Published in final edited form as:

Methods. 2019 April 15; 159-160: 90–95. doi:10.1016/j.ymeth.2019.01.016.

Time-Resolved Analysis of Transcription through Chromatin

Han-Wen Chang^{1,*}, Fu-Kai Hsieh^{2,*}, Smita S. Patel³, and Vasily M. Studitsky^{1,4,**}

¹Fox Chase Cancer Center, Philadelphia, PA 19111, USA

²Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA

³Department of Biochemistry & Molecular Biology, Robert Wood Johnson Medical School, Rutgers University, Piscataway, NJ, 08854, USA

⁴Biology Faculty, Lomonosov Moscow State University; Moscow 119992, Russia.

Abstract

During transcription along nucleosomal DNA, RNA polymerase II (Pol II) pauses at multiple positions and induces formation of multiple intermediates that aid in maintaining proper chromatin structure. To describe the kinetics of this multiple-step reaction, we utilized a computational model-based approach and KinTek Explorer software to analyze the time courses. Here we describe the stepwise protocol for analysis of the kinetics of transcription through a nucleosome that provides the rate constants for each step of this complex process. We also present an example where this time-resolved approach was applied to study the mechanism of histone chaperone FACT action during Pol II transcription through a single nucleosome by comparing the rate constants derived in the presence or in the absence of FACT.

Keywords

transcription; factors; chromatin; nucleosome; kinetics

1. Introduction

RNA polymerase II (Pol II) pauses at multiple positions during transcript elongation on nucleosomal DNA forming structurally distinct intermediates [1-6] that play important roles in the maintenance and recovery of proper chromatin structure during transcription (see [6] for review). To study this process, we utilized the well-studied “minimal” *in vitro* system supporting a single round of transcription by yeast Pol II through a single positioned nucleosome [1]. The pausing pattern recapitulates the Pol II pausing observed during transcription of genes bodies in the cells [7-9], which plays important roles in regulation of gene expression [6, 8]. The following critical factors regulate pausing of Pol II during

^{**}To whom correspondence should be addressed. Tel: 215-728-7014; Fax: 215-728-4333; Vasily.Studitsky@fccc.edu.

^{*} - equal contribution

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

transcription through chromatin: (1) DNA sequences; (2) post-translational modifications of Pol II; (3) post-translational modifications of histones; (4) protein factors, such as transcription factors, histone chaperones and ATP-dependent chromatin remodelers [1, 2, 10-15]. One well-studied factor that facilitates Pol II transcription through a nucleosome by relieving all major nucleosomal pauses is the human histone chaperone FACT (FACilitates Chromatin Transcription) [6, 10, 15, 16].

Previously we have described methods to evaluate structures of Pol II elongation complexes by DNA footprinting [17] and to monitor the dynamics of Pol II elongation complexes by single particle FRET [18]. Using these methods, we studied the structures and dynamics of the Pol II elongation intermediates paused at specific nucleosomal positions. Here we describe a complementary approach that provides kinetic parameters characteristic for each step of the reaction during ongoing Pol II transcription through a nucleosome. Accurate measurement of the kinetics of Pol II transcription through chromatin in the presence and absence of regulatory factors provides both quantitative and qualitative data allowing mechanistic understanding of the effect of these factors on transcription [19]. It is impossible to describe the multiple rate constants using a simple equation because of the complexity of the reactions. We therefore used a model-based approach that employs the KinTek Explorer software to analyze the time-courses of Pol II transcription through a nucleosome described here. The best-fit model suggests that Pol II transcription through chromatin involves formation of multiple productive and non-productive Pol II-nucleosome intermediates [2, 4, 6, 15]. The time-courses of Pol II transcription through a nucleosome are computationally fit to the model to obtain the rate constants of the multiple equilibria alternating between the productive and non-productive Pol II-nucleosome complexes (Fig. 1).

In summary, we describe here a detailed protocol to measure the complex kinetics of Pol II transcription through chromatin involving conducting, quantitating, and model fitting the time-courses. This protocol can be generalized to measure complex kinetics of other processive enzymes moving along DNA or chromatin, such as DNA polymerases [20], as well as for analysis of the mechanism of action of histone chaperones such as FACT [6, 15] or PARP1. To illustrate the method, we included some previously published data on analysis of the kinetics of human FACT-dependent Pol II transcription through a nucleosome [15].

