Structural and biochemical analysis of DNA lesion-induced RNA polymerase II arrest

Juntaek Oh[1], **Jun Xu**[1], **Jenny Chong**[1], and **Dong Wang**[2]

[1]Division of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, United States.

[2]Division of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, United States; Department of Cellular and Molecular Medicine, School of Medicine, University of California, San Diego, La Jolla, CA 92093, United States.

Abstract

Transcription, catalyzed by RNA polymerase II (Pol II) in eukaryotes, is the first step in gene expression. RNA Pol II is a 12-subunit enzyme complex regulated by many different transcription factors during transcription initiation, elongation, and termination. During elongation, Pol II encounters various types of obstacles that can cause transcriptional pausing and arrest. Through decades of research on transcriptional pausing, it is widely known that Pol II can distinguish between different types of obstacles by its active site. A major class of obstacles is DNA lesions. While some DNA lesions can cause transient transcriptional pausing, which can be bypassed by Pol II itself or with the help from other elongation factors, bulky DNA damage can cause prolonged transcriptional pausing and arrest, which signals for transcription coupled repair. Using biochemical and structural biology approaches, the outcomes of many different types of DNA lesions, DNA modifications, and DNA binding molecules to transcription were studied. In this mini review, we will describe the *in vitro* transcription assays with Pol II to investigate the impacts of various DNA lesions on transcriptional outcomes and the crystallization method of lesion-arrested Pol II complex. These methods can provide a general platform for the structural and biochemical analysis of Pol II transcriptional pausing and bypass mechanisms.

Keywords

RNA polymerase II; X-ray crystallography; DNA lesion; transcription

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Correspondence to: Dong Wang.

1. Introduction

RNA polymerase II (Pol II) is a key enzyme complex in eukaryotic gene expression that is responsible for synthesizing the precursors of mRNA, snRNA and microRNA [1, 2]. During transcription elongation, Pol II elongation complex translocates along the template DNA and frequently encounters numerous kinds of modifications and lesions that occurs on a daily basis [3]. While some DNA lesions can cause transient transcriptional pausing, bulky DNA damage can cause prolonged transcriptional pausing and arrest, which signals for transcription coupled repair. Defects of transcription coupled repair and prolonged transcription arrest can attribute a variety of diseases and aging [4].

Ever since the determination of the first structures of a 10-subunit Pol II core [5, 6] and a complete 12-subunit Pol II with subcomplex Rpb4/7 [7, 8], dozens of Pol II structures in complex with a wide range of transcription factors or DNA binding molecules, lesions, and modifications have been reported [9–30]. With these results, now we have a better understanding of how Pol II reads out template DNA strand and incorporates the correct nucleotide substrate during transcriptional elongation, and how Pol II recognizes and deals with different types of DNA modifications. While small DNA lesions can lead to transient pausing and error-prone transcriptional bypass, bulky DNA lesions can induce persistent transcriptional stalling and provide a strong signal for the recruitment of repair factors to initiate transcription coupled repair (TCR) or the degradation of stalled Pol II by ubiquitylation [31–35].

Combining structural and biochemical approaches in the past decade, we have gained important insights into understanding the mechanism of normal transcription process as well as transcriptional pausing/arrest and bypass. For example, the bridge helix, a conserved motif featuring a long α-helix, dictates the positioning of template strand DNA and separates the upstream DNA-RNA hybrid from the downstream DNA duplex. It is suggested that the bridge helix plays important roles in Pol II forward and backtracking translocation [19, 36, 37]. During forward translocation, the downstream template nucleobase needs to cross over the bridge helix to reach +1 position. During backtracking, the template nucleobase at +1 position also needs to cross over the bridge helix in the opposite direction to reach the downstream channel. We found that this crossing over of the bridge helix serves as an important check point for DNA lesion detection. Indeed, several DNA-lesion arrested Pol II complexes are trapped in the half-translocation intermediate state in which the DNA lesions are located above the bridge helix [10, 20, 26, 38].

In this mini-review, we will describe the methods of how *in vitro* transcription assays are carried out to investigate the effect of various DNA modifications on Pol II elongation. We will also describe the methods for the structural determination of Pol II with DNA modification or lesions. This mini-review is aimed to provide an overview for those who are interested in understanding the impact of DNA modifications or DNA lesions on Pol II transcription.

