Methods 159–160 (2019) 70–81

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Biochemical methods to characterize RNA polymerase II elongation complexes

J. Brooks Crickard[1], Joseph C. Reese*

Department of Biochemistry and Molecular Biology, Center for Eukaryotic Gene Regulation, Penn State University, University Park, PA 16802, United States

ARTICLE INFO

Keywords:
RNA polymerase II
Elongation complex
Footprinting
Crosslinking

ABSTRACT

Transcription of DNA into RNA is critical for all life, and RNA polymerases are enzymes tasked with this activity. In eukaryotes, RNA Polymerase II (RNAPII) is responsible for transcription of all protein coding genes and many non-coding RNAs. RNAPII carries out the remarkable feat of unwinding the stable double-stranded DNA template, synthesizing the transcript and re-forming the double helix behind it with great precision and speed. *In vitro,* RNAPII is capable of carrying out templated RNA chain elongation in the absence of any accessory proteins. However, in cells, the transcription of genes is influenced by several factors, including DNA structure, chromatin, co-transcriptional processes, and DNA binding proteins, which impede the smooth progression of RNAPII down the template. Many transcription elongation proteins have evolved to mitigate the complications and barriers encountered by polymerase during transcription. Many of these elongation factors physically interact with components of the RNAPII elongation complex, including the growing RNA transcript and the DNA template entering and exiting RNAPII. To better understand how transcription elongation factors (EFs) regulate RNAPII, elegant methods are required to probe the structure of the elongation complex. Here, we describe a collection of biochemical assays to interrogate the structure of the RNAPII elongation complex of *Saccharomyces cerevisiae* that are capable of providing insights into the function of EFs and the elongation process.

1. Introduction

Transcription of protein coding genes is essential to all domains of life, and mis-regulation of any step in transcription can lead to a number problems including death, developmental defects, cancer and diseases [1]. Transcription across genes can be divided into three stages: initiation, elongation, and termination. The stages of transcription are loosely defined by the position of RNA Polymerase II (RNAPII) within the gene body, the maturity of the emerging RNA transcript, and the collection of proteins bound to RNAPII [2–4]. Each one of these phases is biochemically unique, and also includes transitions between phases. We have developed a number of biochemical assays to characterize *Saccharomyces cerevisiae* RNAPII during the elongation phase of transcription, which we will refer to as RNAPII elongation complexes or ECs. Here we describe biochemical assays used to identify how proteins involved in transcription elongation interact with RNAPII and regulate the biochemical properties of RNAPII during the elongation phase of transcription. Some of the methods described here are unique, while others are derivations of classical methods [5] that have been forgotten. The methods described here use yeast proteins, but the exceptional conservation of RNAPII and its EFs makes them applicable to all eukaryotic species.

1.1. RNAPII enzyme

RNAPII is a twelve-subunit complex that has a size of approximately 700 MDa [6]. The subunits of RNAPII are named Rpb1-Rpb12, based on their molecular weights and migration in gels. The primary enzymatic reactions carried out by RNAPII, RNA extension and RNA nucleolytic cleavage, occur within an active site formed from the two largest subunits, Rpb1 and Rpb2 [6]. Rpb1 and Rpb2 are analogous to B and B' of prokaryotic RNAPs [7], and form a crab claw-like structure that wraps around DNA. Rpb1 and Rpb2 also combine to form an RNA exit channel for the transcript [6,8,9]. This structural organization is universally conserved in all forms of RNA polymerases including single subunit RNAP's, such as T7 RNAP, in which this organization occurs within a single polypeptide chain [10]. The Rpb1 subunit contains a mobile clamp domain that can move relative to Rpb2. This occurs through

* Corresponding author at: Center for Eukaryotic Gene Regulation, Biochemistry and Molecular Biology, Penn State University, 463A North Frear Laboratory, University Park, PA 16802, United States.
E-mail address: Jcr8@psu.edu (J.C. Reese).
[1] Current address: Department of Biochemistry & Molecular Biophysics, Columbia University, New York, NY 10032, United States.

https://doi.org/10.1016/j.ymeth.2019.01.011
Received 3 December 2018; Received in revised form 15 January 2019; Accepted 21 January 2019
Available online 24 January 2019
1046-2023/

conformational shifts in hinges located in Rpb1, and movement of the clamp is important for both initiation and the transition to elongation [11]. The dynamics of the clamp are regulated by the Rpb4/7 subunits that form the "stalk" of the RNAPII. Rpb4/7 lie on the backside of the Rpb1 clamp domain, and Rpb4/7 are also positioned to interact with and influence the processing the nascent transcript as it emerges from RNAPII [11–15]. This organization makes the Rpb1 clamp-Rpb4/7 interface an ideal target for factors that process RNA [16], or control the Rpb1 clamp [11,15]. In fact, several EFs target this region and have the potential to influence nucleic acids-RNAPII interactions. The methods described here can be used to understand the complex relationship between the protein and nucleic acid components of the RNAPII EC and how elongation factors affect the structure of the EC. In addition, this collection of methodologies can be applied to address biological questions related to collisions between RNAPII and molecular obstacles, i.e. nucleosomes and DNA binding factors, encountered during the transcription cycle. Finally, the strategies presented here can address fundamental questions related to competition between initiation factors and elongation factors that arise during the process of promoter escape, and in higher eukaryotes during promoter proximal pausing.

1.2. Nucleic acid structure

Enzymes that synthesize nucleic acids have evolved to bind to and function on specific nucleic acid structures. In the case of RNAPII, this structure is known as the transcription bubble. Recent high-resolution structures of the RNAPII open- and elongation complexes have provided tremendous insights into how polymerase opens and maintains the transcription bubble [17]. The transcription bubble consists of two strands of DNA, a template strand and a non-template strand. The bridge helix of Rpb1 separates the two DNA strands for a length of approximately 12–14 nt [17]. In the active site, the template strand forms an 8–9 nucleotide RNA-DNA hybrid. As RNAPII translocates, a ribonucleotide is added to the 3′ end of the transcript and the RNA-DNA hybrid moves one position along the template [18]. For this to occur, the RNA-DNA hybrid is displaced on the 5′ side of the growing transcript, a process that is facilitated predominantly by Rpb1 [17,18]. The RNA is then redirected through the RNA exit channel where it emerges in proximity to the base of the Rpb1 clamp, and Rpb4/7 [18]. After disassociation of the transcript from non-template strand in the transcription bubble, the template strand remains single stranded for another 6–7 nucleotides before it can rejoin the non-template strand. Closure of the transcription bubble is aided by the arch and wedge domains of Rpb2. It should be noted that there is an 130° angle in the path of DNA within RNAPII that aids in the maintenance of the transcription bubble [17]. The nucleic acid structures within RNAPII are very complex, and controlling ssDNA, RNA, and dsDNA in the EC is essential for transcription elongation. RNAPII is generally very good at this, but errors do occur. EFs can affect the formation and structure of the transcription bubble, including separation of DNA, re-annealing of DNA, and the exit of RNA from polymerases. Thus, methods to probe the structure of the nucleic acid scaffold and detect interactions between the scaffold and subunits of EFs are essential to understand transcription mechanisms.

1.3. Elongation factors

Most transcription elongation factors (EFs) assist RNAPII to complete transcription by increasing processivity, synthesis rate or resolving arrest. In some cases, elongation factors can act to negatively regulate transcription elongation such as the Negative Elongation Factor (NELF). Different classes or types of elongation factors use distinct strategies to accomplish this. EFs can directly influence RNAPII by incorporating into the EC through contacts with polymerase and the nucleic acid scaffold, as is the case with Spt4/5 (DSIF), NELF, and TFIIS [3,16,19–22]. Another class acts indirectly through the manipulation of chromatin structure, including histone chaperones, histone modifying enzymes and ATP-dependent chromatin remodeling complexes [23–26]. For our purposes we will focus on EFs that bind to RNAPII. Recent advances in cryo-Electron Microscopy have revealed how some transcription elongation factors interact with the elongating form of RNAPII. These structures have revealed the spatial interaction between DSIF [8], NELF (Negative Elongation Factor) [19], yPaf1c (Polymerase associated factor) [27], TFIIS, and Spt6 [27] with polymerase. While these structures provide a wealth of information about interactions between RNAPII and EFs, the biological implications of these structural observations will still need to be deduced from biochemical and biological experimentation.

As revealed through prior biochemical analysis, some transcription factors interact with the nucleic acid scaffold. Spt4/5 (DSIF) contacts most components of the RNAPII EC nucleic acid scaffold and the clamp of Rpb1. DSIF binds the non-template strand, the emerging RNA transcript, and upstream DNA [20,28–30]. Granted, not all elongation factors will interact as extensively with the nucleic acid scaffold as Spt4/5; however, Spt4/5 provides an excellent case study in how proteins promote elongation through interaction with the nucleic acids.

2. Materials and methods

2.1. RNAPII purification

Producing large amounts of high-quality RNAPII is essential for the biochemical characterization of elongation complexes. The procedure described below is a compilation of other methods that have been described previously, combining the best features of each [31–33]. The procedure described here isolates the 12 subunit RNAPII using a tandem-affinity tag on the C-terminus of Rpb4. The cells are grown to saturation, which increases the number of cells and leads to the purification of the hypo-phosphorylated version of the polymerase, which is extracted more easily from the chromatin pellet and can be used to prepare differentially phosphorylated CTD using kinases. The use of a protease depleted strain reduces the cleavage of the CTD. Finally, the dual ion-exchange chromatography produces preparations essentially free of other tightly-bound elongation factors, such as Spt4/5 (DSIF), TFIIS and TFIIF. The procedure typically yields over 2 mg of RNAPII from 12 L of culture.

