that are cross-linked to one of the native cysteines. Complexes cross-linked to Cys 94 should be resistant to the sink (a free sink promoter cannot effectively compete with a locally tethered promoter), whereas the complexes containing promoter DNA accessible in trans should be completely inhibited by the trap. In the bead-isolated control experiment with wild type enzyme and thiol modified DNA there is no increase in the ratio of 12- and 13-mer products relative to 20-mer, and transcription from these complexes is completely inhibited by the addition of the sink, suggesting that this residual level of transcription is occurring in trans (data not shown). This result raises the possibility that some of the cross-linked complexes in the experiment with the A94C mutant are cross-linked via one of the native cysteines. Transcription in trans would lead to full-length transcripts.

To assess whether some of the 20-mer RNA observed in Fig. 5B is being synthesized by complexes transcribing in trans, similar assays were run but with increasing concentrations of promoter sink. The results shown in Fig. 6 show that challenging the cross-linked, bead-isolated complexes with promoter sink leads to only a small decrease in 20-mer synthesis. This small decrease must reflect a similarly small population of incorrectly cross-linked complexes operating in trans. At the end point of this titration, the ratio of 12- and 13-mer products relative to 20-mer increases to 2.9, which should now reflect only those complexes cross-linked via Cys-94. Titration of sink into enzyme and thiol-modified DNA incubated previously under the oxidizing conditions described above but not bead-isolated similarly shows a final maximal ratio of 2.7 (data not shown). Together, these data show that bona fide cross-linked complexes terminate transcription at positions 12 and 13—and 75%
of the time. Only 25% escape to form competent elongation complexes.

As a final control to show that the effects of the cross-link are reversible, DTT was added to the transcription assay at a final concentration of 50 mM. For bead-isolated cross-linked complexes, the addition of DTT restored the ratios of 12- and 13-mer product to 20-mer product, indicating that the 20-mer product is indeed produced by properly cross-linked enzyme-DNA complexes. Transcription assay with increasing sink:DNA concentration.

FIG. 6. Use of a promoter sink to “remove” incorrectly cross-linked complexes. A promoter sink was titrated into the cross-linked complex. Transcription between improperly cross-linked complexes and naked DNA is efficiently inhibited by the addition of promoter sink, whereas the correctly cross-linked complex is resistant. A, titration curve showing a maximum of 290% of 12- and 13-mer product to 20-mer product, indicating that the 20-mer product is indeed produced by properly cross-linked enzyme-DNA complexes. B, transcription assay with increasing sink:DNA concentration.

DISCUSSION

Despite dissimilarities in sequence and structure, all RNA polymerases are remarkably similar in that they transition through an abortive cycling phase prior to entering the more stable elongation phase (4, 5, 8, 9, 33). This transition from an unstable initiation complex to a stable elongation complex occurs after synthesis of ~10 bases. Understanding this conserved transition is critical for understanding the mechanisms and regulation of transcription.

T7 RNA polymerase, like the multi-subunit bacterial enzyme, binds promoter DNA, melts open the initiation region, and begins transcription while maintaining initial promoter contacts (13, 14). At some point during the transition from an unstable initiation complex to a stable elongation complex, promoter contacts are lost (2, 10, 34).

Recently, two different research groups have published crystal structures of T7 RNA polymerase elongation complexes derived from synthetic RNA-DNA scaffolds (28, 29). Both crystal structures show a dramatic rearrangement of the N-terminal domain, including a region of the portion of the enzyme responsible for promoter recognition and binding (3, 21). In the elongation complex, two of the three promoter binding elements have moved as a rigid body with respect to the C-terminal domain. This rigid body movement might allow the enzyme to bring downstream DNA into the active site (extending the footprint downstream) while maintaining upstream promoter contacts (and the upstream footprint). Tahirov et al. have proposed a model for a late initiation complex in which an 8-mer RNA can be formed while two of the three initial promoter contacts are retained (28). In this model, contacts are retained between the AT-rich recognition loop and the −17 to −14 region of the promoter DNA, as well as between the intercalating loop and the promoter DNA at −5. This model also predicts that the initially melted bubble remains open when the polymerase reaches position +8 but that the bubble collapses and the promoter contacts are lost on translocation beyond positions +9 to +11. This interpretation is supported by biochemical studies (11, 35) that additionally suggest that promoter release may not simply occur at one defined/precise position. A more recent model suggests that the rigid body rotation is preceded by a simple back translocation of the promoter binding element. In this case, promoter contacts can also be retained during initial translocation.