2.   Biochemical analysis of the impacts of DNA lesions on RNA Pol II transcription pausing/arrest, bypass and transcriptional fidelity

2.1   Purification of RNA polymerase II

Yeast RNA polymerase II is purified as previously reported [9, 15, 24]. Briefly, cells are first resuspended in lysis buffer and then lysed by French press and followed by centrifugation. The cleared lysate is transferred to a new container and Pol II is precipitated by the addition of 50% saturated ammonium sulfate solution. The pellet is then dissolved in Ni buffer and purified by Ni-NTA affinity chromatography. After washing with high-salt buffer (Ni buffer with 1000 mM KCl and 7 mM imidazole) and Ni wash buffer, the protein is eluted with Ni wash buffer containing 100 mM imidazole and no protease inhibitors. The eluted protein is diluted with MonoQ buffer and then purified by anion exchange chromatography (MonoQ, GE healthcare) using a gradient ranging from 150 mM to 1500 mM KOAc. The last elution peak is collected and concentrated. For the strains containing recombinant protein A tag, Pol II is first purified using IgG sepharose resin (GE Healthcare)[16, 39, 40]. After cleavage of protein A tag by TEV protease, Pol II is further purified by Hi-Trap Heparin and Mono Q columns (GE Healthcare). For biochemical analysis, purified protein is stored in aliquots of elongation buffer and stored at −80 °C or liquid nitrogen. For crystallization study, Pol II is precipitated by adding saturated ammonium sulfate to 55% concentration at 4 °C. After centrifugation, the majority of supernatant is discarded, leaving a small remaining amount to protect the protein from air. The protein is then flash frozen and stored in −80 °C or liquid nitrogen.

In addition to direct purification [21, 27, 39, 40], 12-subunit RNA Pol II can also be reconstituted by mixing 10-subunit core RNA Pol II (strain name: CBC010   4 Protein A-tagged) and Rpb 4/7 subcomplex [7, 8]. Rpb 4/7 is expressed in Rosetta 2 (Novagen) and purified using Ni-NTA affinity chromatography (Qiagen). Ten-subunit core RNA Pol II and Rpb 4/7 are mixed at a molar ratio of 1:4 in elongation buffer and purified by size exclusion chromatography to remove excess Rpb 4/7. Buffers used in this method are summarized in Table 1.

2.2   Purification of site-specific DNA lesion-containing DNA template

There are two conventional methods for obtaining site-specific DNA lesion-containing DNA template. If lesion-modified nucleoside phosphoramidites are available, the DNA-lesion containing oligonucleotides can be synthesized using a solid-phase DNA synthesizer via phosphoramidite chemistry [20, 26]. Otherwise, DNA lesion can be formed in a post-synthesis manner [11, 16]. The short oligonucleotides are directly reacted with chemical agents. Extensive purification steps are often required to separate DNA-lesion containing oligonucleotides from undamaged oligonucleotides using HPLC (ion exchange and reverse phase) and PAGE purification [11, 16]. The identities and purities of these lesion-containing oligonucleotides can be confirmed by electrospray ionization-mass spectrometry (ESI-MS) and tandem MS (MS/MS) analyses. For the long DNA-lesion containing DNA template (for full-bubble scaffold), additional preparation steps are required starting from a short DNA-lesion containing oligos via DNA ligation or primer extension [20, 26].

2.3 Preparation of Transcription Scaffold

The promoter-dependent transcription initiation is very inefficient *in vitro* and requires general transcription factors. To overcome this technical barrier, a synthetic transcription RNA:DNA scaffold is used to assemble Pol II elongation complex. This system allows us to achieve 100% transcription elongation efficiency *in vitro* and quantitatively measure the kinetic impact on transcription by site-specific DNA lesions at base resolution.

The scaffold can be further divided into two classes: mini-scaffold and full-bubble scaffold (Fig. 1). The mini-scaffold represents a part of the transcription bubble that harbors 8–9 bases of the DNA-RNA hybrid upstream and 18–19 bases of the double-stranded DNA downstream, whereas the full-bubble scaffold contains an intact transcription bubble (matched or mismatched) with a preformed short DNA-RNA hybrid (see Table S1 for examples). Both systems are widely used for biochemical and structural studies. Here, we will describe the assembly of both systems.