2.1.1. Materials

5X TEZ: 250 mM Tris-HCl, pH 7.5 at RT, 5 mM EDTA (500 mM stock, pH 8), 50 μM $ZnCl_2$ (100 mM stock in 10 mM HCl), 50% glycerol.

Lysis buffer: 5XTEZ + 5% DMSO, 4 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 mM benzamidine-HCl, 10 mM DTT. PMSF is added at different steps in the protocol.

TEZ500KAc: 1X TEZ + 500 mM potassium acetate, 0.4 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.4 μg/ml aprotinin, 2 mM benzamidine-HCl, 2 mM DTT.

TEZ200KAc: 1XTEZ + 200 mM potassium acetate, 0.2 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.2 μg/ml aprotinin, 1 mM benzamidine-HCl, 2 mM DTT.

TEZ500ammonium sulfate: 1XTEZ + 500 mM ammonium sulfate, 0.2 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.2 μg/ml aprotinin, 1 mM benzamidine-HCL, 2 mM DTT.

Low Q buffer: 25 mM Tris-HCl, pH 7.5, 100 mM ammonium sulfate, 1 mM EDTA, 10 μM $ZnCl_2$, 10% Glycerol, 1 mM DTT.

High Q buffer: 25 mM Tris-HCl, pH 7.5, 650 mM ammonium sulfate, 1 mM EDTA, 10 μM $ZnCl_2$, 10% Glycerol, 1 mM DTT.

0.2 M PMSF in ethanol.

Biospec beadbeater fitted with a stainless steel chamber and cooling jacket (Biospec).

0.5 mm glass beads (Biospec).

Kontes chromatography columns, 1.5 cm × 5 cm and 1.5 cm × 10 cm.

Sepharose 4B (GE Life Sciences).
Human IgG-sepharose (GE Life Sciences).
Vivaspin 6 100KDa MWCO.
1 ml Hi-Trap SP-FF column (GE Life Sciences).
MonoQ 5/50 Tricorn column (GE Life Sciences).
Strain JR1413 (MATa ade2-11; his3-11, 15; leu2-3,112; ura3-1; trp1-1; can1-100; RPB4-TAP::TRP1; pep4::HIS3; prb1::LEU2; prc1::HISG).

2.1.2. Methods

Growth and harvest of cells:

1. Inoculate two 200 ml cultures of YP (1% yeast extract, 2% Bactopeptone) supplemented with 4% dextrose and 20 μg/ml adenine sulfate with three colonies from a freshly grown plate. Grow for 24 h at 30 °C.
2. Add 30 ml of the starter culture to 1 L of 1.5X YP supplemented with 4% dextrose and 20 μg/ml adenine sulfate. Prepare 12 L total. Grow at 30 °C for 22–24 h while shaking at 240 rpm. The OD_{600} reading of the culture is typically between 7 and 8.
3. Pour into 1L centrifuge bottles and collect the cells by spinning at 5000 rpm in an SLC6000 rotor (Sorvall) for 10 min. Drain the pellet well and determine the weight of the pellets. Typically, ~360 g wet weight is obtained from 12 L.
4. Place cells on ice and immediately add 1 ml of ice-cold Lysis Buffer (5X TEZ + 5% DMSO) per 5 g of cell pellet (e.g. ~6 ml per bottle). Add 100 μl 0.2 M PMSF to each bottle. Resuspend on ice using a wide-bore pipet. Transfer the cell suspension from three, 1L cultures into a single 250 ml centrifuge bottle.
5. Add 1 ml 0.2 M PMSF to each bottle, swirl and snap freeze in liquid nitrogen. Store at −80 C.

Extract preparation

The following processes 6 L of the culture. Perform cell lysis in two batches to lyse the entire 12 L of cell culture.

1. Thaw cells in an ice-cold water bath, mixing and swirling constantly. Once most of the suspension thaws, resuspend using a 25 ml plastic pipet. Add 200 μl of PMSF, swirl.
2. Break cells using a Bead Beater fitted with a 450 ml stainless steel chamber. Load the cell suspension from 6 L of cells, ~200–220 ml, into the chamber. Slowly fill with cold (−20 C) dry 0.5 mm glass beads up to near the top. Mix with a plastic pipet to liberate air trapped in the beads. You should add just enough beads so that a drop or two of suspension leaks out when you assemble the rotator assembly. Avoid introducing air into the chamber.
3. Pack the cooling jacket with layers of ice, rock salt, ice, rock salt. Add some cold distilled water “to wet it”. Place a thermometer in the cooling jacket to monitor the temperature during lysis. It should read between −5 and −10 °C.
4. Break the cells using 20 cycles of 30 s on/90 s off. Replenish or supplement the ice-salt mixture during the process.
5. Rinse grinding chamber and cooling jacket with distilled water and blot dry to remove the salt.
6. Place Nitex screen in a large funnel and place it in a cold 1L flask. Pour the slurry from the chamber into the funnel, trying to avoid pouring in too many beads, and let drain. Add 200 ml TEZ500KAc to the chamber, mix well and add this to the funnel. Let it drain through, washing the beads. Add 2 more amounts (~150 ml each) of TEZ500KAc to wash the beads, stirring the beads carefully, for a total of 500 ml. Once all of the crude lysate is collected, add 1 ml of 0.2 M PMSF, mix.
7. Pour into 250 ml screw-top centrifuge bottles and spin in a JA-15 rotor (Sorvall) at 6000 rpm for 10 min.
8. Pour off the supernatant and loose sediment into a cold beaker. Distribute into ultracentrifuge tubes for the Ti45 rotor (Beckman).
9. Spin the lysate at 40,000 rpm for 60 min at 4 °C.
10. Remove the supernatant with a pipet, taking care to avoid the white lipid layer on top and the loose brown sediment above the pellet. It is better to sacrifice some of the supernatant (6–8 ml per tube), rather than risk taking these layers. Pass the supernatant through gauze pads to filter out particulates. Pool extracts from the two batches of bead beating. This procedure yields approximately 900 ml of extract at 9 mg/ml protein.

IgG affinity chromatography and elution from IgG

Prepare and run the columns in a cold room.

1. Prepare a pre-filter of a 3 ml Sepharose 4B in a 1.5 cm × 5 cm column. This pre-filter is plumbed in series before the IgG column to catch lipids and other debris before the sample enters the IgG column.
2. Prepare a 5 ml bed volume column of human IgG-sepharose in a 1.5 cm × 10 cm column.
3. Equilibrate in TEZ200KAc, at least 10 column volumes (CV) (no protease inhibitors).
4. Load the lysate overnight using a peristaltic pump at a flow rate of 0.8 ml/min. Save the flow-through fraction.
5. Remove the sepharose 4B pre-filter and proceed to the washing step.
6. Add 5 ml of TEZ500KAc to the IgG column and let this pass through.
7. Wash 1: 100–125 ml TEZ200KAc, with protease inhibitors added. Close the column and add 5 ml of TEZ200KAc. Gently stir the beads to break up the lipid layer on the surface (the pre-filter doesn’t catch all of it). Wash at a flow rate of about 2 ml/min.
8. Wash 2: 100–125 ml TEZ500KAc at 2 ml/min.
9. Wash 3: 100 ml TEZ500ammonium sulfate at 2 ml/min.
10. Wash 4: 50 ml Low Q buffer supplemented with 1 mM benzamidine-HCl, 0.5 μg/ml pepstatin A, 0.2 mM PMSF. No leupeptin.
11. Add 1 ml of Low Q buffer containing 6 μg of recombinant 6HIS-TEV protease (contained in 25 mM Tris-HCl, 1 mM imidazole, 1 mM $MgCl_2$, 10% glycerol, pH 7.4 and 2 mM DTT) and let this flow into the column.
12. Close the bottom of the column. Add 3 ml low Q buffer containing 45 μg 6XHIS-TEV protease. Completely resuspend the beads using a pipet to make a uniform slurry. Digest overnight at 4°C.
13. Mix the beads once more and let them settle.
14. Collect the sample in a 15 ml tube.
15. Wash and collect four 3 ml fractions in low Q buffer supplemented with 1 mM benzamidine-HCl, 0.5 μg/ml pepstatin A, 0.2 μg/ml leupeptin, 0.2 mM PMSF and 1 mM DTT.
16. Perform a Bradford assay on 10 μl of each fraction. Pool the peak fractions.
17. Wash the column with 15 ml 1XTEZ 500 mM ammonium sulfate, 10 ml Low Q buffer without DTT. Store in this buffer until regeneration.

Ion-exchange chromatography

The sample is purified by ion-exchange chromatography, passing it through a SP-ion exchange column to remove Spt4/5 and TFIIF (Craig Kaplan, personal communication) directly onto a MonoQ column. The 12 subunit RNAPII is separated from the small amount of free Rpb4/7 dimer on the MonoQ column. The polymerase eluates at a concentration of ammonium sulfate of ~ 0.30–0.33 M at 1.5–3 mg/ml per peak fraction.