2. Materials

2.1 Miscellaneous Items

1. Low adhesion tubes (USA Scientific).
2. Gel extraction kit (Omega Bio-Tek).
3. Dialysis membranes (Spectra/Por; molecular weight cutoff of 6,000-8,000).
4. Ni-NTA agarose (Qiagen).
5. 3 MM chromatography paper (Whatman).

2.2 Plasmids, Oligonucleotides and commercial DNA products

1. Plasmid pGEM-3Z-603. *See Note 1.*

5. T4 DNA ligase (Promega).
6. Purified yeast RNA polymerase II [22]. *See Note 4.*
7. Purified recombinant human FACT complex [10].

2.5 Buffers and reagents

1. Phenol and Chloroform (Sigma-Aldrich).
2. TE buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.
3. Core Reconstitution Buffers (CRB) 1–6: all buffers contain 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 5 mM β -mercaptoethanol, 0.1% NP-40, and NaCl at the following concentrations: buffer 1, 2 M; 2, 1.5 M; 3, 1 M; 4, 0.75 M; 5, 0.5 M; and 6, 0.01 M.
4. TB buffer: 20 mM Tris-HCl (pH 8.0), 5 mM MgCl_2 , 2 mM β -mercaptoethanol, and various concentrations of KCl. For example, TB40 contains 40 mM KCl.
5. Acetylated BSA (Sigma-Aldrich).
6. Imidazole (Sigma-Aldrich).
7. Polyethylene glycol (PEG)-8000 (Sigma-Aldrich).
8. Ethylenediaminetetraacetic acid (EDTA).
9. RNA loading buffer (RLB): 95% formamide, 10 mM EDTA, 0.1% SDS, and 0.01% bromophenol blue and xylene cyanol dyes.
10. 40% (w/v) acrylamide/bis-acrylamide solution (19:1).
11. Urea.
12. 10% (w/v) ammonium persulfate.
13. N, N, N', N'-Tetramethylethylenediamine (TEMED).
14. 5X TBE stock solution: 445 mM Tris base, 445 mM boric acid and 10mM EDTA (pH 8.0).

2.7 Software

1. OptiQuant software. *See Note 5.*
2. KinTek Explorer program (KinTek) [23, 24].

³The concentrations of unfolded purified histones are determined by measuring OD₂₇₆ value followed the published protocol [28]. The unfolded histones are then refolded to obtain the histone H2A/H2B dimers and H3/H4 tetramers [28].

⁴In this protocol, the purified His-tagged yeast core RNA polymerase II is typically utilized. The presence of His-tag allows immobilization of the Pol II EC on Ni²⁺-NTA agarose. The immobilized Pol II can be “walked” to every desired position along the DNA by adding different subsets of NTPs to the reaction. The non-tagged yeast or human Pol II can also be immobilized on avidin beads using end-biotinylated DNA template.

⁵The OptiQuant software can be substituted by any equivalent software allowing quantitation of the labeled RNA, such as Image Quant.

3. Methods

The following experimental system was utilized to study the mechanism of Pol II transcription through chromatin and the mechanism of FACT action during this process. The system is assembled from highly purified components (core yeast Pol II and precisely positioned mono-nucleosome comprising of purified DNA containing a strong nucleosome positioning sequences [1, 25] and *X. laevis* recombinant histones [21]); it was described in detail previously [2, 6]. The nucleosome assembly procedure is based on a published protocol [26]. This experimental system faithfully recapitulates many features of chromatin transcribed *in vivo* [1, 2, 5, 6, 11-13, 22, 27]. The hFACT protein complex was purified and assembled as described [10].

