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Size of the Unwound Region of DNA in *Escherichia coli* RNA Polymerase and Calf Thymus RNA Polymerase II Ternary Complexes

H.B. GAMPER AND J.E. HEARST

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Based almost entirely on studies with the bacterial enzyme, a general model has emerged for the ternary complex between DNA, RNA, and RNA polymerase. A central feature of this model is the existence of an enzyme-stabilized, unwound region in the DNA double helix that permits the coding strand to specify the complementary RNA sequence through hydrogen bonding. Helix unwinding first occurs at the promoter site in conjunction with formation of the open-promoter complex. Strand separation in this complex has been directly observed by analysis of the unwinding angle (Saucier and Wang 1972; Wang et al. 1977) and DNA-sequencing technology (Siebenlist 1979). Recent studies indicate that 12–15 bp of DNA are unwound between the Pribsnov box and the site of initiation (Melnikova et al. 1978; Siebenlist et al. 1980). It is believed that the size of the unwound region does not change during elongation and that the RNA-DNA hybrid associated with the coding strand is approximately 12 bp long (Kumar and Krakow 1975).

We are interested in refining the structure of the RNA polymerase ternary complex so as to understand better the mechanisms of pausing and termination. Our approach has been to determine the size of the DNA unwound region in *Escherichia coli* RNA polymerase and calf thymus RNA polymerase II (pol II) ternary complexes formed on superhelical SV40 DNA. Polymerase-induced unwinding has been quantified by relaxing the ternary complexes and electrophoresing the deproteinized DNA. Analysis shows that the unwound region is 17 ± 1 bp (580 ± 30°) long in the *E. coli* complex and at least 10–11 bp (360°) long in the calf thymus complex. The results with the bacterial enzyme allow us to propose a topological model for transcription. This model has been described in detail elsewhere (Gamper and Hearst 1982).

**EXPERIMENTAL PROCEDURES**

*E. coli* holoenzyme was obtained from M. Chamberlin (University of California at Berkeley) and A.-Y.M. Woody (Colorado State University). Calf thymus pol II was purchased from Bethesda Research Laboratories and diluted before use with an equal volume of 50 mM Tris-HCl (pH 7.9) buffer containing 0.1 mM (NH₄)₂SO₄, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 50% glycerol. Nicking-closing enzyme consisted of the fraction-I supernatant of lysed HeLa cell nuclei described by Vosberg et al. (1975). Superhelical SV40 DNA was purified from lytically infected TC-7 African green monkey kidney cells as outlined by Hallick et al. (1978).

The formation and analysis of *E. coli* RNA polymerase transcriptional complexes, including the electrophoretic determination of mean superhelicity, have been described previously (Gamper and Hearst 1982). Binary complexes of pol II were formed by incubating 0.5 μg of SV40 DNA with pol II for 5 minutes at 37°C in 9 μl of 100 mM Tris-HCl (pH 7.9), 25 mM (NH₄)₂SO₄, 3 mM MgCl₂, and 0.5 mM DTT. Initiation complexes were formed by making the reaction buffer 200 μM each in ATP, GTP, and CTP and incubating it an additional 5 minutes at 37°C. Finally, ternary complexes were formed by diluting the reaction mixture sixfold with 50 μl of 100 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 150 mM NaCl, and 1 mM spermidine containing 200 μM each ATP, GTP, and CTP, 12 μM UTP, and diluted nicking-closing extract. RNA synthesis and enzymatic relaxation of the DNA was allowed to proceed for 15 minutes at 37°C. Binary and initiation complexes were relaxed in the same buffer containing the appropriate supplement of NTPs.

When pol II transcriptional complexes were analyzed directly by gel electrophoresis, the enzymatic relaxation (and RNA synthesis) was terminated by making the reaction mixtures 1% in SDS. The DNA was then precipitated with ethanol, dissolved in electrophoresis buffer containing 3.5% sucrose and 0.0035% bromphenol blue, and loaded onto a horizontal 1% agarose gel immersed in 40 mM Tris-HCl and 5 mM MgCl₂. The samples were electrophoresed at 4°C for 35 hours across a 1.2 V/cm voltage gradient. The gel was stained with ethidium bromide and photographed on Polaroid type-665 positive/negative film.