1. Soak a Vivaspin 6 100KDa MWCO unit in low Q buffer for 1hr. Spin for 5 min at 3 K in Beckman J6 centrifuge using a swinging bucket rotor. Discard the liquid.
2. Add 6 ml of the pooled fraction. Spin at 3 K in the J6 centrifuge until 1.5–2 ml is left. Add the remaining sample. Repeat. Concentrate to a

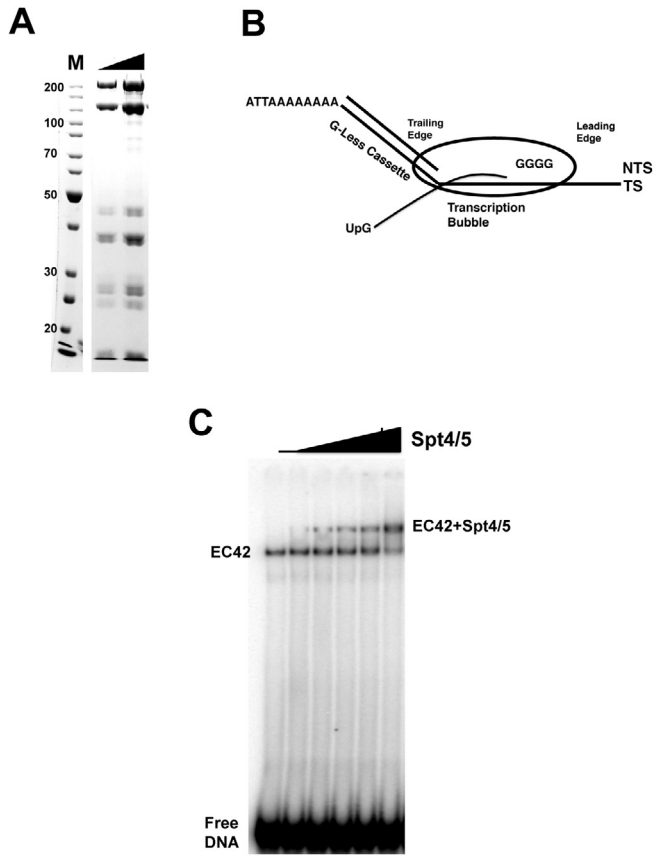

Fig. 1. Reconstitution of RNAPII elongation complexes. (A). Example of an RNAPII preparation. One and three micrograms of RNAPII was separated on an 8% NuPage gel and stained with coomassie blue. The numbers on the left indicate the molecular weights of the standard, M. (B). Schematic diagram of EC formed from end-initiated templates. Transcription is initiated at the 3′ extension using UpG dinucleotide and transcription occurs across a G-less cassette and then it arrests when it encounters a series of four G nucleotides. (C). EMSA assay of Spt4/5 binding to radiolabeled ECs. End-labeled EC42 template was prepared as described in Section 2.2. Increasing amounts of Spt4/5 (0.3, 1, 3, 10, 30 nM) were titrated into the binding assay containing (5 nM RNAPII) and the samples were separated on a native gel. Recombinant Spt4/5 was expressed in *E. coli* and purified as described in reference 20. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

volume just below 2 ml.

3. Spin the sample at 14,000 rpm in a centrifuge for 10 min at 4 C. Transfer supernatant to a new tube.
4. Purify on an AKTA FPLC system or equivalent, fitted with a 1 ml HiTrap SP-sepharose and MonoQ 5/50 column arranged in series and equilibrated in Low Q buffer without protease inhibitors.
5. Apply the sample at a flow rate of 0.25 ml/min and then wash with 3 ml Low Q buffer.
6. Remove the HiTrap SP-sepharose column and wash the MonoQ column at 1 ml/min with 3 ml Low Q buffer.
7. Elute RNAPII with a 15 ml gradient of 0–100% High Q buffer at 1 ml/min, collecting 0.5 ml fractions.
8. RNAPII typically elutes in 2 fractions. Aliquot and snap freeze in liquid nitrogen. Store at −80 °C. An example of an RNAPII preparation is shown in Fig. 1A.

2.2. Production of transcription templates with 3′ ssDNA overhangs for end-initiated RNAPII transcription

Elongation complexes formed from promoter-based initiation are more natural but typically use cell extracts as the source of proteins and RNAPII. The ECs are isolated by high salt washes [34]. Clearly, this system has been quite useful to characterize elongation factors, but since they are formed from extracts the purity of these ECs are uncertain. An alternative approach is to use a DNA substrate which bypasses the need for promoter-based initiation, and it has been known for many years that promoter-less initiation of RNAPII can be achieved through the utilization of a 3′ single stand extension in the presence of UpG [20,35,36]. Early studies using C-tailed templates raised questions about this approach because of the propensity of these templates to form ECs with extended RNA-template strand hybrids (R-loops) that displace the non-template strand [37,38]. However, the extent to which this occurs is influenced by the sequences of the 3′ tail and that encompassing the single strand-double strand junction [37,38]. Others and we have shown that the template described here is not prone to these artifacts [20,35]. This protocol details how to prepare ECs for use in gel shift assays, DNA footprinting and crosslinking approaches. The template contains a G-less cassette of varying length followed by a tract of G bases, which arrests RNAPII in the absence of GTP (Fig. 1B). Initiation using dinucleotides is increased by using Mn^{2+} during EC formation, but once ECs are formed excess Mg^{2+} is added back in later parts of most procedures. This is especially important when conducting run-off experiments. Suitable templates can be prepared either by annealing two oligonucleotides (EC42) or using PCR followed by the ligation of a tail to generate longer templates (EC70). Both methods are described below. It should also be noted that the terminology EC42 and EC70 refer to the length of the G-less cassette downstream of the start site of transcription. For example, EC42 has 42 base pairs of DNA upstream of RNAPII and 42 nucleotides of nascent RNA produced.

2.2.1. Materials

Gamma-^{32}P ATP > 7000 Ci/mmol, end-labeling grade (MP Biomedical).

EC42 Template strand: 5′GCCACCGCGGTCTAGAGGATCCCCGGGAGTGGAATGAGAAATGAGTGTGAAGATAGAGGAGAGATCAAAAAAATTA 3′.

EC42 Non-template strand: 5′CTCCTCTATCTTCACACTCATTTCTCATTCCACTCCCGGGGATCCTCTAGACCGCGGTGGC 3′.

Plasmid pBS-TT150 [28,36].

EC70 Forward PCR primer: 5′ GATGGAATTCTTGGCAGATCTTATCCTCTCCTCACCTCTC 3′.

EC70 Reverse PCR primer: 5′ biotin-CGGATAACAATTTCACACAGGA 3′

Tail sequence: 5′ P-GATCAAAAAAAATTA-3′.

15% Denaturing 8 M Urea PAGE in 1X TBE.

100% formamide (Ultrapure, JT Baker).

50-, 140- and 250 mM sodium phosphate buffer pH 7.5.

Sep-Pak C-18 cartridge (Waters).

Gel extraction buffer (500 mM NH_4OAc, 10 mM $MgCl_2$).

Phenol:Chloroform:Isoamyl alcohol (24:25:1).

Pfu polymerase.

Bgl II (NEB).

NEB Buffer #3 (NEB).

Antarctic Phosphatase (NEB).

Antarctic Phosphatase buffer (NEB).

10X ligase buffer (Promega).

T4 DNA ligase (prepared locally).

Siliconized glass wool.

10X Polynucleotide kinase buffer and Poly-Nucleotide Kinase (NEB).

Sephadex G-50 prepared in 0.1X TE.

12% native PAGE (19:1 acrylamide:bis-acylamide) in 0.5X TBE.

2.2.2. Methods

Gel purification of oligonucleotides

1. Dissolve single-stranded oligonucleotides in 100% formamide, heat and then purify 25 nMol on 15% denaturing urea-PAGE (Poly Acrylamide Gel Electrophoresis) in 1XTBE. Run gels "warm" at 15 W for 2 h.
2. Remove the gel from the plates and locate the band by UV shadowing over a Kodak intensifying screen and excise the band from the gel.
3. Macerate the gel slice and incubate in 3 ml gel extraction buffer (500 mM NH_4OAc, 10 mM $MgCl_2$) overnight at room temperature in a 15 ml screw cap tube with agitation.
4. Centrifuge the sample and save the supernatant. Wash the gel slice with an additional 3 ml of gel extraction buffer.
5. Pass the solution through an activated Sep-Pak C-18 cartridge. Wash with extraction buffer and elute DNA with two 750 µl aliquots of 60% methanol in distilled water.
6. Vacuum-dry the DNA, resuspend in 40 µl of Tris-EDTA pH 8.0 and quantify using a Nanodrop instrument.

Labeling of non-template strand (NTS) oligo and annealing

1. Mix 150 pmol of the non-template strand oligo with 10X Polynucleotide Kinase (PNK) buffer (NEB), 1 µl of gamma-^{32}P-ATP (> 7000 Ci/mmol), 10 units of PNK and bring the volume to 20 µl using TE + 50 (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 50 mM NaCl).
2. Incubate for 1 h at 37 °C, and then at 65 °C for 20 min.
3. Add an equal molar amount of template strand oligo and anneal the two strands by incubating at 95 °C for 5 min followed by gradual cooling in 10 °C steps for 5 min at each step.
4. Pass the DNA through a G-50 spin column to remove unincorporated nucleotide. The DNA can be purified directly on native PA gel or subjected to hydroxyapatite chromatography first.

Hydroxyapatite Purification of DNA (optional)

We have found that it is difficult to remove all of the single-stranded oligos for the duplex by gel purification. The small amount of contaminating single-stranded DNA reduces the quality of DNA footprinting studies, especially permanganate footprinting, where even a small amount of ssDNA produces a signal and higher background. To overcome this, we purify the templates by hydroxyapatite chromatography before gel purification.

