Nuclease Cleavage of the Upstream Half of the Nontemplate Strand DNA in an Escherichia coli Transcription Elongation Complex Causes Upstream Translocation and Transcriptional Arrest*

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We tested the susceptibility of nucleic acid strands in a halted transcription elongation complex to digestion by micrococcal nuclease (MN). The 16-nucleotide nascent RNA was protected within RNA polymerase. A 27–28-nucleotide template strand DNA fragment also was resistant to MN digestion. However, the upstream half of the nontemplate DNA within this region was digested rapidly by MN, suggesting that the nontemplate strand emerges from the RNA polymerase near the middle of the melted transcription bubble with the bases oriented away from the enzyme surface. MN cleavage of the exposed nontemplate DNA shifted polymerase backward, making it unable to extend the RNA chain. However, the MN-trimmed G16 complexes could be reactivated by GreB-stimulated cleavage of the nascent RNA. These results favor a model of transcriptional arrest involving upstream slippage of RNA polymerase along the RNA and DNA chains. They also suggest that the exposed segment of nontemplate DNA may directly or indirectly stabilize the lateral position of the transcription complex along the DNA.

Transcribing RNA polymerase contacts ~30 bp of DNA, although the length of DNA protected from cleavage agents can vary by as much as ~10 bp in transcription complexes halted near initiation, pause, termination, or arrest sites (1–9). An ~18-bp segment of DNA from near the upstream edge to the middle of this footprint is melted to form the transcription bubble, as indicated by both topological and chemical reactivity assays (7, 9–12). The reactive region also can vary by ~5 bp in some halted complexes (7, 9, 11), although this has yet to be tested by topological measurement. To maintain the transcription bubble, the nontemplate DNA strand must be separated from the template strand as polymerase moves forward and then reanneal after the template strand is transcribed. Previous findings led us to suggest that, whereas strand separation occurs within polymerase, ~10–15 bp upstream from its forward edge on DNA, the strands reanneal passively as the template DNA exits upstream (Refs. 11 and 13; see Fig. 1).

This hypothesis predicts that a part of the nontemplate DNA may be exposed on the outside of the transcription complex, rather than protected within it. The chemical reactivity of the nontemplate DNA is consistent with this view. Within the transcription bubble, bases on the nontemplate strand are highly reactive with single strand-specific reagents such as K\textsubscript{2}MnO\textsubscript{4}, dimethylsulfate, or diethylpyrocarbonate (7, 9, 11, 12), whereas the phosphodiester backbone is mostly protected from cleavage by HO\textsuperscript{−} (hydroxyl radical) (3, 7). The template DNA in contact with RNA polymerase also is protected from HO\textsuperscript{−} generated in solution; however, it is readily cleaved by HO\textsuperscript{−} generated from the intercalating reagent 1,10-phenanthroline copper, whereas the corresponding nontemplate DNA is not (14–16). This suggests that interactions of the template strand bases with polymerase or with RNA mediate high-affinity binding of 1,10-phenanthroline copper, but that nontemplate strand bases either are loosely associated with polymerase or held in a channel accessible to small reagents such as K\textsubscript{2}MnO\textsubscript{4} but not to the larger 1,10-phenanthroline copper. Although these findings are consistent with the idea that part of the nontemplate strand lies on the surface of RNA polymerase, they fall short of establishing it unambiguously or delineating which part is exposed. Furthermore, although the major sources of transcription complex stability recently were localized to contacts to the nascent RNA, the template DNA strand near the active site, and ~10 bp of duplex DNA in front of the transcription bubble (17), the possible role of the nontemplate DNA in stabilizing the position of polymerase along the DNA is unknown.

To address these questions, we examined the susceptibility of the nontemplate DNA to cleavage by micrococcal nuclease (MN). MN cleaves both RNA and DNA endonucleolytically by Ca\textsuperscript{2+}-mediated attack of OH\textsuperscript{−} at the 5′-position of the phosphodiester bond, yielding 3′-mononucleotides or -oligonucleotides (18–20). Bases are bound without sequence specificity within a large (~10 Å wide) hydrophobic pocket, whereas the scissile and 3′-phosphates make hydrogen bond contacts to MN near the rim of the pocket. These properties make MN an ideal probe for exposure of bases in the transcription complex.