For isolation of relaxed pol II ternary complexes on nitrocellulose, the reaction volume was scaled up threefold. Enzymatic relaxation and RNA synthesis were terminated by adding a 2.5-fold volume of 10 mM NaH₂PO₄ (pH 7.4), 1.25 mM NaCl, 50 mM EDTA, and 250 μg/ml of heparin. After 10 minutes at 4°C, each sample was diluted to 1.0 ml with 10 mM NaH₂PO₄ (pH 7.4), 0.6 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 10 μg/ml of heparin, filtered across nitrocellulose at a flow rate of 4 ml/min, and washed with 3 ml of the same solution. The DNA was recovered by shaking each filter in 0.8 ml of 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 0.2% SDS at room temperature for 40 minutes. After adjusting the solution to 0.2 mM NaCl and adding 40
µg of tRNA carrier, the DNA was precipitated with ethanol and analyzed by gel electrophoresis.

RESULTS

E. coli RNA Polymerase

At low ratios of enzyme to DNA, E. coli RNA polymerase initiates transcription from either of two adjacent thymidine residues at positions 2556 and 2555 on the early strand of SV40 (Reisbig and Hearst 1981). The open-promoter complex readily forms at this preferred promoter site and is converted into a ternary complex by the addition of all four NTPs. Since termination and reinitiation does not occur under the conditions of our assay (data not shown), the number of active polymerases per SV40 DNA molecule can be determined by [γ-32P]ATP end-labeling of the transcript.

Both binary and ternary transcriptional complexes have been analyzed for their ability to unwind DNA. The methodology involves relaxing superhelical DNA containing one or more transcriptional complexes to covalently closed, relaxed circles, denaturing the reaction mixtures with SDS, and electrophoresing the DNA through agarose under conditions that permit resolution of the topoisomeric bands. Figure 1 shows the gel patterns for SV40 DNA relaxed at 37°C (A) or 5°C (B) in the presence of a range of ternary complex concentrations.

Covalently closed DNA is unique in that its topological winding number (α) is a fixed integer. This dictates that any change in helicity (β) of the molecule be balanced by an equal and opposite change in superhelicity (τ). Since ternary complexes locally unwind the DNA, denaturation of the enzymatically relaxed SV40 complexes permits the helix to reform and generates a slight superhelicity in the DNA. The polymerase-dependent shift in mean superhelicity of the DNA samples in Figure 1 is obvious. The direction of shift is opposite in the two gels because the corresponding DNAs have superhelicities of different handedness.

Unwinding can be quantified by tracing the photographic negative and determining a τ for each DNA sample in the gel from peak-area measurements. The shift in τ relative to that for a DNA control relaxed in the absence of polymerase is equivalent to the helical unwinding induced by a binary or ternary complex. An unwinding angle can be calculated for the complexes when these data are combined with end-labeling data on the number of active polymerases bound per SV40 genome. The results of such an analysis for binary and ternary complexes relaxed at 37°C (A) and at 5°C (B) are shown in Figure 2. At the two temperatures, the ternary complex possesses an unwinding angle of 17.0 bp (580°) and 18.0 bp (610°), respectively. The binary complex exhibits a temperature-dependent unwinding characteristic of the conversion between open- and closed-promoter complexes (Wang et al. 1977). At 37°C the open-promoter form unwinds 17.0 bp of DNA (580°), whereas at 5°C no unwinding is observed with the equivalent of the closed-promoter form.

The unwinding angles determined with DNA end-labeling data have been verified by a semindependent approach based on nitrocellulose filter trapping. This technique takes advantage of the stability of RNA polymerase ternary complexes at low temperature (Rhodes and Chamberlin 1974) and in the presence of high salt (Richardson 1966). When ternary complexes are filtered through nitrocellulose in the presence of 0.6 M NaCl and 100 µg/ml of heparin, they are selectively retained with an efficiency of approximately 60% (Table 1). By filtering relaxed complexes formed at a low ratio of enzyme to DNA, the retained DNA has one ternary complex per genome, and the shift in τ of that DNA relative to a control relaxed in the absence of polymerase should equal the unwinding angle of the complex. When DNA from such an experiment is recovered from the filter and electrophoresed, the

Figure 1. DNA unwinding induced by E. coli RNA polymerase ternary complexes at 37°C (A) and 5°C (B). Ternary complexes were formed on SV40 DNA at 18°C employing enzyme-to-DNA ratios of 0 (lane 1), 0.75 (lane 2), 1.50 (lane 3), 2.25 (lane 4), 3.00 (lane 5), and 3.75 (lane 6). The complexes were then enzymatically relaxed to equilibrium at 37°C (A) or 5°C (B), and the deproteinized DNA was electrophoresed in 1% agarose.
observed unwinding angle is 19 bp (645°) at 37°C and 17 bp (580°) at 5°C. Furthermore, the recovered DNA forms a tight Gaussian distribution in the gel, indicating that the size of the unwound region in the ternary complex is homogeneous. This conclusion is supported by the observation that the unwinding angle of the ternary complex is independent of its position on the SV40 genome (data not shown).

