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Mapping the *Escherichia coli* transcription elongation complex with exonuclease III

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Summary

RNA polymerase interactions with the nucleic acids control every step of the transcription cycle. These contacts mediate RNA polymerase recruitment to promoters; induce pausing during RNA chain synthesis, and control transcription termination. These interactions are dissected using footprinting assays, in which a bound protein protects nucleic acids from the digestion by nucleases or modification by chemical probes. Exonuclease III is frequently employed to study protein-DNA interactions owing to relatively simple procedures and low background. Exonuclease III has been used to determine RNA polymerase position in transcription initiation and elongation complexes and to infer the translocation register of the enzyme. In this chapter, we describe probing the location and the conformation of transcription elongation complexes formed by walking of the RNA polymerase along an immobilized template.

Keywords

Transcription; RNA polymerase; Footprinting; Translocation

1. Introduction

Footprinting (FP) techniques have been widely employed in studies of protein-nucleic acid interactions and nucleic acid structures. The basic concept is that protein binding or formation of a secondary structure will protect an affected nucleic acid region from attack by a protein nuclease or a chemical probe, generating a footprint. Exonuclease III (Exo III) is one of the most frequently employed enzymes to probe protein interactions with the double-stranded DNA. Exo III catalyzes the processive removal of the mononucleotides from the 3'-hydroxyl termini of duplex DNA (1). The free DNA is fully digested by Exo III, eliminating potential background problems caused by the presence of unbound DNA molecules. This is especially important when the protein binding to its DNA target does not proceed to saturation (2). When a protein binds to the DNA, the advance of Exo III is blocked. The lengths of the resultant DNA fragments indicate the boundaries of the DNA region protected by the protein of interest. If one of the DNA strands is labeled (radioactively or fluorescently), the protected region can be visualized after electrophoresis in a denaturing sequencing gel.

The RNA synthesis by the DNA-dependent RNA polymerase (RNAP) is a crucial step in the flow of genetic information. Among the transcription systems, the one mediated by the *Escherichia coli* RNAP has been most thoroughly studied. The precise control of the RNAP location along the DNA is essential for the proper regulation of every step of transcription and may contribute to RNA folding and protein synthesis. In some instances, it is imperative to map RNAP location on DNA template with a single nucleotide precision as well as to monitor changes in its conformation.

Exo III FP has been broadly applied to analyze diverse *E. coli* transcription complexes. Straney and Crothers carried out Exo III FP to study the conformation of promoter complexes during transcription initiation (3). Heumann and colleagues first observed a dramatic reduction of the RNAP-protected region upon transition from initiation to elongation, whereas the RNAP conformation appeared to remain largely constant during elongation (4). Interestingly, the authors found that Exo III performed better in mapping the upstream boundary of the RNAP footprint than the downstream one. In the 1990s, several groundbreaking insights into the mechanism of transcription can be attributed in part to the Exo III FP. During mapping of transcription elongation complexes (TECs) halted at different positions along the template, Goldfarb and Landick groups observed RNAP footprints of different sizes, suggesting that the enzyme advances discontinuously when approaching a pause or a termination signal (5,6). Subsequent studies demonstrated that these apparently non-monotonous movements were due to different propensities of RNAP to backtrack along the nucleic acid chains (7,8), a movement that can be favored by the processive action of Exo III advancing from the downstream direction, emphasizing the importance of the experimental conditions (7). Exo III FP can also be applied to visualize other conformational changes in transcription complexes. For example, Kulbachinskiy and colleagues observed “scrunching” during σ -induced pausing, wherein the upstream protection boundary remained unchanged while the downstream protection boundary advanced upon addition of nucleotides to the nascent RNA (9). Finally, Exo III is sufficiently precise to study subtle movements of the enzyme, such as between a pre-translocation and a post-translocation states of RNAP in the course of a nucleotide addition cycle (10,11), and to elucidate the molecular mechanisms of RNAP inhibitors that affect the RNAP translocation (12,13). Besides the characterization of bacterial RNAP, Exo III FP has been used in studies of RNAP from other domains of life (14,15). The technique of immobilizing TECs is crucial to the studies of transcript elongation as it allows step-wise synchronized movements of RNAP along the DNA until the RNAP reaches a chosen position. DNA or RNAP is first immobilized on a suitable resin (the nickel-histidine and streptavidin-biotin interactions are most frequently employed, but other approaches can also be used). Following the formation of a stable halted TEC, the resin is washed extensively to remove the unincorporated NTPs, and a subset of NTPs required to advance the RNAP to the next position, in single- or multiple-nucleotide steps dictated by the DNA template sequence, is added (16). Selected TECs can be analyzed by a method of choice, including Exo III FP to monitor the RNAP position and evaluate changes in its conformation. Simultaneously, the position of the RNAP active site is monitored by labeling the nascent RNA (7,17).