3.1 Preparation of DNA templates

1. The 603R primer was ³²P-end-labeled using T4 polynucleotide kinase (NEB) and 0.1–0.3 µCi γ-[³²P] ATP for 40 min at 37°C with subsequent enzyme inactivation for 20 min at 65°C in the TB40.
2. The 603 DNA template for transcription was PCR amplified from pGEM-3Z-603 plasmid using Taq DNA polymerase (NEB) with the unlabeled forward primer Uplong1 (containing TspR1 site; see Note 6) and labeled reverse primer 603R.
3. The PCR products were extracted by mixing with equal volume of 1:1 (v/v) phenol:chloroform, and then precipitated with ethanol. The ethanol-precipitated DNA pellets were washed with 70% ethanol and re-suspended in 1X CutSmart® Buffer (NEB).
4. The purified DNA products were digested with *TspR1* (NEB) at 65 °C for 4 h or overnight.
5. The *TspR1*-digested sample was resolved by gel electrophoresis using 2% agarose gel containing 4M urea. The 231-bp DNA fragment was visualized on transilluminator using long UV wavelength lamp (312 nm) and excised from the gel after EtBr staining.
6. The DNA fragments were extracted from the gel using Gel extraction kit (Omega Bio-Tek) following the manufacturer instructions and eluted with 30–50 µl of TE buffer.
7. DNA concentrations were determined by measuring A₂₆₀; purified DNA fragments were stored at –20°C.

3.2 Nucleosome reconstitution

1. Six Core Reconstitution Buffers (CRB1–6) were cooled to 4°C.
2. 0.5–3 µg of single end-³²P-labeled 603 DNA template was mixed with purified *X. laevis* recombinant histones in the CRB1 buffer. Additionally, salmon sperm

⁶.DNA digested with *TspR1* produces 9-nt long single-stranded overhangs that enhance the efficiency of ligation between the DNA ends of the Pol II elongation complex and the nucleosomal template.

dsDNA (Sigma-Aldrich) was added in 2-fold weight excess to the DNA fragment.

3. For nucleosome assembly using purified histones, H3 and H4 histones were added at 2-fold molar excess to DNA, while H2A and H2B were added at 3-fold molar excess.
4. The total volume of the reaction was adjusted to 40–100 μ l to achieve 10–100 ng/ μ l concentration of DNA.
5. The samples were then dialyzed against CRB1, CRB2, CRB3, and CRB4, each for 1 h, CRB5 for 2.5 h, and CRB6 overnight at 4°C.
6. The assembled nucleosomes were stored at 4°C.
7. The quality of the DNA-labeled nucleosomes obtained after reconstitution was analyzed by native PAGE (Fig. 2).

3.3 Time-Course Pol II Transcription in the absence/presence of human FACT

1. The low adhesion microcentrifuge tubes were used in all steps.
2. 200 pmol of ssDNA oligonucleotide, Template DNA strand 50T, was phosphorylated by T4 polynucleotide kinase (NEB) with 1 mM ATP in TB40 buffer for 20 min at 37°C and then subsequent enzyme inactivation for 20 min at 65°C.
3. The 200 pmol of phosphorylated 50T DNA oligonucleotide and 9-nt RNA primer were mixed in TB40 and annealed to make the RNA: DNA duplex. The annealing reaction was conducted for 10 min at 45°C; then the temperature was decreased by 2°C every 2 min to room temperature.
4. To form the active EC, the RNA: DNA duplex was incubated with 3-fold molar excess of yeast core Pol II in TB40 for 15 min at room temperature.
5. The formation of the active EC was terminated by adding 3-fold molar excess of unlabeled non-template DNA strand 59NT for 15 min at room temperature.
6. 20 μ l of Ni²⁺-NTA agarose (50% suspension in alcohol) was washed with 1 ml TB40 for three times, incubated in the presence of 0.5 mg/ml of acetylated BSA for 10 min, and washed with 1 ml TB40 two times. The volume was adjusted to 25 μ l.
7. To immobilize the EC on Ni²⁺-NTA agarose, The Pol II EC prepared in step 5 was mixed and incubated with Ni²⁺-NTA pretreated in step 6 for 15 min at room temperature.
8. Immobilized EC was washed once with TB40, twice with TB300, and twice with TB40. The volume was adjusted to 25 μ l.
9. The EC was eluted from Ni²⁺-NTA agarose by adding the imidazole to final concentration of 100 mM and incubating for 5 min at room temperature.