Calf Thymus RNA Polymerase II

In the presence of MgCl2, purified pol II from both plant and animal sources initiates nonspecific RNA synthesis with low efficiency (Mandel and Chambon 1974a,b; Dynan and Burgess 1981). The purified enzyme lacks one or more factors required for promoter recognition and efficient formation of an open complex with DNA. In the absence of these as yet unidentified factors, initiation occurs preferentially at nicks (Lewis and Burgess 1980) and at single- to double-strand junctions (Lilley and Houghton 1979). Supercollapsed DNA is also a good substrate for pol II (Lescure et al. 1978b). Torsional strain in the molecule is believed to facilitate open-complex formation by the enzyme. Nonspecific initiation off the form-I substrate can be further enhanced by carrying out the reaction in the presence of MnCl2 and (NH4)2SO4 (Mandel and Chambon 1974a). These conditions further destabilize the helix and favor its unwinding by the enzyme.

RNA synthesis off nicked or superhelical substrates can lead to aberrant transcription by pol II. With nicked DNA, the 3' side of the nick can actually prime RNA synthesis, resulting in the generation of a covalent linkage between the noncoding DNA strand and the RNA transcript (Lewis and Burgess 1980). With superhelical DNA it is reported that pol II is unable to displace the transcript off the coding strand, leading to extensive R-loop formation (Lescure et al. 1978b; Lilley and Houghton 1979). We have utilized a three-step reaction scheme to obtain high levels of initiation off superhelical DNA without subsequent R-loop formation. Purified pol II from calf thymus is incubated with SV40 DNA at 37°C in the presence of MnCl2 and (NH4)2SO4. After 5 minutes, ATP, GTP, and CTP are added to the binary reaction mixture to form initiation complexes. Elongation is then started by the simultaneous addition of the fourth nucleotide, UTP, together with HeLa nicking-closing enzyme in a buffer containing MgCl2 and NaCl. Elongation thus proceeds off a relaxed DNA substrate, and R-loop formation is avoided. When RNA synthesis is monitored by the uptake of [α-32P]UTP into acid-insoluble product, over 99% of the transcriptional activity is inhibited by 1.0 μg/ml of α-amanitin. This level of inhibition is diagnostic for a type-II RNA polymerase.

Benzoylated, naphthoylated DEAE (BND)-cellulose column chromatography has been used to verify an absence of R loops on the DNA transcribed by pol II. This column matrix, which retains nucleic acids in 0.3 M NaCl, releases double-stranded DNA in 1.0 M NaCl and single-stranded DNA and RNA in 1.0 M NaCl and 1% caffeine. Figure 3 shows that 96% of the relaxed DNA and 78% of the superhelical DNA from SDS-disrupted ternary complexes elute with 1.0 M NaCl. This indicates that nearly all of the relaxed and most of the superhelical ternary complexes release RNA when pol II is denatured.

For comparison, the elution profiles of SV40 DNA from SDS-disrupted, relaxed and superhelical, E. coli

<table>
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<th>Table 1. Selective Retention of E. coli and Calf Thymus RNA Polymerase Ternary Complexes on Nitrocellulose</th>
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<td><strong>Sample</strong></td>
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Free SV40 3H-labeled DNA or the same DNA containing approximately 0.7 E. coli polymerase or 2–3 calf thymus pol II transcriptional complexes per genome was filtered through nitrocellulose in the presence of 0.6 M NaCl and 10 μg/ml of heparin. Approximately 40% of the DNA in the E. coli ternary complex sample and 45% of the DNA in the corresponding pol II sample was retained by the filter. Different nicking-closing extracts were used to relax the two DNA reaction series, and this accounts for the high background obtained with the relaxed calf-thymus-treated substrates.
polymerase ternary complexes are shown in Figure 4. The *E. coli* enzyme displaces the transcript off the coding strand on both relaxed and superhelical DNA substrates (Richardson 1975). The R-loop formation observed with the superhelical substrate occurs during disruption of the ternary complex and is driven by the release of torsional strain in the DNA helix. By analogy with the *E. coli* enzyme, pol II under our assay conditions probably maintains a short RNA-DNA hybrid even on superhelical substrates, with R-loop formation occurring only at the time of complex disruption.