In this chapter, we describe a protocol for using Exo III to map the *E. coli* RNAP walked through a short regulatory sequence on an immobilized template.

2. Materials

This section lists proteins, reagents, buffers and equipment necessary for these experiments. In some cases, as noted, the source of the reagent is critical; in most, many commercial sources should prove adequate even though we did not test them.

2.1. Supplies

1. 1.7 mL siliconized microcentrifuge tubes.
2. PCR purification kit. We use QIAquick PCR purification kit (Qiagen) and while other kits should also be suitable, templates purified by kits from some suppliers (*e.g.*, Promega) inhibit *in vitro* transcription reactions with *E. coli* RNAP.
3. G-50 spin columns. We use Illustra MicroSpin (GE Healthcare).
4. A standard kit for DNA sequencing. We use SequiTherm II Excel kit (Epicentre).
5. Low retention, sterile, RNase/DNase free micropipette tips.
6. Magnetic streptavidin-coated resin. We use Invitrogen Dynabeads M-280.

2.2 Nucleotides

1. FPLC-purified rNTPs (we use rNTPs from GE Healthcare; *see* Note 1).
2. Adenylyl (3'-5') uridine (ApU) RNA dinucleotide (*see* Note 2).
3. ³²P labeled nucleotides.
4. dNTPs.
5. DNA oligonucleotides for template preparation.

2.3 Proteins

1. T4 polynucleotide kinase supplied with 10X buffer.
2. *Taq* DNA polymerase supplied with 10X buffer.
3. RNAP core enzyme and σ factor purified as in (18); *see* also chapter XX.
4. Exonuclease III.

2.4 Transcription and footprinting solutions

2.4.1. Stock solutions—Solutions are treated with DEPC (solutions 1-4) or prepared with DEPC-treated ultra-pure H₂O (resistivity of 18.2 M Ω , MilliQ); (solutions 5-7), unless otherwise indicated, and filtered through a 0.45 μ m filter. Following DEPC treatment (0.1%,

¹In our hands, FPLC-purified NTPs from GE Healthcare (formerly Pharmacia) work best for stepwise walking of RNAP.

²Although RNAP can initiate using single NTPs, their K_m are relatively high. Using a dinucleotide primer allows one to initiate transcription at low [NTPs], a condition essential for limiting readthrough during formation of a halted TEC.

1 mL/L solution) overnight with stirring, autoclave the solution (for solutions 1-4 only, to remove traces of DEPC) and store at room temperature.

1. 1 M Tris-HCl (pH 7.9).
2. 1 M KCl.
3. 4 M NaCl.
4. 1 M MgCl₂.
5. 2M Tris-acetate (pH 8.0).
6. 2M Na acetate.
7. 1M Mg acetate.
8. 1 mg/mL heparin.
9. 0.5 M EDTA: Weigh 93.06 g EDTA disodium salt dihydrate, add 350 mL DEPC-treated H₂O and NaOH pellets. When the solution pH is close to 8.0 and all EDTA is dissolved, adjust pH with 10 M NaOH solution to pH 8, filter.
10. 10X TBE: Weigh 216 g Trizma base, 110 g Boric acid, dissolve in 1750 mL ultra-pure H₂O, add 80 mL 0.5 M EDTA, adjust the final volume to 2 L, filter.
11. 1 M DTT: Weigh 1.5425 g DTT; dissolve in 10 mL ultra-pure H₂O, filter through a syringe microfilter (0.22 µm), aliquot 100 µL/tube, and freeze at -20°C.

2.4.2. Working solutions—The buffers and water for *in vitro* transcription are prepared with DEPC-treated ultra-pure H₂O. All pipette tips, falcon tubes, and microcentrifuge tubes used in buffer preparation and *in vitro* transcription are RNase-free. The RNAP storage buffer, 10X TGA2 buffer and 2X stop buffer are aliquoted and stored at -20 °C. The wash buffers are stored at 4 °C.