We found that rapid MN cleavage of nontemplate DNA within the transcription bubble causes RNA polymerase to slide upstream along the DNA and RNA chains into an arrested state. An arrested RNA polymerase remains bound to the DNA and nascent transcript but is unable to extend the RNA chain because its 3′-OH has relocated outside of the active site (4, 8, 21–27). Either active site slippage relative to the RNA or RNA slippage relative to the active site generates complexes in which the antiarrest factors GreA or GreB can stimulate internal cleavage of the RNA chain (28), thus restoring the ability of polymerase to extend it. We consider the implications of our findings for the mechanism of arrest under “Discussion.”
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FIG. 1. DNA sequence of the G16 transcription complex and summary of previous studies of halted elongation complexes (11, 13). The boundaries of the G16 complex as measured by ExoIII footprinting are indicated by arrows. A summary of the reactivities of G16 (13) and 12 other halted elongation complexes (11) to KMnO4, and diethylpyrocarbonate, reagents that are specific for unstacked bases, is plotted above and below the strands (arbitrary scale with 1 equal to strong reactivity). The protected region of template DNA probably reflects an ~8 bp RNA-DNA hybrid (11), although protection by strong interaction with RNA polymerase has not been ruled out.

MATERIALS AND METHODS

Reagents and Enzymes—ApU was purchased from Sigma; polynucleotide kinase from New England Biolabs; micrococcal nuclease from Boehringer Mannheim; purified NTPs from Pharmacia Biotech Inc.; and [α-32P]GTP and [γ-32P]ATP from Amersham Corp. RNA polymerase was purified by the method of Burgess and Jendrisak (29) with the modifications described previously (28). Grea and GreB were purified by after overproduction from plasmids pDNL279 and pGF103 as described previously (6, 28).

Preparation of DNA Templates—DNA templates for transcription reactions were synthesized by polymerase chain reaction amplification from plasmid pC185 (28) with primers that hybridize upstream and downstream from the T7A1 promoter-leader segment; primer 947, 5′-GTGGTTTAAATTTGAGCCG (50 bp downstream). The polymerase chain reaction product is 142 bp long and yields a 50-nt-long runoff transcript (see Fig. 1).

Transcription Complex Formation, Purification, and MN Digestion—G16 transcription elongation complexes (halted prior to addition of U17) were formed by incubation of 25 pmol of Escherichia coli RNA polymerase holoenzyme (Eσ20) and 20 pmol of DNA in 50 μl of 20 mM Tris acetate, pH 8.0, 20 mM NaCl, 20 mM MgCl2, 14 mM β-mercaptoethanol, 20 μg of acetylated bovine serum albumin/ml, 2% (v/v) glycerol. After exchanging to MN buffer, CaCl2 was added to G16 complex to a final concentration of 1 mM. G16 complexes were then digested with 10 units of MN/μl (final) at 37°C for the time desired. Digestions were stopped by adding 5 μl EDTA (final) to the mixture.

Agarose Gel Electrophoresis of Transcription Complexes—G16 complexes before and after MN digestion were electrophoresed through a 50 × 75-mm 4% NuSieve agarose gel (FMC) cast on the hydrophilic side of a 1.5-mm thick gel slice and stained with 0.1% w/v Coomassie Brilliant Blue R (Sigma) in 30% acetic acid for 60 min and destained by gentle shaking overnight submerged in a covered tray of 20% methanol, 10% acetic acid, also containing a small piece of open cell polystyrene foam. Adherence to Gelbond film is essential to prevent disintegration of the agarose gel, which becomes brittle in the stain and destain solutions.

Recovery, End Labeling, and Sequencing of the MN-digested DNA—To recover the MN-digested DNA, the digested G16 complexes were extracted once with phenol-chloroform, the aqueous layer was then extracted once with chloroform, and the DNA was recovered by ethanol precipitation.

To end label the recovered DNA products, 1 pmol of MN-digested DNA (MN digestion generates 5′-OH) was incubated with 15 units of polynucleotide kinase (New England Biolabs) in buffer provided by the manufacturer and 2 μl of [γ-32P]ATP in a final volume of 15 μl at 37°C for 30 min. The end-labeled DNA was purified away from the free [γ-32P]ATP by centrifugation through a pre-equilibrated 150-μl Sepharose G-10 spin column (Pharmacia) at 500 × g for 5 min. The end-labeled DNA then was mixed with an equal volume of loading buffer (2 × TBE, 50% (w/v) urea, 0.25% (w/v) each xylene cyanol and bromphenol blue), heated at 95°C for 5 min, and resolved on a 20% polyacrylamide (19:1 acrylamide:bisacrylamide), 7M urea, 0.5 × TBE gel. The gel was then exposed to Kodak XAR-5 film to locate the labeled, single strand DNA. The radioactive bands were then excised as small gel slices and soaked in 1% SDS, 0.5 μ m ammonium acetate overnight at 37°C for elution. The supernatants were separated from the gel slices, extracted with phenol-chloroform, and then extracted to dryness with 1-butanol. The pellets containing the labeled, single strand DNAs were washed once with 70% ethanol, dried under vacuum, and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Chemical sequencing of purified 5′-end-labeled single strand DNAs was performed as described by Maxam and Gilbert (32). The sequencing products were then resolved on a 20% polyacrylamide sequencing gel (19:1 acrylamide:bisacrylamide) and visualized by exposing on Kodak XAR-5 films.