Figure 5 shows the effect of pol II transcriptional complexes on the local topology of the SV40 template. Binary, initiation, and ternary complexes are formed with graded amounts of the enzyme, as described in Experimental Procedures. These complexes are then relaxed to equilibrium at 37°C with HeLa nicking-closing enzyme. After deproteinization and ethanol precipitation, the DNA is electrophoresed through agarose. Not unexpectedly, the binary reaction mixture (Fig. 5, lanes A–D) fails to unwind DNA in the relaxation buffer. In the presence of MgCl₂ and physiological salt, pol II is unable to form a detectable level of open binary complex. In contrast, the initiation and ternary complexes (Fig. 5, lanes E–H and I–L, respectively) exhibit an enzyme-dependent unwinding of the helix. The magnitude of unwinding is approximately the same for the two complexes.

In the last three lanes of Figure 5, the effect of varying the reaction protocol has been examined. The sample in

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**Figure 3.** BND-cellulose chromatography of deproteinized SV40 DNA from superhelical (a) and relaxed (b) pol II ternary complexes. Superhelical and relaxed SV40 ⁴H-labeled DNA containing two to three pol II ternary complexes per genome was disrupted with SDS, extracted with phenol, and precipitated with ethanol. The pellet was dissolved in 0.10 M Tris-HCl (pH 7.2) and 10 mM EDTA (TE buffer) and applied to a 0.5-ml column of BND-cellulose. DNA was eluted in 1.0-ml fractions from the column with TE buffer containing 1.0 M NaCl (1); 1.0 M NaCl and 1% caffeine (2); and 1.0 M NaCl, 1% caffeine, and 1% SDS (3).

**Figure 4.** BND-cellulose chromatography of deproteinized SV40 DNA from superhelical (a) and relaxed (b) *E. coli* RNA polymerase ternary complexes. The protocol was identical with that described in Fig. 3 except that the SV40 DNA contained one to two *E. coli* RNA polymerase ternary complexes per genome.
Figure 5. Effect of pol II binary, initiation, and ternary complexes on the local topology of DNA. Pol II binary (lanes A-D), initiation (lanes E-H), and ternary (lanes I-L) complexes were formed as described in Experimental Procedures. The complexes were relaxed to equilibrium at 37°C, and the deproteinized DNA was electrophoresed in 1% agarose. For each of the three complexes, from left to right, 0.5 μg of SV40 DNA was treated with 0, 0.6, 1.2, and 1.8 μl of pol II extract. The last four samples in the gel show the effect of variations in experimental protocol as described in the text. Except for the sample in lane M, which lacked pol II, the DNA in lanes N-P was treated with 2.0 μl of pol II extract.

The ternary complex unwinding angle has been determined by using the nitrocellulose filter trapping technique. Like the bacterial enzyme, pol II forms a very stable ternary complex resistant to polyanions and high salt (Lescure et al. 1978a,b; Dynan and Burgess 1981). When filtered through nitrocellulose in the presence of heparin and 0.6 M NaCl, DNA that contains a pol II ternary complex is selectively retained on the filter (Table 1). Since the number of active enzymes per microliter of extract is not known for the pol II preparation, several ternary complex reaction mixtures are formed with graded amounts of the enzyme. After relaxation at 37°C, each is passed through nitrocellulose, and the \( \bar{\tau} \) of the retained DNA is determined by gel electrophoresis. In Figure 6 the \( \Delta \bar{\tau} \) of the DNA samples relative to a DNA control relaxed in the absence of pol II is plotted versus the amount of pol II extract in the reaction mixture. As the amount of pol II decreases, \( \Delta \bar{\tau} \) decreases to a plateau value. In the plateau region, there is less than one ternary complex per SV40 genome in the reaction mixture and \( \Delta \bar{\tau} \) is assumed to represent the ternary complex unwinding angle. On the basis of a helical repeat of 10.6 bp per turn and assuming that 10% of the filter-retained DNA does not possess a ternary complex (see Table 1), an unwinding angle of 10-11 bp (360°) is estimated for the pol II ternary complex at 37°C.