To prepare the mini-scaffold, a ratio of 1:3:3 of P^{32}-labeled RNA, DNA-lesion containing DNA template strand, and non-template strand DNA are mixed in the elongation buffer. 5'-P^{32} labeled RNA oligo is generated using T4 PNK (NEB). Final concentration of the labeled RNA is 200 nM. The reaction mixture is annealed by incubating at 95 °C in a thermocycler or heat block for 2 min and slowly cooling down to room temperature (23 °C). (Note: If the DNA lesion is heat sensitive, lower temperature and shorter incubation time are needed for denaturation and annealing. For DNA lesions that are light-sensitive, the tubes are wrapped in aluminum foil).

The full-bubble scaffold requires a two-step assembly. In the first step, 1:1 molar ratio of labeled RNA and DNA-lesion containing DNA template strand are mixed and annealed as described, with a final concentration of 200 nM each. The non-template DNA strand is added after the incubation with Pol II (see section 2.4 step a).

2.4 *In vitro* transcription assay

a. For the transcription assay with mini-scaffold, a minimum of 2- to 5-fold excess amount of Pol II is incubated with pre-assembled mini-scaffold in elongation buffer (EB, See Table 1) at room temperature for at least 10 min to ensure the formation of a stable Pol II elongation complex. For the transcription assay with full-bubble scaffold, we use a slightly modified annealing method (Fig. 1). First, 1:1 molar ratio of P^{32}-labeled RNA and template strand DNA are annealed. This DNA-RNA hybrid is mixed with 2.5 molar excess of 12-subunit Pol II and incubated for 10 min at room temperature, followed by 2 min at 37 °C. Subsequently, after the addition of 5'-biotinylated non-template strand DNA, the reaction mixture is incubated for 5 min at 37 °C and then for 20 min at room temperature. Final concentrations are 100 nM of RNA and template strand DNA, and 250 nM of Pol II and non-template strand DNA. To remove unbound RNA and Pol II, the elongation complex is incubated with streptavidin magnetic beads (NEB) for 30 min at room temperature and washed successively with EB, EB

with 0.3 M NaCl, EB with 1 M NaCl, EB with 0.3 M NaCl, and EB. The assembled Pol II elongation complex is ready for transcription assay.

b. To start the transcription reaction, an equal volume of Pol II elongation complex in (a) is mixed with various concentration of rNTPs or single nucleotide. Final reaction condition is 100 nM of Pol II, 20 nM of scaffold, and varying concentration of NTPs in elongation buffer. For the RNA cleavage assay, TFIIS is incubated with/without NTPs and mixed with elongation complex when the reaction starts, because pre-incubation of TFIIS and elongation complex without NTPs can lead to the degradation of labeled RNA, especially for the damaged scaffold. For reaction with Rad26, 3 mM of dATP is also included with NTPs to facilitate the ATPase activity (Fig. 2B).

c. For each designated time point, four volumes of the quench-loading buffer (see Table 1) is added to the reaction mixture to stop the reaction. Time point zero can be prepared by adding quench-loading buffer to the elongation complex without NTPs. The KinTek RQF-3 Quench-Flow instrument is needed for rapid time points in pre-steady state kinetic study. After quenching, the samples are incubated at 95 °C for at least 5 min to denature the protein complex and scaffold. After denaturation, the RNA transcripts samples are ready for denaturing urea/PAGE gel analysis.

d. The quenched products (RNA transcripts) are analyzed by denaturing urea/PAGE gel in $1\times$ TBE at 200 V for 2.5–3.5 h. 8–16% of acrylamide can be used depending on the predicted size of RNA product. RNA transcripts are visualized using a storage phosphor screen and Pharos FX imager (Bio-Rad).