1. Dilute the annealed DNA to 1 ml with 50 mM sodium phosphate buffer pH 7.5 and then pass over a 250 µl hydroxyapatite bed volume column equilibrated in the same buffer and packed into a disposable plastic column from Bio-Rad.
2. Wash the column with 6 ml of 140 mM sodium phosphate buffer (pH 7.2) to remove ssDNA.
3. Elute the dsDNA with 250 mM sodium phosphate buffer (pH 7.2) in 250 µl fractions.
4. Ethanol precipitate the DNA and resuspend in 50 µl of TE.
5. Pass the sample over a G-50 spin column to remove residual phosphate, which precipitates with ethanol.
6. Separate DNA on a 12% native polyacrylamide gel run in 0.5X TBE.
7. Visualized the band by shadowing on an intensifying screen and excise from the gel.
8. Place gel slice in a 0.65 ml tube with a hole pierced in the bottom into a 1.7 ml tube and spin at 10,000 g for 5 min. This step extrudes the gel slice through the hole to break it up.
9. Incubate the crushed gel in 250 µl gel extraction buffer (500 mM NH_4OAc, 10 mM $MgCl_2$) overnight with agitation.
10. Centrifuge at 10,000 g for 5 min and remove the supernatant.
11. Add 250 µl of gel extraction buffer to the gel pieces.
12. Place the gel slice buffer suspension from step 15 in a 0.65 ml tube containing a hole punched in the bottom and stuffed with siliconized glass wool. Place in a 1.7 ml tube and centrifuge at 10,000 g for 30 sec and then combine the two extractions.
13. Concentrate the DNA by ethanol precipitation, wash with 70% ethanol, dry and resuspend in 40 µl of TE.

Method for generating long DNA templates by PCR and ligation

This method is an alternate strategy to produce tailed templates with longer transcripts, for example when preparing templates used in transcription elongation experiments. A PCR product is produced from a plasmid, and a single-stranded oligo is ligated to the end to produce a 3′ extension. Templates with a radiolabeled non-template strand are produced by using a biotinylated reverse primer. The biotin on the 5′ end blocks the template strand from phosphorylation by polynucleotide kinase.

1. PCR is performed using the plasmid pBS-TT150 as a template and EC70 forward and reverse primers. A 1 ml scale PCR reaction generates 30–60 µg of product. It is essential to use a thermostable proofreading polymerase, such as *Pfu* polymerase, because the adenine base added to the end of the DNA by *Taq* polymerase interferes with downstream steps.
2. Use 55 °C annealing temperature for the first five cycles and 58 °C for 20 cycles. The three-degree lower annealing temperature is used in the first five cycles because the *Bgl II* site added to the forward primer does not hybridize to the template.
3. Purify the product by Phenol:chloroform extraction, followed by ethanol precipitation.
4. Digest the PCR product with 20 U Bgl II in a volume of 50 µl overnight at 37 °C.
5. Purify DNA on a 2% agarose gel and isolate the DNA using a Qiagen gel extraction kit. Elute the DNA in 0.1X TE.
6. Treat the DNA with 25 U Antarctic phosphatase in 30 µl for 2–3 h. Heat-inactivate the phosphatase for 15 min at 75 °C.
7. Add 7 µl 10X ligase buffer, a 10:1 M ratio of the single-stranded tail oligo to the PCR product and 3 U T4 DNA ligase. Incubate overnight at 15C.
8. Gel purify the template on an agarose gel as described in step 5. Elute the DNA in a volume of 0.1X TE to obtain a concentration of 200–250 ng/µl.
9. End-label 1 µg of the DNA in a 50 µl reaction containing 10 units polynucleotide kinase and 1 µl gamma-^{32}P ATP (> 7000 Ci/mmol). Incubate at 37 °C for 1 h.
10. Remove unincorporated nucleotide using a G50 spin column. Measure the activity of the probe using a scintillation counter.

2.3. Analysis of factor binding by electrophoretic mobility shift assay (EMSA)

Many elongation factors form tight complexes with the EC. It is useful to determine if a factor binds directly to RNAPII incorporated into an EC, and when combined with mutagenesis of the factor of interest, can provide valuable insights into how elongation factors bind to ECs. The procedure described here produces an end-labeled DNA template. However, another method that has been described elsewhere [28,35,36] incorporates the label into the RNA transcript during transcription through the G-less cassette. By altering the length of the G-less cassette, one can produce ECs with different transcript lengths to determine the minimum length required for elongation factor binding. An example of the application of this method using Spt4/5 is shown in Fig. 1C.

2.3.1. Materials

End-labeled EC42 tailed template (Section 2.2).

Transcription buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 0.5 mM DTT, 10% glycerol, 0.3 mM UpG, 0.1 µg/µl BSA, 1 unit/µl RNasin)

Salmon sperm DNA, 5 mg/ml.

Nucleotide mix 1 (0.4 mM ATP, 0.4 mM UTP, 0.4 mM CTP, 0.02 mM

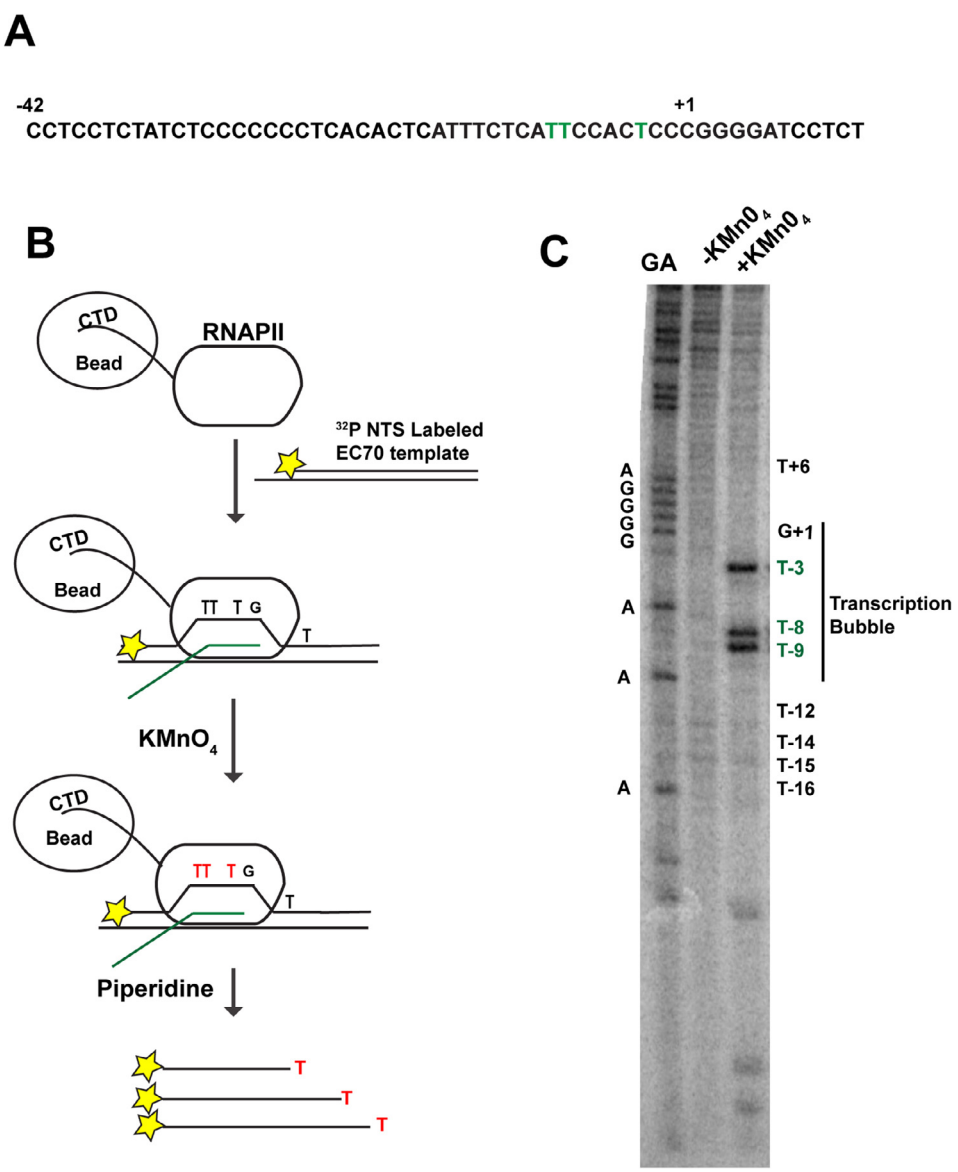

Fig. 2. Permanganate footprinting of RNAPII ECs. (A). The sequence of the non-template strand around the transcription bubble on EC70. Reactive "Ts" in the transcription bubble are labeled in green. (B). Schematic of the EC enrichment and $KMnO_4$ treatment. (C). $KMnO_4$ footprint of the EC70 complex. A GA marker was loaded onto the gel. Reactive thymine bases are colored in green, and the black line indicates the estimated location of the transcription bubble. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3′O-MeGTP).

4% native PAGE in 0.5X TBE.

2.3.2. Method

1. Mix 75 ng of ^{32}P end-labeled template with 150 fmol of RNAPII in 15 µl transcription buffer and incubate for 5 min.
2. Initiate transcription by the addition of 5 µl of nucleotide mix 1. The final concentration of all nucleotides is 100 µM, except 3′ O-MeGTP, which is 5 µM. Incubate at 30 °C for 20 min.
3. Then add 5 µl transcription buffer or EF diluted in the same buffer. Incubate for 20 min at 30 °C.
4. Add 250 ng of salmon sperm DNA to act as a competitor for non-specific interactions that might occur between the elongation factor and DNA/RNA. Incubate for 5 min.
5. Resolve samples on a 4.5% native gel in 0.5X TBE that was pre-run for 1 h prior to loading. Run at 200 V for 2 h at room temperature.
6. After two hours dry the gel and expose to a phosphorimager screen.