DISCUSSION

The DNA unwound region in the E. coli RNA polymerase open-promoter and ternary complexes is 17±1 bp at 37°C. This compares favorably with the estimate of 15 bp made by Melnikova et al. (1978) but is one-third larger than the value of 12 bp, arrived at by

Figure 6. Shift induced by pol II in the mean superhelicity of SV40 DNA recovered from nitrocellulose filters. Pol II ternary complexes on SV40 DNA were formed with the indicated volumes of extract and filtered through nitrocellulose as described in Experimental Procedures. The shift in mean superhelicity of the recovered DNA was measured relative to a DNA sample relaxed in the absence of polymerase.
Siebenlist (1979) for the open-promoter complex. In agreement with earlier observations (Saucier and Wang 1972; Melnikova et al. 1978), both complexes have unwound regions of identical size. Furthermore, the size of the unwound region in the ternary complex is independent of its position on the SV40 genome. Together, these data indicate that the DNA unwound region is rigidly controlled by the polymerase.

A topological model of the ternary complex consistent with the unwinding angle data is shown in Figure 7. Two windase centers bracket the catalytic site and induce a rotation in the DNA helix on either side of the enzyme, as well as in the RNA-DNA hybrid. The rotation is concerted and results in a separation of the DNA helix at the leading unwindase site and its formation again at the lagging rewindase site after displacement of the RNA transcript. In the unwound region, the noncoding DNA strand and the RNA-DNA hybrid interact with two separate binding domains on the polymerase, which converge at the windase centers. A central feature of this model is the rotation of the DNA double helix and not the RNA polymerase. As a result, the model predicts that simultaneous convergent or divergent transcription requires a swivel in the intervening DNA such as could be provided by a topoisomerase. It further predicts that hindrance of DNA rotation, such as through the covalent addition of a large solid support to the helix, will inhibit transcription. These predictions are being tested.

During in vitro transcription, E. coli RNA polymerase moves discontinuously along the helix, pausing for long periods of time at certain distinct positions. One causative factor appears to be the presence in the DNA of a hyphenated dyad symmetry element with a center of symmetry 16 to 20 nucleotides and a start 5 to 10 nucleotides upstream from the pause or termination site (Rosenberg and Court 1979; Farnham and Platt 1981). The three alternative secondary structures that could arise from such a symmetry element are shown in Figure 8. Only the RNA hairpin (Fig. 8b) (Adhya and Gottesman 1978; Farnham and Platt 1980) and the bridged RNA-DNA intermediate (Fig. 8c) (Gamper and Hearst 1982) are consistent with an unwound region of 17 bp. Formation of a DNA hairpin on the noncoding strand (Fig. 8d) (Hobom et al. 1979) would lead to a much greater unwinding of the DNA helix and so is considered unlikely.

Due to the inability of purified pol II to form stable open-promoter complexes under the relaxation conditions, we have been unable to determine an unwinding angle for this complex. In contrast, the pol II ternary complex is a remarkably stable species, similar in many respects to the corresponding E. coli complex. It clearly is able to displace the nascent RNA transcript off the DNA coding strand and maintains an unwound region equivalent to one turn of helix. The length of the unwound region is surprisingly small considering the size and complexity of the pol II enzyme. Nonetheless, it is reasonable to assume that the topological model outlined in Figure 7 also applies to pol II. One observation that suggests this is the case involves an examination of the termination site for transcription of the eukaryotic

**Figure 7.** A topological model for the E. coli RNA polymerase ternary complex. The two oval regions represent the binding domains in the enzyme for the RNA-DNA hybrid and the noncoding DNA strand. (►) Windase centers; (<) catalytic site.

**Figure 8.** Alternative secondary structures for the expression of a hyphenated dyad symmetry element during pausing and termination. The palindromic regions are represented by a bar on the RNA.
histone gene. This site is highly conserved and contains a hyphenated dyad symmetry element upstream from the stop point (Birchmeier et al. 1982). The sequence of this palindromic region and the corresponding region of the tryptophan attenuator are shown in Figure 9. In each case the distance between the dyad center and the stop site is given above the arrows.

Figure 9. Potential RNA hairpin structure at the tryptophan attenuator site (a) and the histone gene termination site (b). The number of nucleotides between the dyad center and the stop site is given above the arrows.

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