e. For intrinsic transcript cleavage assays, the Pol II complex is assembled as described above in a 20 mM Tris-HCl (pH 7.5) buffer without Mg^{2+}. Intrinsic cleavage is initiated after the addition of Mg^{2+}. Final concentrations for intrinsic cleavage are 20 mM Tris-HCl (pH 9.0), 100 nM Pol II, 25 nM scaffold, and 50 mM $MgCl_2$. The reaction is stopped by adding four volumes of quench loading buffer at various time points and analyzed by denaturing PAGE. For TFIIS-stimulated transcript cleavage assays, Pol II complex is assembled as aforementioned in a 20 mM Tris-HCl (pH 7.5) buffer without Mg^{2+}. The solution is then mixed with an equal volume of solution containing TFIIS and $MgCl_2$ in elongation buffer. The final reaction solution contains 100 nM Pol II, 25 nM scaffold, 1 μM TFIIS, and 5 mM $MgCl_2$. The reactions are quenched at various time points by the addition of an equal volume of quench loading buffer. The quenched products are again analyzed by denaturing urea/PAGE and visualized using a storage phosphor screen and Pharos FX imager (Bio-Rad).

f. For detailed single-turnover kinetic analysis, non-linear regression data fitting is performed using Prism 6. The time dependence of product formation is fit to a one-phase association equation to determine the observed rate (k_{obs}). The substrate concentration dependence is fit to a hyperbolic equation to obtain values for the maximum rate of NTP incorporation (k_{pol}) and apparent K_d ($K_{d,app}$) governing NTP binding as previously described. The specificity constant

is determined by $k_{\mathrm{pol}}/K_{\mathrm{d,app}}$. These kinetic parameters are used for measuring the impacts of DNA lesion on nucleotide incorporation and transcriptional fidelity in a quantitative manner.

g. Troubleshooting: If a high level of unextended RNA is detected, check the Pol II activity with undamaged DNA template as a control. If Pol II is active, increase Pol II concentration or preincubation time to make sure all scaffolds formed Pol II elongation complex. If the bands are smeared, increase denaturation time. Please also use RNase-free water to prepare buffers. The same method can be used with other eukaryotic and prokaryotic RNA polymerases.

3. Structural analysis of DNA-lesion arrested Pol II complexes

Crystallization methods are essentially the same as previously reported [9, 15, 26]. However, optimization is needed to determine the best crystallization conditions for different types of DNA lesion-arrested Pol II complex (Fig. 3). The optimization procedures include designing different lengths and sequences of DNA and RNA scaffold for structural studies, changing initial protein concentration, crystallization buffer concentration, ratios of protein:crystallization buffer, and variation of pH, salt, detergents, and temperature. Note: If DNA lesion is light-sensitive, the trays are covered with aluminum foil. If DNA lesion is sensitive to redox, special consideration is needed for choosing compatible reducing agents in the buffer.

3.1 Preparation of DNA-lesion arrested Pol II complexes

a. For complexes the mini-scaffold, mix 1:2:2 molar ratio of DNA-lesion containing template DNA, non-template DNA, and RNA oligonucleotides in elongation buffer. Final concentration of template DNA can vary but is usually adjusted to 0.1 mM. Annealing is performed by incubating at 65 °C for 5 min and slowly cooling down to 25 °C.

b. Centrifuge ammonium sulfate precipitated Pol II to remove residual supernatant and dissolve in elongation buffer to final concentration of 5 μM.

c. Add five molar equivalents of prepared mini-scaffold to purified Pol II in elongation buffer. Final concentrations of Pol II and template DNA are 2.5 μM and 12.5 μM, respectively. Incubate elongation complex at room temperature for 30 min, followed by at least 30 min on ice.

d. Ultrafiltration is performed to change buffer and exclude excess scaffold, by using 100 kDa molecular cut-off centrifuge filters (Merck). We add two equal volumes of exchange buffer to elongation complex and centrifuge to concentrate. This process is repeated 3 times. Final concentration is set to 6–8 mg/ml by Bradford assay (Bio-rad). Prepared elongation complex can be directly used for crystallization, or flash-frozen and stored in −80 °C for crystallization repeat and optimization.