2.4. Preparing immobilized ECs

Because of the low efficiency of elongation complex formation, some experiments require the enrichment of ECs from the more abundant free template. For example, free templates in DNA footprinting experiments produces high background and obscures the detection of protein-induced protection. We developed an immobilization strategy to enrich for DNA bound by active ECs [20]. The approach involves immobilizing RNAPII onto protein-A beads through the CTD of Rpb1 using 8WG16 antibody. As we describe below in other sections of this paper, this immobilization strategy can be used to improve the quality and sensitivity of multiple EC mapping techniques.

2.4.1. Materials

EC70 tailed template. End-label on the non-template strand.

8WG16 monoclonal antibody ascites fluid (Biolegend).

Protein A sepharose magnetic beads (GE Life Sciences).

Magnetic separator stand (Dynal).

RNAPII blocking buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl,

0.250 μg/μl BSA, 10% glycerol).

Bead wash buffer (50 mM Tris-HCl pH 8.0, 200 mM KCl, 0.01 mM $ZnCl_2$, 0.1 μg/μl BSA 10% glycerol).

IgG wash buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 0.01 mM $ZnCl_2$, 0.1 μg/μl BSA, 10% glycerol).

Reaction buffer (20 mM HEPES-KOH pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 0.01 $ZnCl_2$, 0.5 mM DTT, 0.1 μg/μl BSA, 2 mM $CaCl_2$, 10% glycerol).

Transcription buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 0.5 mM DTT, 10% glycerol, 0.3 mM UpG, 0.1 μg/μl BSA, 1 unit/μl RNasin).

Nucleotide mix (0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP).

2.4.2. Method

1. Wash Protein A sepharose magnetic beads three times with RNAPII blocking buffer.
2. Add 50 μl of RNAPII blocking buffer and 1 μl of 8WG16 ascites fluid to 10 μl of magnetic beads. Incubate with agitation for 1 h at room temperature.
3. Wash beads 3 times with 100 μl of bead wash buffer and twice with IgG wash buffer.
4. Re-suspend beads in 30 μl of transcription buffer containing 2 pmol of RNAPII and incubated for 1 h at 4 °C with gentle agitation
5. Wash beads 3 times with 100 μl IgG buffer, and re-suspend in the appropriate volume of reaction buffer for the assay to be performed.

2.5. $KMnO_4$ footprinting of ECs

The transcription bubble is a major feature of ECs. Thymidine bases in single-stranded DNA are more prone to chemical oxidation than those in double-stranded DNA, which allows $KMnO_4$ to be used to probe the size and location of the transcription bubble in RNAPII. Whereas single-stranded DNA is hyper-reactive to $KMnO_4$, double-stranded DNA displays low reactivity. Since RNAPII utilizes only a fraction of templates [39], the much more abundant double-stranded free template increases the background in footprinting experiments. To overcome this, we have developed a method that conducts $KMnO_4$ footprinting on immobilized ECs (Fig. 2).

2.5.1. Materials

EC70 tailed template with the non-template strand ^{32}P-end-labeled (Section 2.2).

8WG16 monoclonal antibody ascites fluid (Biolegend).

Protein A sepharose magnetic beads (GE Life Sciences).

Magnetic separator stand (Dynal-Invitrogen).

RNAPII blocking buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 0.250 μg/μl BSA, 10% glycerol).

Bead wash buffer (50 mM Tris-HCl pH 8.0, 200 mM KCl, 0.01 mM $ZnCl_2$, 0.1 μg/μl BSA 10% glycerol).

IgG wash buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 0.01 mM $ZnCl_2$, 0.1 μg/μl BSA, 10% glycerol).

Reaction buffer (20 mM HEPES-KOH pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 0.01 $ZnCl_2$, 0.5 mM DTT, 0.1 μg/μl BSA, 2 mM $CaCl_2$, 10% glycerol).

Transcription buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 0.5 mM DTT, 10% glycerol, 0.3 mM UpG, 0.1 μg/μl BSA, 1 unit/μl RNasin).

Nucleotide mix (0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, Optional: 0.05 mM O-me-GTP).

$KMnO_4$ Stop Solution (20 mM EDTA, 250 mM 2-mercaptoethanol (BME), 300 ng/μl salmon sperm DNA, 10% piperidine).

Formamide loading solution (90% formamide, 1 mM EDTA, bromophenol blue, xylene cyanol).

Salmon sperm DNA, 5 mg/ml.

Molecular biology grade glycogen 2 mg/ml (Thermo-Fisher).

Piperidine (Sigma-Aldrich).

Potassium permanganate (99% Pure, Alfa Aesar).

Water-saturated butanol.

10% 8 M-urea PAGE (19:1 acrylamide:bis-acylamide) in 1X TBE.

2.5.2. Method

1. Immobilize RNAPII onto beads as described above in Section 2.4.
1. Add 75 ng of DNA template to 150 fmol of immobilized RNAPII in 15 μl transcription buffer. Incubate for 5 min.
2. Add 5 μl of nucleotide mix. Incubate 20 min at 23 °C.
3. Collect the beads on a magnet and wash 6 times with 100 μl of IgG wash buffer) and twice with reaction buffer lacking DTT. Reducing agent must be washed out prior to adding $KMnO_4$.
4. Resuspend beads in 15 μl of reaction buffer (-DTT) and add salmon sperm DNA to 100 ng/μl.
5. Add 5 μl nucleotide mix to achieve a final concentration of 100 μM. Nucleotides are added to prevent reiterative rounds of RNAPII backtracking and transcript cleavage.
6. Add $KMnO_4$ to 5 mM and incubate for 2 min at room temperature.
7. A separate reaction of naked DNA is treated with KMnO4. Add a 1:9 dilution of the starting template and treat as described in steps 4–6. 1/9th roughly correlates with the amount of template incorporated into ECs on the beads.
8. Quench the reaction with 2 volumes of $KMnO_4$ stop solution.
9. Heat the samples to 90 °C for 10 min to induce cleavage.
10. Collect the beads on the magnet and save the supernatant.
11. Extract the supernatant with 500 μl of water- saturated butanol and centrifuge at 10,000 g for 2 min.
12. Collect the aqueous phase (bottom), add 4 μg glycogen and concentrate by ethanol precipitation.
13. Resuspend the pellet in formamide loading buffer containing 0.1% SDS.
14. Resolve the DNA on 10% urea-PAGE. Dry the gel and expose to a phosphorimager screen.

2.6. DNaseI footprinting of RNAPII ECs

DNase I has been used to footprint RNAPII on DNA. It is critical to saturate binding sites in DNAseI footprinting experiments because even a small amount of unbound template prevents the visualization of the protected region. As discussed above, only a fraction of the templates are incorporated into ECs. The method described here combines two purification techniques to improve the quality of the footprint. First, ECs are formed on immobilized RNAPII and then eluted from the beads using recombinant GST-CTD. This step greatly reduces the amount of free DNA in the sample. Next, we adapted a classical procedure used to footprint preinitiation complex components developed by Steve Buratowski [5]. The eluted ECs are treated with DNaseI in solution and then separated on native gels. The bands corresponding to free DNA and the EC are eluted from the gel and analyzed on denaturing gels. An example of our use of this procedure is shown in Fig. 3.

2.6.1. Materials

EC70 template with an end-labeled non-template strand (Section 2.2).

8WG16 monoclonal antibody ascites fluid (Biolegend).

Protein A sepharose magnetic beads (GE Life Sciences).

Magnetic separator stand (Dynal-Invitrogen).

RNAPII blocking buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 0.250 μg/μl BSA, 10% glycerol).

Bead wash buffer (50 mM Tris-HCl pH 8.0, 200 mM KCl, 0.01 mM $ZnCl_2$, 0.1 μg/μl BSA 10% glycerol).

IgG wash buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 0.01 mM $ZnCl_2$, 0.1 μg/μl BSA, 10% glycerol).