1. RNAP storage buffer: 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 50% glycerol.
2. 10X TGA2: 200 mM Tris-acetate (pH 8.0), 200 mM Na acetate, 20 mM Mg acetate, 140 mM β-ME, 1 mM EDTA, 50% glycerol.
3. High salt wash buffer: 500 mM KCl, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.9), 1 mM β-ME.
4. Low salt wash buffer: 20 mM KCl, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.9), 1 mM β-ME.
5. 2X stop buffer: 24 g Urea, 20 mL H₂O, 0.1 g Bromophenol blue, 0.1 g Xylene cyanol, 100 µL 0.5 M EDTA, 5 mL 10X TBE buffer. Heat at 65 °C to dissolve the urea. Heat 15 min at 65 °C before use.

2.5 Gel electrophoresis

We prepare two stock acrylamide solutions, 15% and 0%, to pour denaturing gels ranging from 4 to 15%. Above 15%, gels are difficult to dry. We store the stock solutions at 4 °C for up to 3 months and mix them at the desired ratio just before use (*see* Note 3).

1. To make 0% solution, weigh 420.42 g urea and slowly add it to 500 mL ultra-pure H₂O on a magnetic stirrer hot plate. Stir at 65 °C until urea is dissolved. Adjust the volume to 1 L with H₂O. Add 12 g TMD-8 mixed bed resin hydrogen and hydroxide form (we purchase it from Sigma) and continue stirring for 30 min. Filter through a bottle top filter (0.45 µm) and degas using a vacuum pump for at least 30 min. Store at 4 °C (*see* Note 4).
2. Prepare 15% solution as above, substituting 500 mL 30% (w/v) acrylamide/bis-acrylamide (19:1) for 500 mL ultra-pure H₂O.
3. N,N,N',N'-Tetramethylethylenediamine (TEMED).
4. Ammonium persulfate: prepare 10% (w/v) solution in H₂O. Aliquot 200 µL/tube, store at -20 °C.

2.6. Equipment

1. Plastic pipettes and micropipettes of various volumes.
2. Table top centrifuge.
3. PCR cycler.
4. Heating blocks set at 37 °C, 90 °C, and 65 °C (optional); (*see* Note 5).
5. Hot plate/stirrer.
6. Vacuum pump.
7. Vertical gel electrophoresis system and power supply.
8. Matching sets of glass plates and spacers (*see* Note 6).
9. Gel dryer.
10. Phosphor screen and cassette.
11. Phosphorimaging system (we use FLA 9000; GE Healthcare).

3. Methods

3.1. Preparation of transcription templates

The DNA template is generated by PCR amplification of an appropriate plasmid using two oligonucleotides, one of which is 5' labeled with biotin for immobilization and the other – with [³²P] phosphate for visualization. Primers can be obtained from many sources; standard

³The gel for footprinting should be made freshly (less than 24 hours) before electrophoresis.

⁴We find that de-ionizing and degassing the gel solutions improve the footprint resolution.

⁵We prefer using heating blocks (*e.g.*, filled with sand) with radiolabeled samples over a water bath.

⁶To facilitate the separation of the plates after electrophoresis, the shorter plate may be siliconized after 15 to 20 electrophoreses.

desalting is sufficient. In the experiment described here, we used pIA349 vector (19) that encodes a T7A1 promoter followed by a U-less initial transcribed region and an *ops* pause signal (*see* Fig. 1). In this chapter, we use a 211 bp template (transcribed region 119 bp) for the FP assay (*see* Note 7).

The template was generated with 2 primers:

Top: 5'-GGAGAGACAACTTAAAGAGACT-3'.

Bottom: 5'-CCACCATCATCACCATCATCCT-3'.

The choice of primer labeling is dictated by the identity of the strand probed.

3.1.1. Radiolabeling primer

1. Mix 2 μ L, 100 μ M primer with 2 μ L H₂O, 1.5 μ L T4 polynucleotide kinase, 1.5 μ L 10X T4 polynucleotide kinase buffer and 8 μ L [γ -³²P]ATP (10 mCi/mL, 6000 Ci/mmol).
2. Incubate 45 min at 37 °C.
3. Terminate the labeling reaction by heating 3 min at 95 °C.
4. Add 10 μ L H₂O to the mixture in order to fit the minimum loading volume of G-50 column (*see* Note 8).
5. Prepare the G-50 column by gently vortexing it and centrifugation at 735 g for 1 min.
6. Place the column into a new microcentrifuge tube.
7. Load the sample onto the column and elute the DNA by centrifugation at 735 g for 2 min.