10. The eluted EC was ligated with 100–200 ng of the nucleosomal template in the presence of 100 μ M ATP, 1% PEG-8000, and 50 units of T4 DNA ligase in a volume of 50 μ l at 16°C for 1–2 h.
11. RNA transcript was pulse-labeled during the formation of EC-5 (the Pol II EC with the active center of Pol II localized 5 bp upstream of promoter-proximal nucleosomal boundary). The EC-5 was formed in the presence of 1 μ M α -[³²P] GTP and 2 μ M unlabeled CTP and 10 μ M unlabeled ATP for 10 min at room temperature.
12. The formation of EC-5 was completed by adding non-labeled GTP to final concentration of 10 μ M for 5 min at room temperature.
13. The RNA-labeled paused EC-5 was chased to complete the transcription in the presence of 400 μ M NTPs for different time intervals (0, 5, 10, 15, 20, 25, 30 and 60 seconds) in TB150.
14. To evaluate the effect of FACT, 0.1 μ M of hFACT was added simultaneously with the NTPs during the chase.
15. The reaction was terminated by adding EDTA to final concentration of 20 mM.
16. The pulse-labeled RNA transcript was extracted with one volume of 1:1 (v/v) phenol:chloroform, precipitated with ethanol, washed with 70% ethanol, dried, and dissolved in 5 μ l of RLB (RNA loading buffer).
17. RNA samples in RLB were heat denatured for 3 min at 100°C and analyzed by denaturing PAGE (8% polyacrylamide gel (19:1 acrylamide: bis) containing 8M urea) electrophoresis in 0.5xTBE buffer for 1–1.5 h at 2000 V.
18. Gel was dried on Whatman 3MM paper and exposed to a PhosphorImager screen at room temperature overnight (Fig. 3) (*see Note 7*).

3.4 Kinetic Analysis

To start the kinetic analysis using the KinTek Explorer program, models of Pol II transcript reaction were developed. In the absence of hFACT, Pol II naturally pauses and/or arrests at multiple positions in the nucleosome at physiological salt conditions (150 mM KCl). Three models were considered (Fig. 4). Model 1 is a “minimal model” in which the polymerase successively and productively progresses to a new position on the nucleosomal template. Model 2 invokes a non-productive state at each pause position. Thus, Model 2 proposes that the polymerase can either progress to the next productive position as in Model 1 or partition irreversibly as a non-productive complex at each nucleosomal position. Model 3 is similar to Model 2, except the non-productive state is reversible. Thus, Model 3 proposes that the polymerase can either progress to the next position as in Model 1 or partition reversibly to a non-productive complex at each position along nucleosomal DNA. The best model was selected after comparative analysis described below. The obtained experimental data did not

⁷ Since the template contains a small fraction of free DNA (<10%) (Fig. 2), some run-off transcripts (K) appear much faster than expected (Fig. 3). The small amounts of contaminating DNA (5–10% of all templates) do not considerably affect the outcome of the analysis.

fit to Model 1 [15]. Although Model 2 fits to the experimental data reasonably well, a better fit was provided by the Model 3 (Fig. 4), suggesting that Model 3 simulates the reaction of Pol II transcription through a nucleosome more accurately. A key observation that distinguished Model 2 and Model 3 is the following: although most nucleosome-specific complexes paused for a short time intervals (<1 min), the paused complexes could be eventually extended into longer transcripts, suggesting that the formation of the non-productive complexes is a reversible process, as hypothesized in Model 3 [15].

1. The imaging data (Fig. 3) were quantified using OptiQuant software. The pausing regions in the nucleosome were grouped based on the cut-off in the Pol II reaction in the absence of hFACT. The intensities of the signals were presented as the percentage of the sum of all groups (the groups A to K in the Fig. 3). The background noise was subtracted from the signals.
2. The rate constants were obtained using KinTek Explorer software according to the manufacturer instruction. The procedure is briefly described below.
3. The model 3 (Fig. 4) was input using the KinTek Explorer software and the numerical values for the species A to K (Fig. 3) were inserted.
4. The starting concentration of species A was introduced manually and was assumed to be 100%. *See Note 8.*
5. The units and ranges of the rate constants were pre-defined. The KinTek Explorer software manual suggests that: (1) The units of time are seconds and units of concentrations are micromoles; (2) Typically the rate constants are within the range of 0.01 and 100 $\mu\text{M}^{-1}\text{s}^{-1}$
6. The experimental data were imported based on the manufacturer instruction. The quantitative data from step 1 were input in the software.
7. The experimental data were processed to model fitting in the KinTek Explorer program. After model fitting, the simulation results were explored (Fig. 5).
8. The accuracy of the model fitting was evaluated by the sigma (standard deviation) or p value (<0.01), or by the visual examination of the alignments in the model fitting charts. KinTek Explorer includes FitSpace tool, which samples the key parameters around the best values and provides confidence contours and pairwise parameter dependencies to assess whether the fitted parameters are well-constrained.
9. The rate constants for each step were obtained after model fitting (Fig. 6).
10. Each dataset was processed from hands-on experiment to model fitting at least three times. For each reaction, a single fitting was processed to obtain one set of rate constant. The experiments are repeated three times; thus, three sets of rate constants were obtained, averaged, and calculated with the standard deviations.