Reaction buffer (20 mM HEPES-KOH pH 7.8, 100 mM KCl, 5 mM

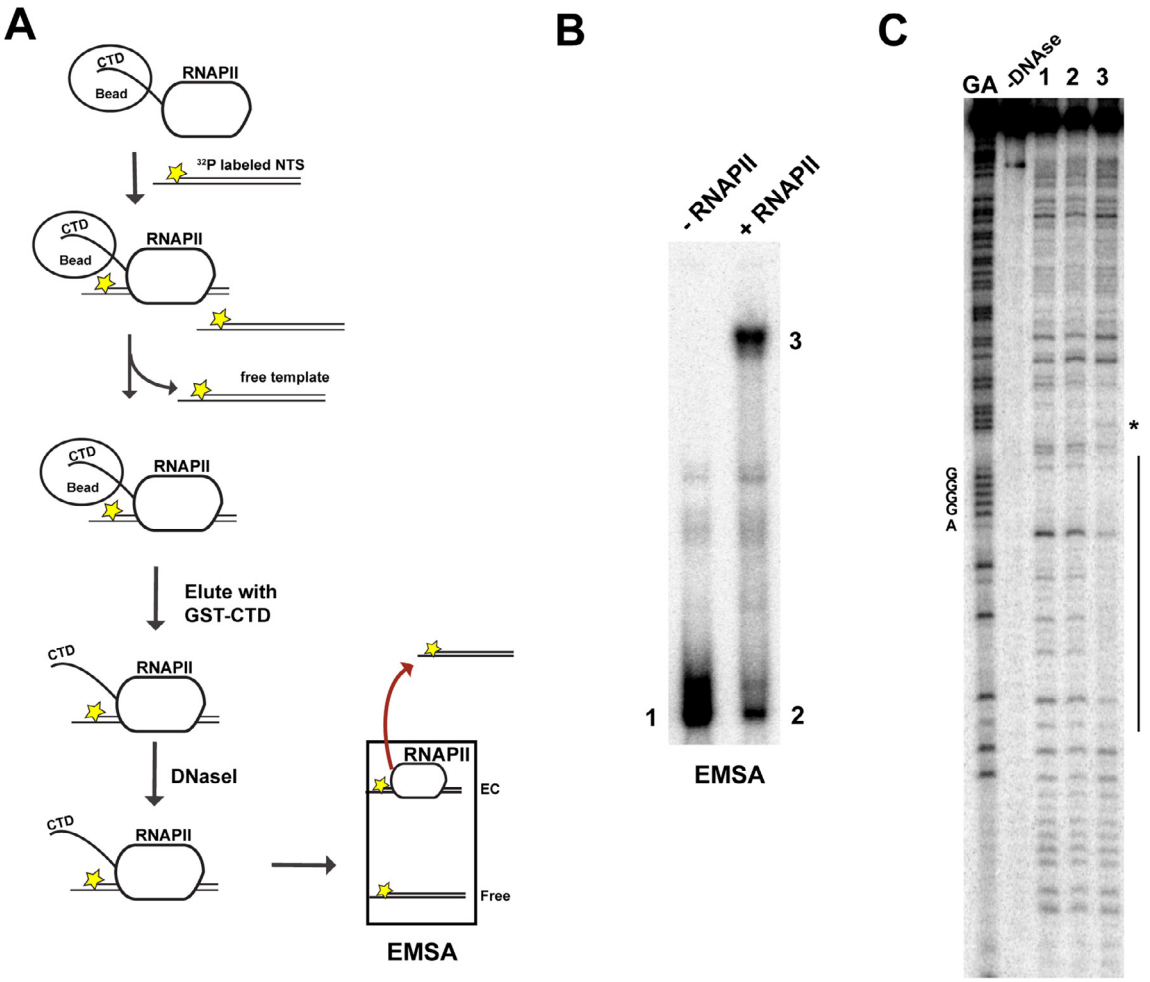

Fig. 3. DNaseI footprinting of RNAPII ECs. (A). Schematic of the DNase I footprinting procedure. (B). Isolation of DNAseI-treated ECs from gels. ECs were formed on a template with a G-less cassette (EC70), subjected to the enrichment and DNaseI digestion procedure described in panel A and separated on a native gel. Naked DNA was treated in parallel. Bands, labeled 1–3, were purified from the gel and run on the denaturing gel displayed in panel C. (1) Treated free DNA; (2) free DNA in the EC preparations; (3) EC. (C) DNase1 footprint of EC70 complexes. Lane 1 contains a GA marker; lane 2 contains undigested DNA; lane 3 naked DNA; lane 4 free DNA in EC preparations; lane 5 EC DNA. The footprint of RNAPII is highlighted by on the right of the gel. The asterisk marks a hypersensitive site ahead of RNAPII.

$MgCl_2$, 0.01 $ZnCl_2$, 0.5 mM DTT, 0.1 μg/μl BSA, 2 mM $CaCl_2$, 10% glycerol).

Transcription buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 0.5 mM DTT, 10% glycerol, 0.3 mM UpG, 0.1 μg/μl BSA, 1 unit/μl RNasin).

Nucleotide mix (0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 0.05 mM O-me-GTP).

Recombinant GST-CTD 0.5 mg/ml in GST-Buffer (25 mM Tris-Cl pH 7.5, 100 mM NaCl, 0.01% TritonX-100, 10% Glycerol).

10X DNaseI buffer (100 mM $MgCl_2$, 50 mM $CaCl_2$, 100 mM Tris-HCl pH 8.0).

DNase I 10 units/μl in DNase I buffer + 50% glycerol (Roche).

4.5% native PAGE in 0.5X TBE.

Gel extraction buffer (500 mM NH_4OAc, 10 mM $MgCl_2$).

Siliconized glass wool.

Molecular biology grade glycogen 2 mg/ml (Thermo-Fisher).

Formamide loading solution (90% formamide, 1 mM EDTA, bromophenol blue, xylene cyanol).

Salmon sperm DNA, 5 mg/ml.

10% 8 M urea-PAGE (19:1 acrylamide:bis-acylamide) in 1X TBE.

2.6.2. Method

1. Immobilize RNAPII onto beads as described above in Section 2.4.
2. Add 75 ng of DNA template to 150 fmol of immobilized RNAPII in 15 μl transcription buffer. Incubate for 5 min.
3. Prepare a naked DNA sample by treating, 75 ng of free DNA in solution with DNaseI, and then 1/10th of this was loaded on the native page for purification. Loading this amount assumes that only 10% of the DNA is immobilized and recovered through the various steps in the procedure.
4. Add 5 μl of nucleotide mix and incubate 20 min at 23 °C.
5. Collect the beads on a magnet and wash 6 times with 100 μl of IgG wash buffer and twice with reaction buffer. Re-suspend the beads in 15 μl of reaction buffer.
6. Add 5 μl nucleotide mix to achieve a final concentration of 100 μM of each nucleotide. Nucleotides are added to prevent RNAPII from moving backward by reiterative rounds of backtracking and transcript cleavage.
7. Elute the elongation complexes from the beads by adding 1 μg GST-CTD for 10 min at 30 °C.
8. Collect the beads on a magnet and remove the supernatant.
9. Add 10 X DNase I buffer to a final concentration of 1 × and 1.4 miliunits of DNase I and incubate for 1 min at room temperature. Add EDTA to 10 mM EDTA to stop the digestion.
10. Load samples onto a 4.5% native PAGE prepared in 0.5x TBE. Run for 2 h at 200 V.
11. Remove the gel from the plates and wrap in plastic. Place

radioactive ink markers onto the gel to orient the gel in the next step. Expose to x-ray film for 1 h.
12. Overlay the developed x-ray film on the gel and use it as a template to excise the bands corresponding to the EC and free DNA (see Fig. 3B).
13. Place gel slice in a 0.65 ml tube with a hole pierced in the bottom into a 1.7 ml tube and spin at 10,000 g for 5 min. This extrudes the gel slice through the hole to break it up.
14. Extract DNA overnight in 250 µl gel extraction buffer +1% SDS.
15. Centrifuge at 10,000 × g for 5 min and collect the supernatant.
16. Wash the gel fragments with 250 µl of gel extraction buffer, centrifuge again and combine the liquid with the first extraction.
17. Place the gel slice-buffer suspension from step 16 in a 0.65 ml tube containing a hole punched in the bottom and stuffed with siliconized glass wool. Place in a 1.7 ml tube and centrifuge at 10,000 × g for 30 s to collect the liquid and then combine the extractions.
18. Add 4 µg of glycogen and concentrate by ethanol precipitation. Dry the pellet and resuspended in formamide loading buffer.
19. Heat sample at 90 °C and resolve on a 10% urea-PAGE gel.

2.7. Site-specific DNA photocrosslinking

A number of elongation factors bind to DNA in the nucleic acid scaffold, which is important for their transcription regulatory activities. For example, Spt5 in DSIF contacts DNA exiting RNAPII and the NTS (Non-template strand) in the transcription bubble (20). Site-specific photoaffinity crosslinking is one method to map contacts between elongation factors and DNA in ECs. A photoreactive nucleotide is incorporated adjacent to a radiolabeled nucleotide in the template through primer-directed DNA synthesis (Fig. 4A). In our experiments to examine the binding of Spt4/5 to ECs, we chose sites on the DNA up and downstream of RNAPII, as well as within the transcription bubble (Fig. 4B and C). The position of the probe can be changed by varying the positions of the radiolabeled and nucleotide analog pair. We highly recommend showing that incorporation of the probe does not interfere with the binding of the target protein, such as by EMSA. An example is shown in Fig. 4D. Furthermore, the label-transfer method can identify the subunit of an elongation factor complex that contacts DNA by running the crosslinked material on SDS-PAGE protein gels (Fig. 4E). In this method, we describe how to generate probes into the NTS of DNA and carry out UV-crosslinking of proteins to DNA in ECs.

2.7.1. Materials

EC42 Template Strand (oligo gel purified).

5′GCCACCGCGGTCTAGAGGATCCCCGGGAGTGGAATGAGAAATGAGTGTGAAGATAGAGGAGAGATCAAAAAAATTA 3′.

EC42 non-template strand (oligo, gel purified), size corresponding to desired position of photo-reactive nucleotide.

5-Iodo-dCTP (Sigma-Aldrich).

alpha-^{32}P-dATP (6000 Ci/mmol, MP Biomedical).

dATP, dCTP, dTTP, dGTP nucleotide mix, 25 mM each.

Klenow (exo-) fragment of DNA polymerase 10U/µl (NEB).

NEB buffer # 2 (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 1 mM DTT).

G-50 prepared in TE.

12% native PAGE in 1X TBE.

Gel extraction buffer (500 mM NH_4OAc, 10 mM $MgCl_2$).