3.1.2. PCR amplification

1. Assemble a reaction mixture from the following components:
 - H₂O to 200 μ L.
 - 2 μ L biotin labeled primer (100 μ M).
 - All of the radiolabeled primer (about 30 μ L) prepared in 3.1.1.
 - 20 μ L 10X *Taq* polymerase standard buffer.
 - 16 μ L dNTP mix (2.5 mM of each dNTP).
 - 2 μ L pIA349 vector (10 ng/ μ L).
 - 1 μ L *Taq* DNA polymerase (5 U/ μ L).
2. Amplify using a standard cycle, *e.g.*,

⁷When designing a template, it is imperative to predict the region of protection. DNA fragments shorter than 100 nucleotides provide the best resolution.

⁸Based on our experience, the minimum loading volume of G-50 column is 25 μ L, not the 12 μ L recommended by the manufacturer.

- Initial incubation at 94 °C for 2 min.
 - 30 cycles: 10 s at 94 °C, 10 s at 58 °C, 10 s at 72 °C.
 - Final extension: 2 min at 72 °C.
3. Purify the PCR product using a kit.
 4. Measure the DNA concentration on a 1% agarose gel (*see* Note 9).

3.2. Immobilization of the DNA template

1. For a 100 µL reaction with about 60 nM DNA template, 15 µL of magnetic streptavidin-coated beads is required (*see* Note 10).
2. Place the tube on the magnetic particle separator to pellet beads and remove the supernatant (usually, 20 - 40 s is enough to separate the supernatant from the beads).
3. Wash the beads with 200 µL H₂O; repeat at least twice.
4. Wash the beads with 200 µL 1X TGA buffer; repeat three times.
5. Resuspend the washed beads in 100 µL of 1X TGA buffer.
6. Add DNA template and mix with the beads. Incubate 25 - 30 min at 37 °C. Every 5 min, resuspend the mixture to prevent the beads from settling at the bottom of tubes.
7. Wash the beads with 200 µL 1X TGA buffer three times. Estimate the DNA concentration using Geiger counter (*see* Note 11).

3.3. The formation of the halted TEC

The analysis of post-initiation events in transcription requires the synchronization of TECs – if initiated from a promoter, RNAP molecules will move along the DNA template at different rates. The TECs are synchronized by halting the elongating RNAP 20 nts or further downstream from the promoter, which is achieved by omitting one or more NTPs from the starting reaction, as determined by the initial transcribed region. In the template used here, a 37 nts long RNA can be synthesized if transcription is initiated with the ApU primer in the absence of UTP.

1. Assemble a 100 µL reaction mixture from the following components:
 - H₂O to 100 µL.
 - 10 µL 10X TGA2 buffer.

⁹To measure the concentration of the radiolabeled template, several dilutions of an unlabeled PCR product at a known concentration are loaded on an agarose gel alongside the radiolabeled product, stained with an intercalating dye, visualized, and analyzed by densitometry.

¹⁰Although the 15 µL beads in 100 µL of 60 nM DNA far exceeds the volume recommended by the manufacturer, we found that this amount is the minimum for this assay (3 walking cycles) because of the beads loss during washing. The more washing cycles, the larger starting volume of beads is required.

¹¹The binding efficiency is far less than 100%, between 33% and 60% in our hands.

- 10 μ L 10X initiation NTP mixture (25 μ M ATP and GTP, 10 μ M CTP) (*see* Note 12).
 - 1.5 μ L 10 mM ApU.
 - 3 μ L RNAP holoenzyme (if using 4 μ M stock; the volume depends on the activity of the RNAP preparation, which is usually 50 -70%).
 - If transcription product at each step is to be observed, add 1.5 μ L [α -³²P]CTP (3000 Ci/mmol).
2. Incubate 15 min at 37 °C (*see* Note 13).
 3. Add heparin to 15 μ g/mL and incubate 3 min at 37 °C (*see* Note 14).

3.4. Stepwise walking of immobilized TECs

1. After forming the halted TEC, remove the aliquots for Exo III FP. Usually, 10 - 15 μ L aliquot from the aforementioned mixture is adequate for one FP reaction.
2. Place the magnetic particle separator on ice and the tubes containing the halted TEC on the separator; remove the supernatant completely without disturbing the beads.
3. Resuspend the beads in 300 μ L of high salt wash buffer, wait for all beads to pellet, and remove the supernatant. Repeat three times.
4. Wash the beads with 300 μ L low salt wash buffer as above. Repeat three times.
5. Resuspend the beads in NTP mixture (10 μ M of each NTP required in the next step, *e.g.*, in the first step, from G37 to G40, the mixture is ATP/GTP/UTP; NTPs are diluted in 1X TGA2 buffer).
6. Incubate 2 min at 37 °C to allow for nucleotide incorporation.
7. Repeat steps 1 - 6 for the next walking step.