⁸.Typically, starting concentrations are the concentrations of the substrates in the reaction. KinTek Explorer software allows the starting concentration to be introduced manually.

Acknowledgements

This work was supported by the National Institutes of Health R01GM119398 and R21CA220151 grants to V.M.S., GM118086 grant to S.S.P., and by Russian Science Foundation Grant 14-24-00031.

4. References

- [1]. Bondarenko VA, Steele LM, Ujvari A, Gaykalova DA, Kulaeva OI, Polikanov YS, Luse DS, Studitsky VM, Nucleosomes can form a polar barrier to transcript elongation by RNA polymerase II, *Mol Cell* 24(3) (2006) 469–79. [PubMed: 17081995]
- [2]. Kulaeva OI, Gaykalova DA, Pestov NA, Golovastov VV, Vassilyev DG, Artsimovitch I, Studitsky VM, Mechanism of chromatin remodeling and recovery during passage of RNA polymerase II, *Nat Struct Mol Biol* 16(12) (2009) 1272–8. [PubMed: 19935686]
- [3]. Chang HW, Shaytan AK, Hsieh FK, Kulaeva OI, Kirpichnikov MP, Studitsky VM, Structural Analysis of the Key Intermediate Formed during Transcription through a Nucleosome, *Trends in cell & molecular biology* 8 (2013) 13–23. [PubMed: 25364155]
- [4]. Gaykalova DA, Kulaeva OI, Volokh O, Shaytan AK, Hsieh FK, Kirpichnikov MP, Sokolova OS, Studitsky VM, Structural analysis of nucleosomal barrier to transcription, *Proc Natl Acad Sci USA* 112(43) (2015) E5787–95. [PubMed: 26460019]
- [5]. Chang HW, Kulaeva OI, Shaytan AK, Kibanov M, Kuznedelov K, Severinov KV, Kirpichnikov MP, Clark DJ, Studitsky VM, Analysis of the mechanism of nucleosome survival during transcription, *Nucleic Acids Res* 42(3) (2014) 1619–27. [PubMed: 24234452]
- [6]. Kulaeva OI, Hsieh FK, Chang HW, Luse DS, Studitsky VM, Mechanism of transcription through a nucleosome by RNA polymerase II, *Biochim Biophys Acta* 1829(1) (2013) 76–83. [PubMed: 22982194]
- [7]. Churchman LS, Weissman JS, Nascent transcript sequencing visualizes transcription at nucleotide resolution, *Nature* 469(7330) (2011) 368–73. [PubMed: 21248844]
- [8]. Teves SS, Weber CM, Henikoff S, Transcribing through the nucleosome, *Trends Biochem Sci* 39(12) (2014) 577–86. [PubMed: 25455758]
- [9]. Weber CM, Ramachandran S, Henikoff S, Nucleosomes are context-specific, H2A.Z-modulated barriers to RNA polymerase, *Mol Cell* 53(5) (2014) 819–30. [PubMed: 24606920]
- [10]. Belotserkovskaya R, Oh S, Bondarenko VA, Orphanides G, Studitsky VM, Reinberg D, FACT facilitates transcription-dependent nucleosome alteration, *Science* 301(5636) (2003) 1090–3. [PubMed: 12934006]
- [11]. Ujvari A, Hsieh FK, Luse SW, Studitsky VM, Luse DS, Histone N-terminal tails interfere with nucleosome traversal by RNA polymerase II, *J Biol Chem* 283(47) (2008) 32236–43. [PubMed: 18815126]
- [12]. Hsieh FK, Fisher M, Ujvari A, Studitsky VM, Luse DS, Histone Sin mutations promote nucleosome traversal and histone displacement by RNA polymerase II, *EMBO Rep* 11(9) (2010) 705–10. [PubMed: 20706221]
- [13]. Gaykalova DA, Nagarajavel V, Bondarenko VA, Bartholomew B, Clark DJ, Studitsky VM, A polar barrier to transcription can be circumvented by remodeler-induced nucleosome translocation, *Nucleic Acids Res* 39(9) (2011) 3520–8. [PubMed: 21245049]
- [14]. Pestov NA, Gerasimova NS, Kulaeva OI, Studitsky VM, Structure of transcribed chromatin is a sensor of DNA damage, *Science advances* 1(6) (2015) e1500021. [PubMed: 26601207]
- [15]. Hsieh FK, Kulaeva OI, Patel SS, Dyer PN, Luger K, Reinberg D, Studitsky VM, Histone chaperone FACT action during transcription through chromatin by RNA polymerase II, *Proc Natl Acad Sci USA* 110(19) (2013) 7654–9. [PubMed: 23610384]
- [16]. Gurova K, Chang HW, Valieva ME, Sandleh P, Studitsky VM, Structure and function of the histone chaperone FACT - Resolving FACTual issues, *Biochim Biophys Acta Gene Regul Mech* (2018).
- [17]. Gaykalova DA, Kulaeva OI, Pestov NA, Hsieh FK, Studitsky VM, Experimental analysis of the mechanism of chromatin remodeling by RNA polymerase II, *Methods Enzymol* 512 (2012) 293–314. [PubMed: 22910212]