Siliconized glass wool.

Transcription Buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 0.5 mM DTT, 10% glycerol, 0.3 mM UpG, 0.1 µg/µl BSA, 1 unit/µl RNasin).

Nucleotide mix (0.4 mM ATP, 0.4 mM UTP, 0.4 mM CTP, 0.02 mM 3′-O-MeGTP).

Spectroline short wave UV (300 nm) lamp.

10X DNase I buffer (100 mM $MgCl_2$, 50 mM $CaCl_2$, 100 mM Tris-HCl pH 8.0).

DNase I (Worthington) diluted to 10 units/µl in DNase I buffer.

RNase A 5 mg/ml.

3X SDS-PAGE loading buffer (180 mM Tris-HCl pH 6.8, 15% 2-mercaptoethanol, 6% SDS, 15% glycerol, 0.25% bromophenol blue).

SDS-PAGE running buffer (50 mM Tris-base, 500 mM Glycine, 0.2% SDS, pH 8.3).

2.7.2. Method

1. Short EC42 oligonucleotide primers corresponding to the NTS are designed based on the desired location of the photoreactive group. The oligos are gel purified as described above in Section 2.2.
2. Anneal oligos as described above in Section 2.2, except that a 2-fold excess of the EC42 oligo is used. Annealing reactions are carried out in 20 µl of annealing buffer (25 mM Tris-HCl 7.5, 100 mM NaCl, 10 mM $MgCl_2$).
3. Add 1 µl ^{32}P-alpha-dATP (6000 Ci/mmol), 100 µM 5-Iodo-dCTP, NEB buffer #2, and 25 units of Klenow(-exo) and incubate for 20 min at 37°C. The final volume of this reaction is 30 µl.
4. Add a mix of all dNTP mix to a final concentration of 1 mM and continue the incubation for 40 min to complete strand synthesis.
5. Pass the sample over a G-50 spin column to remove the free nucleotides.
6. Gel purify the probes by 12% native PAGE and isolate the DNA by a crush and soak procedure as described in Section 2.2.2.
7. Resuspend the DNA in 25 µl of TE after ethanol precipitation.
8. Count the amount of radioactivity in a scintillation counter.
9. Analyze a small amount of each probe by urea-PAGE to confirm the quality and to quantify the amount of probe to be added to each crosslinking reaction.
10. Add approximately 50,000 cpm of labeled template DNA and to 1 pmol of RNAPII in transcription buffer in a final volume of 15 µl and incubate for 5 min.
11. Add 5 µl nucleotide mix (ATP, CTP, and UTP) to a final concentration of 100 µM, and 3′ O-Me-GTP to 10 µM. Incubate samples for 20 min at 30 °C.
12. Add 1 µg of salmon sperm DNA.
13. Add 5 µl transcription elongation factor in elongation factor buffer (20 mM HEPES-KOH (7.5), 150 mM KCl, 0.5 mM DTT, 10% Glycerol), or BSA as a control.
14. Pipet sample onto a sheet of parafilm place on a pre-chilled aluminum block in a tray of ice.
15. Irradiate the samples for 10 min at 300 nm wavelength at a distance of 10 cm.
16. Transfer the sample to microfuge tubes and add 2.5 µl 10X DNase I buffer, 1 µl of 10 unit/µl DNase I and 1 µl of 2.5 µg/µl RNase A. Incubate for 45 min at 37 °C.
17. Add 1/3 vol of 3X SDS-PAGE loading buffer and heat to 75 °C for 5 min.
18. Run on SDS-PAGE gels, dry the gel and exposed to phosphorimager screen.

2.8. UV crosslinking of proteins to the emerging transcript.

Some transcription elongation factors and RNA processing factors bind to the emerging transcript. This method uses RNAPII to incorporate a photoactivatable nucleotide derivative and a radiolabeled nucleotide into the nascent RNA produced during transcription through the G-less cassette. The ECs are exposed to short-wave UV to induce crosslinks and the transfer of label to proteins are detected by running the samples on SDS-PAGE gels. This procedure can identify the subunit of the elongation factor that contacts RNA and can be used to analyze the effects of elongation factor mutations on transcript binding [20,28,36].

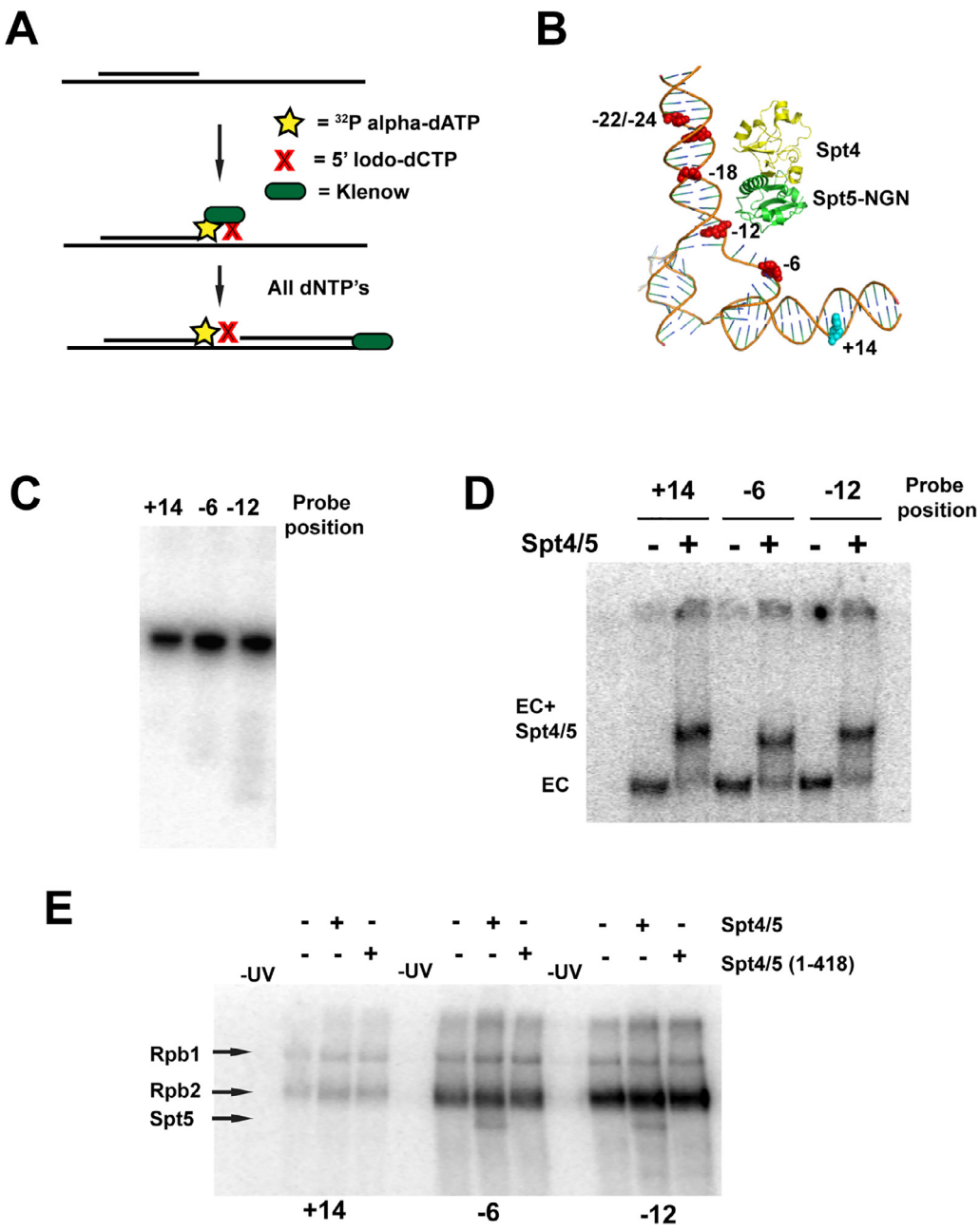

Fig. 4. Site-specific photocrosslinking of ECs. (A). Schematic diagram of the strategy to produce site-specific DNA probes. A single-stranded oligo is used to prime DNA synthesis immediately adjacent to the preferred crosslinking site. A photoreactive nucleotide is incorporated next to a radiolabeled nucleotide by the Klenow fragment of DNA polymerase. The strand is then completed by adding excess nucleotides. (B). A model of the transcription bubble within RNAPII from several crystal structures, including modeling of Spt4/5 (NGN) adjacent to the non-template strand (NTS) (PDB codes, 5C4X, 2EXU, and 3QQC) using PyMol (version 1.7.4 Schrodinger, LLC). Choice of probe sites are indicated. (C). PAGE showing purified labeled templates with probes located at +14, −6, −12, relative to the arrest location designated as +1. (D). EMSA on photoreactive transcription templates in the presence and absence of Spt4/5 (30 nM). (E) Crosslinking and label transfer to Rpb1,2 and Spt5. SDS-PAGE of ECs prepared with probes located at positions +14, −6, −12. As controls, a –UV lane is included for each position, and a mutant version of Spt4/5 (1–418) that does not bind RNAPII is also shown. Both WT and mutant Spt4/5 are at 30 nM and RNAPII is at 5 nM. Black arrows highlight the migration of Rpb1, Rpb2, and Spt5 in the gel.

2.8.1. Materials

EC42 DNA template (Section 2.2).

Transcription buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 0.5 mM DTT, 10% glycerol, 0.3 mM UpG, 0.1 μg/μl BSA, 1 unit/μl RNasin).