3.2 Crystallization of elongation complex and ligand soaking

a.   The hanging drop crystallization plate (Qiagen) is set up by mixing protein and crystallization reservoir solution at 1:1 to 2:1 ratio. 5 mM of fresh reducing agents (such as DTT) is added to the reservoir right before crystallization to prevent the oxidation of Pol II. Crystals are incubated at 22 °C for 4 days to 2 weeks.

b.   The size of crystal is one of the most important factors correlated to diffraction quality. Optimization of crystallization buffer and ratio of protein:reservoir can be done to obtain higher quality crystals. An example of crystal optimization is shown in Figure 3. Ten-subunit Pol II is crystallized with mini-scaffold containing a site-specific 8-oxo-G lesion at the template strand DNA (same scaffold as in Fig. 2A, bottom panel). Initial crystallization buffer is 10 % PEG 6,000 without DTT (Table 1), and 1:1 volume ratio is used. Well-diffracting crystals are obtained by changing the ratio of protein and crystallization buffer to 2:1 and adding additional 5 mM fresh DTT to the crystallization buffer to prevent oxidation.

c.   For the data collection, selected crystals are transferred from crystallization drop to cryo buffer in a step-wise manner. The intermediate buffers are prepared by mixing crystallization buffer and cryo buffer in a ratio of 2:1 and 1:2. The transferred crystals are then incubated overnight at 4 °C and then flash-frozen and stored in liquid nitrogen for remote data collection. In the case of soaking experiments, various ligands are added to the final cryo buffer. If the ligand is nucleotide triphosphate or its analogs, the same or higher concentration of $MgCl_2$ should also be added to prevent Mg^{2+} depletion in the crystal.

d.   Collected datasets can be processed by using iMosflm [41]. CC1/2 around 0.5 is usually used to determine the resolution of each dataset [42]. Initial structure can be determined with PHENIX molecular replacement by using unmodified Pol II elongation complex as a search model [9]. Manual model building and refinement by COOT and PHENIX is necessary to get the final model [43, 44]. Summary of structure determination is depicted in Figure 4.

4.   Conclusion

Investigation of transcriptional dynamics is critical for us to fully understand transcription regulation as well as its interplay with other DNA processes, such as DNA repair and replication. Indeed, the causes of transcriptional pausing and arrest are exceptionally diverse: Pol II complex can pause/arrest through specific interactions with transcription factors (such as promoter proximal-pause), or naturally occurring sequences (such as repetitive sequence or non-B form DNA), or at DNA lesions and modifications [45, 46]. The interplays between Pol II transcription machinery and DNA lesions and modifications dictate distinct transcriptional outcomes. Some of the DNA lesions cause transient transcriptional pausing, which can be bypassed by Pol II itself or with the help from other elongation factors [31], whereas bulky DNA lesions can cause prolonged transcriptional pausing and arrest, which

provides a strong signal for transcription coupled repair. In addition, prolonged transcriptional arrest also leads to the degradation of complex or apoptosis [47].

In this mini-review, we aimed to summarize the current methods of transcription assay and crystallization of DNA lesion-arrested yeast RNA polymerase II. Transcription assay can be used to investigate the transcriptional consequences of modification or DNA binding ligands. For example, the ratio of run-off product, the severity of transcription pausing, pausing position, nucleotide preference, calculating kinetic values, and TFIIS induced cleavage of RNA transcript can be studied by transcription assay. Structural analysis of Pol II and damaged scaffold provides structural snapshots of lesion-arrested Pol II. These combined methods provide novel mechanistic insights into the understanding of how RNA Pol II transcription machinery recognizes different types of lesions and dictates translesion synthesis or transcription coupled repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

1. Description of a combined approach including biochemical transcription assays and structural analysis to investigate the mechanism of DNA lesion arrested RNA Pol II.

2. Method for *in vitro* transcription biochemical assay with RNA Pol II on a DNA lesion-containing template

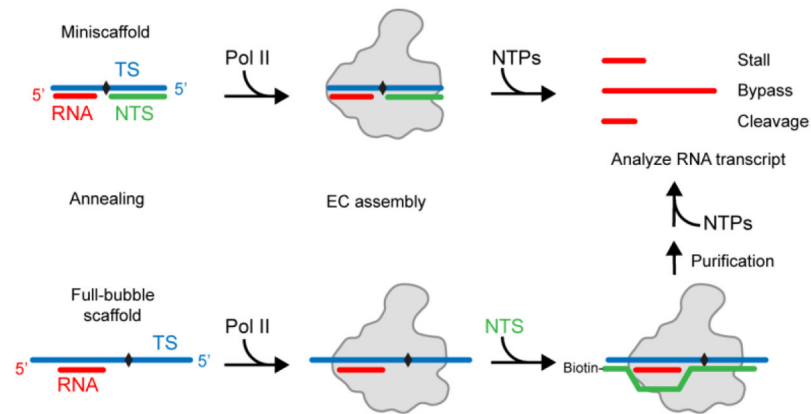3. Crystallization method for DNA lesion-arrested RNA Pol II complex