3.5. Exo III digestion

1. Add 100 units of Exo III to each TEC sample. Mix thoroughly and incubate at 37 °C.
2. Remove aliquots after 0 s (before adding Exo III), 30 s, 60 s and 90 s (*see* Note 15).
3. Quench the reaction by adding an equal volume of 2X stop buffer.

¹²The 10X initiation NTP mixture should be frozen at -20 °C in single-use aliquots. The CTP is subject to deamination and the resultant trace UTP allows RNAP to read through the G37 position. This also applies to the “walking” NTP subsets (section 3.4). Using a template that requires a different subset of starting NTPs can alleviate this problem.

¹³This time is sufficient for all the active RNAP to form the halted complex. After 15 min, the mixture can be transferred to ice. The halted complex usually retains its activity for at least 3 - 4 hours on ice.

¹⁴The heparin treatment is crucial to eliminate non-specifically bound RNAP, which will compound the analysis of the footprint patterns.

¹⁵The Exo III amount and digestion time should be carefully adjusted to avoid “pushing” RNAP backwards (7).

3.6. Electrophoresis

1. Assemble the gel cassette using two side spacers and a strip of Whatman filter paper as a bottom spacer (*see* Note 16). Clamp the plates tightly to ensure watertightness.

To make a 45 cm 6% polyacrylamide sequencing gel, mix 31 mL of 15% gel solution and 40 mL of 0% gel solution. Add 3.75 mL of 10X TBE (to the final 0.5X concentration), 200 μ L 10% ammonium persulfate, and 40 μ L TEMED. Mix well.
2. Slightly raise one side of the gel cassette; slowly load the mixture into the cassette with a syringe (without needle). Insert the comb and cover the gel with plastic wrap.
3. Allow 1 hour for polymerization, attach the cassette to the vertical electrophoresis system. If the system is not equipped with an aluminum plate, attach one to the cassette to allow for uniform heat distribution. Remove the comb and wash the wells with 0.5X TBE immediately.
4. Preheat the gel to 55 °C by running in 0.5X TBE at 130 W (~2,750 V) for 30 min.
5. Heat the samples 2 min at 95 °C, then centrifuge for 1 min at top speed.
6. Wash the wells again and load the samples on the sequencing gel along with DNA sequencing ladders (*see* Notes 17 and 18).
7. Run the gel at 120 W for 10 min, increase the power to 130 W, and run for about 1 hour (*see* Note 19).
8. After electrophoresis, separate the gel plates, transfer the gel to Whatman filter paper, and cover it with plastic wrap.
9. Dry the gel on a gel dryer for 1 hour.
10. Expose the gel to a phosphor screen overnight.
11. Visualize the footprints with phosphor imaging system and analyze the data with chosen software; we use ImageQuant 5.0 (Molecular Dynamics). A representative result is shown in Fig. 2.

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¹⁶Using a paper spacer eliminates the need of removing the bottom spacer and air bubbles that arise in the process.

¹⁷Load the sequencing ladders as close to the sample lanes as possible to minimize the “smiling effect”. Loading several ladder lanes can be helpful.

¹⁸The Exo III activity varies with the sequence. The DNA structure and the identity of nucleotides have been reported to affect the cleavage (20,21). A control sample using the same DNA template in the absence of RNAP should be analyzed in parallel to take Exo III specificity into account.

¹⁹1 hour of electrophoresis on a 6% gel is good for separating DNA fragments between 40 and 110 nucleotides. The electrophoresis time should be adjusted according to the position of the predicted protected region.

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Fig.1. The linear template for *in vitro* transcription

A schematic representation of a linear template pIA349 with a T7A1 promoter, the transcription start site (red arrow, +1) and a U-less transcribed region (+3 to +37; gray) followed by the *ops* element (underlined). The TEC halted at G37 is formed as described in section 3.3. By adding subsets of NTPs (shaded in different colors), the RNAP is walked to G40, G42 and U43 positions.

Fig.2. Exo III footprints of halted TECs
(Top). Schematic of the RNAP (a gray oval) walking along the template, in which the bottom (template) strand is radiolabeled. The RNAP active center (indicated by small white circles) denotes the position of the last added nucleotide. (Bottom) Exo III FP analysis of the halted TEC carried out as described in section 3.5. Samples were loaded alongside a pBR322 DNA digested with *Msp*I and radiolabeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using T4 polynucleotide kinase (M); the fragment lengths are shown. The dashed lines indicate the rear edge of the RNAP in the corresponding TEC. The black triangles point to two representative non-specific blocks to Exo III digestion.