5-Bromo-UTP (Sigma-Aldrich).

Nucleotide labeling mix (4 mM ATP, 2 mM 5-Bromo-UTP, 0.05 mM CTP, 5 μl of alpha-^{32}P-CTP 6000 Ci/mmol, MP Biomedical).

10X DNase I buffer (100 mM $MgCl_2$, 50 mM $CaCl_2$, 100 mM Tris-HCl pH 8.0).

DNase I 10 unit/μl (Worthington).

RNase A 5 mg/ml. mg/ml yeast total RNA.

2.8.2. Method

1. Mix 75 ng of end-labeled template with 150 fmol of RNAPII in 15 μl transcription buffer and incubate for 5 min.
2. Start transcription through the G-less cassette by adding 5 μl of nucleotide mix 1. Incubate at 30 °C for 20 min.
3. Add 5 μl transcription buffer or elongation factor diluted in the same buffer. Incubate for 10 min at 30 °C.
4. Add 1 μg of total yeast RNA. Incubate for 5 min.
5. Pipet the samples onto a sheet of parafilm placed on a pre-chilled aluminum block placed in a tray of ice.
6. Irradiate the samples at 300 nm wavelength for 10 min at a distance of 10 cm.
7. Transfer the samples to a 1.7 ml tube, add 10X DNase buffer to 1X and 10 units DNase I and 2.5 μg of RNase A and incubate at 37 °C for 1 h.
8. Add 1/3 vol of 3X SDS-PAGE loading buffer and boil for 5 min.
9. Separate on SDS-PAGE gels.
10. Dry the gel and exposed to phosphorimager screen.

2.9. Transcript mapping by RNase I protection

The RNA transcript emerges from RNAPII after 17–18 nucleotides

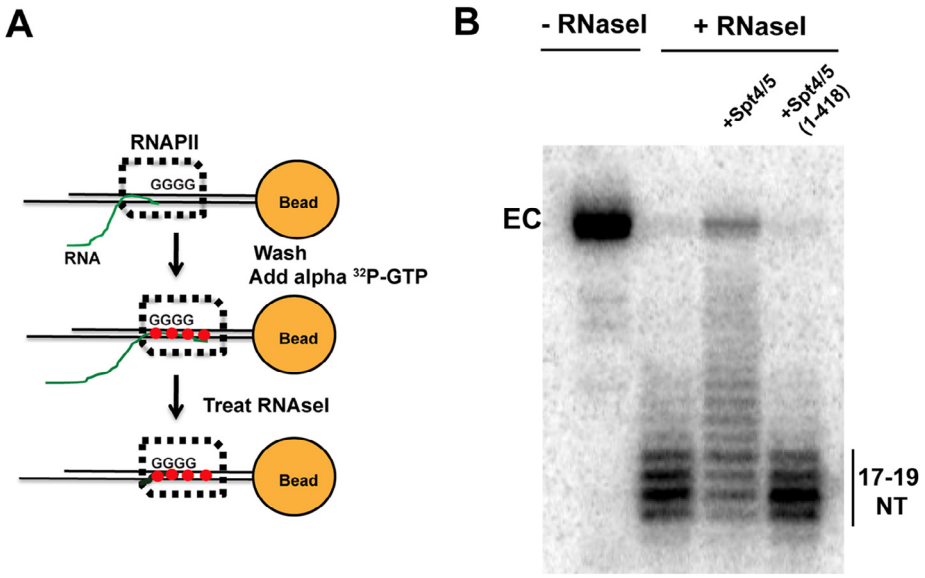

Fig. 5. RNAse I mapping of the transcript in EC42. (A). Schematic of the production of 3′ end-labeled RNA in RNAPII. ECs were formed on immobilized templates by transcription through the G-less cassette with 100 nM RNAPII. The addition of radiolabeled GTP (red circles) labels the RNA on the 3′ end (B). Denaturing gel of transcript length in ECs. ECs with end-labeled transcript were treated as indicated in the panel. Spt4/5, which protects the emerging transcript, was added. Spt4/5 (1–418) is a derivative that does not bind to ECs and was used as a control. WT Spt4/5 was titrated to achieve full protection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

are synthesized. Some elongation factors and RNA binding proteins bind to the emerging transcript as it appears from the RNA exit channel. RNase protection assays can determine if a factor protects the transcript; thus, is located near the RNA exit channel. In this assay, RNAPII transcribes through the G-less cassette and then arrests when it encounters 4C's in the template stand. Immobilization of RNAPII onto beads allows the nucleotides to be washed out and the 3′ end of the RNA is labeled by adding radiolabeled GTP. The ECs are then digested with RNase I in the presence or absence of elongation factor. The binding of the factor extends the length of the protected RNA (Fig. 5).

2.9.1. Materials

EC42 template with biotin synthesized at the 5′ of the template stand.

Streptavidin M − 280 Dynabeads (Invitrogen).

Magnetic Separator stand.

Blocking buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 0.25 μg/μl BSA, 0.1% NP-40, 10% glycerol).

Strep-Wash buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM DTT, 0.02% NP-40, 0.1 μg/μl BSA, 10% glycerol).

Transcription buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 0.5 mM DTT, 10% glycerol, 0.3 mM UpG, 0.1 μg/μl BSA, 1 unit/μl RNasin).

Nucleotide mix (0.4 mM ATP, 0.4 mM UTP, 0.4 mM CTP).

Alpha-^{32}P-GTP 6000 Ci/mmol (MP Biochemicals).

Reaction buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 0.02% NP-40, 0.01 mM $ZnCl_2$, 0.1 μg/μl, 10% glycerol).

RNase If 50 units/μl (NEB).

Stop buffer (65 mM Tris-HCl pH 8.0, 3 mM EDTA, 200 mM NaCl, 0.65% SDS)

Phenol:chloroform:isoamyl alcohol (10:9:1).

Formamide loading solution (90% formamide, 1 mM EDTA, bromophenol blue, xylene cyanol).

Glycogen 2 mg/ml (Thermo-Fisher).

mg/ml yeast total RNA.

15% urea-PAGE (19:1 acrylamide:bis-acylamide).

2.9.2. Method

1. Synthesize the transcription template with biotin on the 5′ end of the non-template strand, which allows for the immobilization of the template on streptavidin-coated magnetic beads.
2. Take 10 μl of streptavidin-coated Dynabeads M-280. This is for 6 samples, scale up if necessary.
3. Add 50 μl of blocking buffer to the beads, then magnetically collect the beads and remove the supernatant. Add another 50 μl of blocking buffer and then add 600 ng of biotinylated transcription template. Incubate for 1 h with occasional mixing.
4. Wash 3 times with 100 μl of Strep-wash buffer. Then re-suspend with 15 μl of nucleotide mix/45 μl of transcription buffer.
5. Add 6 pmol of RNAPII and incubate for 20 min at 30 °C.
6. Collect the beads and wash with 3 times with 100 μl of wash buffer and resuspend in 20 μl of reaction buffer.
7. Add 5 μl alpha-^{32}P-GTP. Incubate at 30°C for 5 min to incorporate the label into the 3′ end of the transcript.
8. Add unlabeled GTP to a concentration of 100 μM and incubate 5 min.
9. Collect the beads and wash 3 times with 100 μl of wash buffer. Resuspend in 45 μl reaction buffer + 15 μl of nucleotide mix (100 μM final concentration for ATP, CTP, UTP)
10. Distribute 10 μl of the bead suspension into separate tubes. Add 10 μl of reaction buffer or elongation factor diluted in the same buffer. Incubate for 5 min and then add 100 ng of yeast total RNA as a carrier.
11. Add RNase If to a final concentration of 1 unit/μl and incubated for 3 min at room temperature. You should optimize the amount of RNase If and digestion times.
12. Terminate the reaction by adding 40 μl of stop solution, and then 1 μl of 1 mg/ml Proteinase K. Incubate at 50 °C for 20 min.
13. Collect the beads using on a magnet and transfer the supernatant to a new tube.
14. Extract with an equal volume of phenol:chloroform:isoamyl alcohol.
15. Add 8 μg of glycogen and 2 μg of tRNA and concentrate by ethanol precipitation.
16. Resuspend pellet in 1 μl H_20 and then add 4 μl of formamide loading dye.
17. Separate the products on a 15% Urea gel at 12.5 W until bromophenol blue dye runs off the gel. Dry the gel and expose to phosphorimager screen

3. Conclusion

Specialized nucleic acid structures play an integral role in all of biology. This is especially true of transcription, where effective

management of the transcription bubble and emerging RNA transcript by RNAPII is essential for proper elongation. The emergence of Cryo-EM as a high-resolution technique is considered a revolution in understanding how transcription factors interact with RNAPII [40]. However, this technique creates snapshots, and it is difficult to use Cryo-EM to analyze a large number of mutants in a cost-effective way. Furthermore, structures need to be validated by biochemistry. *In vitro* transcription, combined with crosslinking and nuclease footprinting, have been used to probe the structure-function relationship of RNAPII and the actions of elongation factors (EFs). Unfortunately, fewer of today's scientists have experience with these techniques; thus, many researchers have abandoned them. We hope that this paper provides a detailed description of highly effective methods to probe EC structure and illustrates the power of using the "lost art" of reconstitution biochemistry to uncover the functions of EFs.

Acknowledgements

We thank Jianhua Fu and Craig Kaplan for sharing their RNAPII purification procedure. Dave Gilmour is thanked for the transcription template and advice during the development of the methods described here. This research was supported by funds from the National Institutes of Health [GM58672 to J.C.R].

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