Figure 1. Schematic representation of investigating the effect of DNA lesions by transcription assay.

To investigate the effect of DNA lesion or modification (black diamond) on transcription, mini-scaffold or full-bubble scaffold is used. Mini-scaffold consists of short template strand, non-template strand and RNA. This miniature transcription bubble is a useful tool in understanding how elongation complex handles obstacles during transcription. Full-bubble scaffold has longer template strand DNA and non-template strand DNA. Additional steps are required for the preparation of elongation complex (EC).
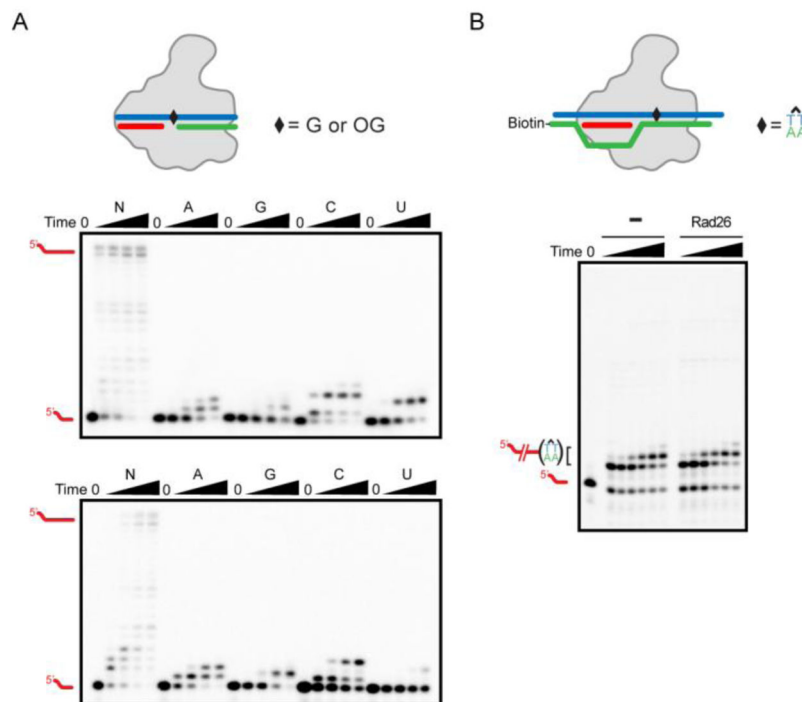
Figure 2. Transcription assay results using mini-scaffold or full-bubble scaffold.
(A) An example of transcription assay is performed by using mini-scaffold harboring non-damaged G (upper panel) or 8-oxoguanine (8OG, bottom panel) in its template strand DNA. Reaction is initiated by adding 50 μM of NTPs, ATP, GTP, CTP, or UTP. Time points are: 0 (without NTPs), 10 sec, 3 min, 30 min, and 2 hr. Note that 8OG can be bypassed by adding A or C, producing full-length transcripts after 3 min, as described in previous paper [13]. (B) Example of full-bubble transcription assay, using the same scaffold in our previous paper [24]. Time points are: 0, 1, 3, 10, 30, and 60 min, respectively. In this experiment, cyclobutane pyrimidine dimer (CPD) is tested to see whether presence of Rad26 can rescue arrested elongation complex (EC). As previously reported [24], Rad26 fails to rescue CPD induced EC arrest. Gel image was processed by Image Lab (Bio-rad). DNA and RNA oligos used in this study are summarized in Table S1.
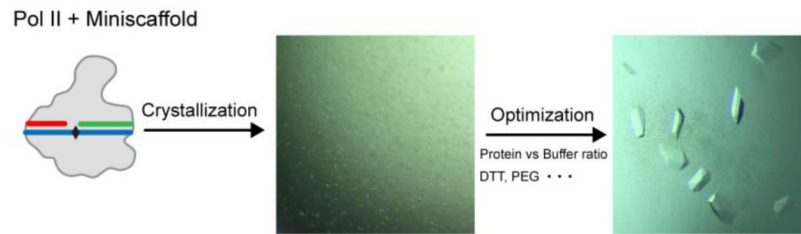
Figure 3. Schematic overview of crystal optimization.
Transcription assay results can be used to design mini-scaffold for crystallization. Crystals of 10-subunit Pol II crystallized with 8OG containing mini-scaffold are shown. Initial crystals are produced by mixing same volume of protein and crystallization buffer (see Table 1) with 10 % PEG 6,000 and without DTT. Optimized crystals were grown in 5 mM DTT buffer with 2:1 protein to buffer ratio.
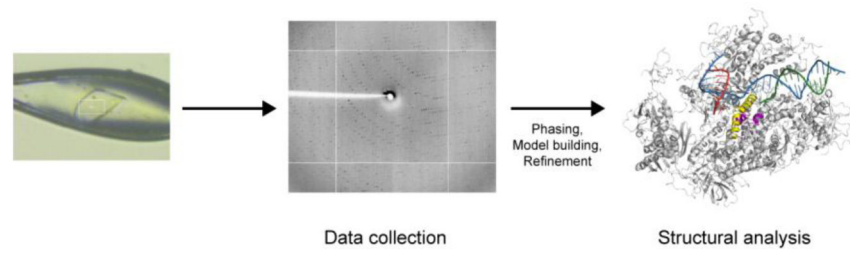
Figure 4. Structure determination of Pol II elongation complex