Elongation of MN-digested G16 Complex—After MN digestion, MgCl2 was added to the trimmed G16 complex to a final concentration of 7 mM. Then the trimmed G16 complex was elongated in the presence of 600 μM all four NTPs for 1, 5, and 30 min at 37°C with or without 0.12 μM GreB.

FIG. 2. MN digestion of G16 elongation complex. G16 complexes were formed and exchanged to MN buffer on purification as described (see “Materials and Methods”). The purified G16 complexes were digested with MN for 0 and 25 s and 2, 4, 8, 15, and 30 min at 37°C, and then electrophoresed through a 4% agarose gel. A, gel stained with ethidium bromide to detect DNA. B, gel stained with Coomassie Brilliant Blue to detect protein.

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RESULTS

The G16 Complex Is Stable after MN Digestion—To test whether the G16 complex is stable after MN digestion, we performed a digestion time course (Fig. 2). After successive intervals, an aliquot of the reaction was removed and stopped by addition of EGTA to 3 mM. We then electrophoresed the samples on a 4% agarose gel (see “Materials and Methods”). After electrophoresis, we stained the gel successively with ethidium bromide to detect the DNA (Fig. 2A) and with Coomassie Brilliant Blue to detect the protein (Fig. 2B). Greater than 90% of the DNA template was incorporated into G16 complex (Fig. 2A, lane 1). A small amount of the DNA was associated with a slower moving complex, probably the result of loading a second polymerase onto the promoter once the G16 complex formed (33). The second polymerase was released from the DNA by 2 min of MN digestion, since only a single MN-trimmed complex was evident at that point (Fig. 2A and B, lane 3). MN digestion was complete after 8 min when all the G16 complex was converted to a faster moving band and most of the ethidium bromide-stainable DNA was lost (Fig. 2A and B, compare lanes 5 and 1).

In contrast, the 16-mer nascent RNA in G16 complexes was completely resistant to digestion by MN (Fig. 3A, lanes 2–7) but not after it was artificially dissociated from the complexes and gel-purified (Fig. 3B, lanes 2–9). Presumably the 16-nt nascent RNA remained within RNA polymerase and was protected from MN attack. Longer nascent RNAs also were cleaved by MN and shortened to a 20–24-nt 3'-protected fragment (data not shown). However, the G16 nascent RNA no longer could be extended even after 2 min of MN digestion (Fig. 3A, lanes 8–13), which was well before the DNA digestion was complete (~8 min, see above). We conclude that MN digestion trims excess DNA from transcription complexes and efficiently converts them to arrested complexes.

To test this interpretation, we treated the G16-arrested complexes with the transcript cleavage factor GreB (Fig. 3C). At low molar ratio (Fig. 3C, lanes 2 and 3), GreB principally cleaved off a 6-nt fragment (Fig. 3C, b), leaving a 10-nt nascent RNA (Fig. 3C, a). A weaker cleavage released a 5-nt fragment, producing an 11-nt nascent RNA. At a higher concentration (Fig. 3C, lanes 4 and 5), GreB shortened these further to a 7-nt nascent RNA (Fig. 3C, c) with release of dinucleotides or trinucleotides (Fig. 3C, d). When GreB treatment was conducted in the presence of NTPs, the same initial cleavage product was evident, but the nascent RNA was rapidly elongated to an ~24-nt runoff RNA (Fig. 3C, lanes 6–8). Treatment of MN-trimmed G16 complexes with GreA yielded similar results, although large 3'-cleavage products were not detected (data not shown). We conclude that G16 complexes were inactivated, but not dissociated, by MN digestion.

The inactivated complexes had features typical of arrested transcription complexes; they could resume elongation only...
after GreB-induced nascent RNA cleavage, which produced large 3'-RNA fragments indicative of relocation of the active site over an internal phosphodiester bond of the nascent RNA. The 10- and 6-nt fragments produced suggest that the active site was positioned over the G10-A11 phosphodiester bond after MN treatment. Interestingly, prolonged incubation at 4°C of G16 complexes that have not been treated with MN also leads to arrest and slow, spontaneous cleavage between A11 and C12 on shifting to 37°C (28). The correlation of large fragment cleavage, arrest, and dwell time was discovered subsequent to publication (data not shown) and has been described by several other groups (5, 8, 21, 23–25, 36). The 1-n-nt difference in cleavage position suggests that MN treatment causes RNA polymerase to slide backward further than occurs spontaneously.