- [18]. Kudryashova KS, Chertkov OV, Nikitin DV, Pestov NA, Kulaeva OI, Efremenko AV, Solonin AS, Kirpichnikov MP, Studitsky VM, Feofanov AV, Preparation of mononucleosomal templates for analysis of transcription with RNA polymerase using spFRET, *Methods Mol Biol* 1288 (2015) 395–412. [PubMed: 25827893]
- [19]. Hsieh FK, Kulaeva OI, Studitsky VM, Experimental analysis of hFACT action during Pol II transcription in vitro, *Methods Mol Biol* 1276 (2015) 315–26. [PubMed: 25665573]
- [20]. Chang HW, Pandey M, Kulaeva OI, Patel SS, Studitsky VM, Overcoming a nucleosomal barrier to replication, *Science advances* 2(11) (2016) e1601865. [PubMed: 27847876]
- [21]. Dyer PN, Edayathumangalam RS, White CL, Bao Y, Chakravarthy S, Muthurajan UM, Luger K, Reconstitution of nucleosome core particles from recombinant histones and DNA, *Methods Enzymol* 375 (2004) 23–44. [PubMed: 14870657]
- [22]. Kireeva ML, Walter W, Tchernajenko V, Bondarenko V, Kashlev M, Studitsky VM, Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription, *Mol Cell* 9(3) (2002) 541–52. [PubMed: 11931762]
- [23]. Johnson KA, Simpson ZB, Blom T, FitSpace explorer: an algorithm to evaluate multidimensional parameter space in fitting kinetic data, *Anal Biochem* 387(1) (2009) 30–41. [PubMed: 19168024]
- [24]. Johnson KA, Simpson ZB, Blom T, Global kinetic explorer: a new computer program for dynamic simulation and fitting of kinetic data, *Anal Biochem* 387(1) (2009) 20–9. [PubMed: 19154726]
- [25]. Thastrom A, Lowary PT, Widlund HR, Cao H, Kubista M, Widom J, Sequence motifs and free energies of selected natural and non-natural nucleosome positioning DNA sequences, *J Mol Biol* 288(2) (1999) 213–29. [PubMed: 10329138]
- [26]. Gaykalova DA, Kulaeva OI, Bondarenko VA, Studitsky VM, Preparation and analysis of uniquely positioned mononucleosomes, *Methods Mol Biol* 523 (2009) 109–23. [PubMed: 19381918]
- [27]. Kulaeva OI, Hsieh FK, Studitsky VM, RNA polymerase complexes cooperate to relieve the nucleosomal barrier and evict histones, *Proc Natl Acad Sci U S A* 107(25) (2010) 11325–30. [PubMed: 20534568]
- [28]. Luger K, Rechsteiner TJ, Richmond TJ, Preparation of nucleosome core particle from recombinant histones, *Methods Enzymol* 304 (1999) 3–19. [PubMed: 10372352]
- [29]. Kireeva ML, Hancock B, Cremona GH, Walter W, Studitsky VM, Kashlev M, Nature of the nucleosomal barrier to RNA polymerase II, *Mol Cell* 18(1) (2005) 97–108. [PubMed: 15808512]