An example of crystal and diffraction pattern of Pol II elongation complex is shown. X-ray diffraction image was collected at synchrotron (beamline 8.2.1, Advanced Light Source, Lawrence Berkeley National Laboratory). An example of structural representation of Pol II elongation complex with mini-scaffold was prepared by Pymol [9]. Color codes are the same as in schematic representation (Fig. 1). Bridge helix and trigger loop are colored as yellow and purple, respectively.

Table 1.

Buffers and reagents used in this study.

Name	Components
Lysis buffer	50 mM Tris-HCl [pH 7.9], 1 mM EDTA, 10 μM $ZnCl_2$, 10% v/v glycerol, 1% v/v DMSO, 10 mM DTT and 1 × protease inhibitors
Ni buffer	20 mM Tris-HCl [pH 7.9], 150 mM KCl, 10 μM $ZnCl_2$, 10% v/v glycerol, 10 mM DTT and 1 × protease inhibitors
Ni wash buffer	20 mM Tris-HCl [pH 7.9], 150 mM KC1, 7 mM imidazole, 10 μM $ZnCl_2$, 10 mM DTT and 1 × protease inhibitors
MonoQ buffer	20 mM Tris-acetate [pH 7.9], 0.5 mM EDTA, 10 μM $ZnCl_2$, 10% v/v glycerol and 10 mM DTT
Elongation buffer	20 mM Tris-HCl [pH 8.0], 40 mM KC1, 5 mM DTT and 5 mM $MgCl_2$
Quench-loading buffer	90% (v/v) formamide, 50 mM EDTA, 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromophenol blue
10X TBE buffer	1 M Tris base, 1 M Boric acid and 20 mM EDTA
TBE-Urea/PAGE	1 × TBE, 7 M urea and 8–16 % (v/v) bis-aerylamide 19:1
Exchange buffer	25 mM Tris [pH 7.5], 20 mM NaCl, 5 mM DTT, 1 μM $Zn(OAc)_2$ and 100 μM EDTA
Crystallization buffer	390 mM Ammonium phosphate [pH 6.0], 5 mM DTT, 5 mM Dioxane and 9–13 % (w/v) PEG 6,000
Cryo buffer	100 mM MES [pH 6.0], 350 mM NaCl, 5mM DTT, 5mM Dioxane, 16 % PEG 6,000 and 17 % PEG400