The Upstream Half of the Nontemplate Strand in the Transcription Bubble of G16 Complex Is Cleaved by MN—In addition to differences in elongation potential and GreB cleavage, MN-trimmed complexes elongated only to position +24, 6 nt short of the boundary of G16 complexes previously defined with ExoIII (Ref. 1; see Fig. 1). To determine whether this reflected differences in the protected DNA strands that remained in the MN-trimmed elongation complex, we analyzed their sequences. After extraction and recovery, the fragments were 5'-end-labeled and resolved on a 20% denaturing gel (see “Materials and Methods”). We observed nine bands ranging from −12 to +30 relative to the transcription start site are shown. The lines above or below that DNA sequence indicate the fragments remaining after MN treatment of G16 complexes. The thickness of the lines represents the relative intensity of the bands.

Comparison of the sequences of bands a–c and g with that of DNA within the G16 complex led to four main observations (Fig. 4B). First, bands a–c corresponded to the template strand DNA, and bands d, e, and g corresponded to the nontemplate strand. Second, all the bands shared the same downstream edge (Fig. 4B), indicating that the downstream DNA in the MN-trimmed G16 complex is tightly and uniquely associated with RNA polymerase. Third, the upstream half of the nontemplate DNA in the G16 complex was much more accessible to MN digestion than was the template strand DNA. Cleavage sites in the nontemplate strand DNA were within the transcription bubble (compare Figs. 1 and 4B), suggesting that the upstream half of the nontemplate strand in the bubble is exposed. Fourth, the upstream half of the nontemplate DNA in the transcription bubble could not be recovered from the MN-trimmed G16 complex. It is likely that this part of the DNA was released from the polymerase after MN cleavage and reduced to mononucleotides and dinucleotides that were lost during EtOH precipitation (see “Materials and Methods”). We conclude that the upstream portion of the nontemplate DNA strand is unnecessary for transcription complex stability, in agreement with the findings of Nudler et al. (17) that only downstream duplex DNA, the nascent RNA, and a short segment of the template DNA near the active site are required for stability of the transcription complex.

**DISCUSSION**

We report here that the upstream half of the nontemplate DNA in the transcription bubble of a G16 halted elongation complex is exposed to attack by MN. The RNA transcript, the template strand DNA, and the other half of the nontemplate strand DNA, however, are relatively protected. MN digestion of
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A current model for transcriptional arrest is that relocation of the active site over an internal phosphodiester bond in the nascent RNA arises when part or all of the RNA polymerase slips backward along the DNA template, blocking subsequent nucleotide addition until transcript cleavage occurs (4, 8, 21–24, 26, 27). Reports that the RNA cleavage interval increases on arrest (5, 8, 21, 23–25) and that cleavage causes upstream shifting of the footprint of RNA polymerase on DNA in a nonarrested complex (13), but not in an arrested transcription complex (22), all favor this model. Here we find that an upstream shift in both edges of the DNA footprint of the polymerase is associated with arrest. Our results thus strongly support the idea that backward translocation of RNA polymerase along DNA and RNA chains can trigger transcriptional arrest. In this case slippage and arrest are caused by nontemplate strand removal, but similar backward slippage on transcriptional arrest also has
been observed without MN treatment for both E. coli RNA polymerase (36) and mammalian RNA polymerase II (37).

A key question now is how these slippage events are related to the apparent discontinuous changes in the RNA and DNA footprints of RNA polymerase (so-called “inchworming”) that have been found to be associated with pausing, arrest, and termination (5, 6, 8). Flexibility in RNA polymerase on encountering certain DNA or RNA sequences could lead to dislocation of the RNA 3′-end from the active site of the polymerase and subsequent sliding of the enzyme. Alternatively, sliding of RNA polymerase along the RNA and DNA chains when complexes are halted for study near regulatory sites may generate the inchworm footprints (36, 37), reflecting a change in the energetic state of the transcription complex that foreshadows pausing, arrest, or termination.

**Regulatory Factor Interaction with the Nontemplate Strand**—The upstream half of the nontemplate strand recently has been found to be the target of a noncanonical activity of the E. coli σ70 initiation factor (38): induction of pausing at position 16 of the λ late transcription unit (39). At least in these complexes, the nontemplate strand is exposed in a way that allows sequence-specific recognition by σ70. Thus, as in our finding that MN can digest this portion of nontemplate DNA (which appears to require outward orientation of the bases), σ70-induced pausing suggests that nontemplate bases are exposed to potential interactions with regulatory factors. These findings raise the speculative possibility that some factors that regulate transcript elongation could target the nontemplate DNA strand, inhibiting or promoting either forward or reverse translocation of the enzyme, and thus favoring pausing, arrest, termination, or rapid transcription, by influencing availability of the nontemplate DNA strand for re-pairing with the template DNA strand upon translocation.

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