By monitoring the melted state of the DNA bases at position −2, Liu and Martin (11) showed that bubble collapse begins when the polymerase translocates to position +9 and is nearly complete by translocation to position +11. In a similar study with exonuclease as a footprinting probe, Brieba and Sousa showed that promoter release may begin as early as position +8 but again suggested that timing of the release is non-homogeneous (35). When stalled at position +7 and then translocated to position +8 by addition of the next incoming NTP, 5–10% of the complexes exhibit a shift in the upstream boundary of exonuclease protection. In complexes walked to position +8, 40% of the complexes show a shift. Taken together, these results support a model in which promoter release begins when the polymerase reaches position +8 and is likely complete when the polymerase translocates to position +11.

To assess the functional importance of promoter release, we have constructed a covalently cross-linked binary complex between T7 RNA polymerase and its promoter. Current models predict that the cross-linked complex should initiate as well as the corresponding non-cross-linked controls (2, 10, 11). In an attempt to determine the position at which promoter release must occur, we allowed the cross-linked complex to transcribe a 20-mer runoff product and compared the products of the cross-linked complex with those of the non-cross-linked complex.


cross-linking Does Not Perturb Transcription through Position +6—As expected, cross-linking of promoter DNA to the promoter binding region of T7 RNA polymerase has no effect on the initiation of transcription. Product profiles from cross-linked complexes in the presence of GTP only (allowing translocation to position +3) or in the presence of GTP and ATP as the substrate (allowing translocation to position +6) are identical to those of the uncross-linked control. This shows clearly that initial promoter contacts need not be released to synthesize up to, at least, a 6-mer product.

Cross-linking Perturbs but Does Not Eliminate Escape to an Elongation Complex—In the cross-linked constructs created in this study, we expect that the loss of promoter contacts and subsequent (or concurrent) initial bubble collapse (from position −4 downstream) should both be impeded. Thus, some

complexes do not proceed to become stable elongation complexes and instead release RNA products 12–13 bases in length. Indeed, ~75% of initiated complexes stop at +12 and +13; only 25% successfully pass this barrier to go on to synthesize full-length RNA products. That 25% escape suggests that the barrier is not absolute.

What is the nature of this barrier and why does transcription stop at positions +12 and +13 rather than positions +8 to +10? A similar increase in 12- and 13-mer transcripts relative to full-length products is observed in transcription from constructs that do not allow normal bubble collapse. An increase in 12- to 13-mer products can be seen in constructs that are nicked on the nontemplate strand in the region of the initially melted bubble, constructs that have an artificially melted (noncomplementary) bubble, and partially single-stranded DNA constructs (15–17). It has been suggested that improper RNA displacement results in a complex that cannot transcribe well beyond position +13 (16). Artificial bubble scaffolds, such as those that were utilized to trap the elongation complex conformation for crystallographic studies, also lack the ability to properly displace the upstream end of the RNA and are similarly unable to make products longer than a 13-mer with any efficiency (33). All of these constructs prevent or weaken the collapse of the initially melted bubble (or of the upstream edge of the bubble in the case of the scaffold) and therefore weaken the ability of the complex to competitively displace the 5′-end of the nascent RNA.

We propose that the increase in the amounts of 12- and 13-mer products from our cross-linked constructs similarly arises from an impairment of bubble collapse, leading to an impairment in the proper displacement of the 5′-end of the RNA. In the current case, however, bubble collapse is impaired by maintenance of the promoter contact, suggesting that promoter release contributes directly to bubble collapse. This is to be expected, because the intercalating loop in promoter-bound complexes is thought to stabilize the melted bubble (15, 17). Release of the promoter during promoter clearance therefore destabilizes the bubble. In either case, incorrect or delayed bubble collapse prevents proper positioning of the 5′-end of the nascent RNA into the RNA exit channel.

A Model for Promoter Escape—Recent studies provide strong evidence that the timing of promoter release is simultaneous with bubble collapse and that a contiguous, complementary, nontemplate strand is required for native RNA displacement. Based on those results and the results presented herein, we believe that a critical event in the formation of a stable elongation complex is bubble collapse, driving initial displacement of the 5′-end of the nascent RNA for correct positioning near the exit channel. Promoter release allows bubble collapse, so limiting promoter release indirectly limits proper RNA displacement. Either the lack of displacement or translocationally delayed displacement prevents proper threading of the RNA into the exit channel. We suspect, therefore, that complexes that do not properly displace the RNA at position +9 can continue to elongate only 3–4 bases further, as in the elongation scaffolds, leading to the production of 12- to 13-mer RNA transcripts.

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