Highlights

- Multiple intermediates are formed during chromatin transcription
- Chromatin transcription intermediates cause pausing of RNA polymerase II (Pol II)
- Pol II pausing in chromatin can be dissected using time-resolved approaches
- Computational approaches provide the rate constants for chromatin transcription
- Action of elongation factors can be studied using the time-resolved approaches

Fig. 1. The experimental approach: analysis of kinetics of transcription through a single positioned nucleosome.

First, RNA is pulse-labeled with P^{32} - α -GTP after limited Pol II transcription *in vitro* as described in [2, 6, 15]; Pol II approaches the nucleosome. Then transcription is conducted for different time intervals. The samples are then analyzed using denaturing gel and the bands in the gel are quantified. The data obtained in several (at least three) independent experiments are fitted to the kinetic model using KinTek software; as a result, the rates of multiple steps that occur during transcription through the nucleosome are determined. The data can be further interpreted in terms of the intermediates that are formed during stepwise Pol II transcription through the nucleosome.

Fig. 2. Analysis of 603 DNA and nucleosome [15].

603 DNA and nucleosome were analyzed by native PAGE. The nucleosome preparations assembled using the protocol described in section 3.2 usually contains minor amounts of dinucleosomes and histone-free DNA (< 10%). M: pBR322 DNA-*MspI* digest.

Fig. 3. Time course of *in vitro* Pol II transcription through a nucleosome in the absence/presence of human FACT

([15], with permission). The *in vitro* Pol II transcription through the 603 nucleosome in the absence or presence of hFACT was conducted at 150 mM KCl for indicated time intervals (0, 5, 10, 15, 20, 25, 30, 60 seconds). After reactions, the pulse-labeled RNA was purified and analyzed by denaturing PAGE. The image was then obtained using phosphorimager and analyzed using OptiQuant software. The position of the nucleosome on the DNA template is indicated. The bands corresponding to nucleosome-specific pausing sites are quantified. The quantified bands are then grouped into 10 clusters of the pausing intermediates (A to J) and the run-off (K). M: pBR322 DNA-*MspI* digest.

Fig. 4. The elongation models for analysis of the quantified data by KinTek Explorer software ([15], with permission). Based on the known mechanism of Pol II transcription through a nucleosome [2, 29], three plausible models of Pol II transcript elongation were considered. MODEL 1 (“minimal”): Pol II can only progress from each position on the nucleosomal DNA to the next position. MODEL 2: Pol II can progress from each position on the nucleosomal DNA to the next position or irreversibly partition into non-productive complexes. MODEL 3: Pol II can progress from each position on the nucleosomal DNA to the next position or switch between productive and non-productive complexes reversibly. Based on the comparative analysis of three models, MODEL 3 provides the best fit of the quantified data using the KinTek Explorer software.

Fig. 5. Data fitting using the KinTek Explorer software ([15], with permission). The quantified experimental data (Fig. 3) were fitted using the MODEL 3 (Fig. 4) and the KinTek Explorer software. The quality of fitting can be evaluated by the estimating the sigma (standard deviation) or p value (<0.01) or by the visual examination of the alignments in the model fitting charts.

Fig. 6. Analysis of kinetics of Pol II transcription through the nucleosome in the presence/absence of human FACT using KinTek Explorer software ([15], with permission). The rate constants of each step of transcription through the nucleosome were determined using the MODEL 3 (Fig. 4) and the KinTek Explorer software. Averages from three independent experiments are shown. The rate constants that are positively affected by FACT more than 7-fold are shown in green. Expected complexes formed at each region [2, 4, 6] are shown. The mechanism of FACT action during the Pol II transcription through a mono-nucleosome was proposed. During Pol II transcription through chromatin, FACT facilitates the rates of transitions from non-productive Pol II elongating intermediates to productive intermediates, thus accelerating the rate of Pol II transcription through chromatin. Blue asterisk indicates DNA-specific paused complex C.