Chapter 2

Preparation and Analysis of Positioned Mononucleosomes

Olga I. Kulaeva and Vasily M. Studitsky

Abstract

Short DNA fragments containing single nucleosomes have been extensively employed as simple model experimental systems for analysis of many intranuclear processes, including binding of proteins to nucleosomes, covalent histone modifications, transcription, DNA repair, and ATP-dependent chromatin remodeling. Here we describe several recently developed procedures for obtaining and analysis of mononucleosomes assembled on 200–350-bp DNA fragments.

**Key words** Nucleosomes, Chromatin, Assembly, Methods

1 Introduction

Various processes in eukaryotic nuclei (such as DNA replication, repair, recombination, and transcription) occur on DNA organized in chromatin. The minimal structural unit of chromatin is the nucleosome core: 147-bp DNA organized in 1 3/4 superhelical coils on the surface of the histone octamer [1]. Short (150–350 bp) DNA fragments containing single-nucleosome cores have been extensively employed recently for analysis of many intranuclear processes, including binding of regulatory proteins to nucleosomes [2], histone modifications [3, 4], transcription of nucleosomal templates [5–8], DNA repair in chromatin [9, 10], ATP-dependent chromatin remodeling [11, 12], and analysis of histone chaperones [13] and nucleosome structure and thermodynamics [14–17]. In many cases such simple model templates faithfully recapitulate important aspects of these processes [2, 5, 6, 9, 12, 13]. At the same time, these experimental systems have the following advantages as compared with more complex polynucleosomal templates: (a) Structure of single positioned nucleosomes and changes in the nucleosome structure during various processes of DNA metabolism can be analyzed with a high resolution. (b) Electrophoretic mobility of mononucleosomes formed on a short DNA fragment (190–350 bp) in a native gel strongly depends on nucleosome

position relative to DNA ends [18, 19] and on its histone composition [20, 21]. Therefore positioning and histone composition of mononucleosomes that are often changed during various processes can be easily monitored by analysis in a native gel. (c) Since the initial sample often contains only one positioned nucleosome, its fate during various processes can be determined with certainty. (d) The mobility of mononucleosomes in a native gel is typically changed upon binding of various protein complexes to the templates. Furthermore, conformationally different complexes having the same protein/DNA stoichiometry often have different mobilities in the gel [21]. Thus analysis of protein binding to nucleosomes and protein-induced changes in the conformation of the complexes are relatively straightforward using mononucleosomal templates.

The fate of histones during progression of various processive enzymes (DNA and RNA polymerases) has been extensively studied using various mononucleosomal templates. In some cases nucleosomes remain associated with DNA during progression of the enzymes. Thus on moderately Pol II-transcribed genes, extensive transcription-dependent exchange of H2A/H2B, but not H3/H4, histones was detected [22–27], suggesting that histones H3/H4 never leave DNA during transcription. The underlying Pol II-type mechanism of transcription through chromatin has been recapitulated in vitro and is conserved from yeast to human [28]. It is characterized by displacement of a single H2A/H2B dimer [29, 30], matching the apparent effect of Pol II passage in vivo [24]. Remarkably, the subnucleosome (DNA-bound histone hexamer (hexasome) formed upon release of H2A/H2B dimer from the octamer) survives Pol II passage and remains at the original position on DNA [29].

Survival of the subnucleosomes at the original position on DNA suggests an interesting possibility that at least histones H3/H4 never leave the template during Pol II transcription. This possibility is consistent with the lack of exchange of H3/H4 histones in vivo [22–27] and with the DNA looping mechanism of Pol II transcription through chromatin observed in vitro [31–33]. Indeed, histone survival during Pol II transcription in vitro is accompanied by formation of a small transient DNA loop (Ø-loop) on the surface of histone octamer including molecule of transcribing Pol II [33]. During formation of the Ø-loop, the recovery of DNA-histone interactions behind Pol II is tightly coupled with their disruption ahead of the enzyme. This coupling is a distinct feature of the Pol II-type mechanism that allows recovery of H3/H4 histones bound at the original position on DNA during transcription [32, 33].

To evaluate whether histones H3/H4 never leave the template during Pol II transcription, the following approach has been utilized (Fig. 1) [34]. If the histone octamer was transiently and fully displaced from DNA, the octamer would rebind to DNA released
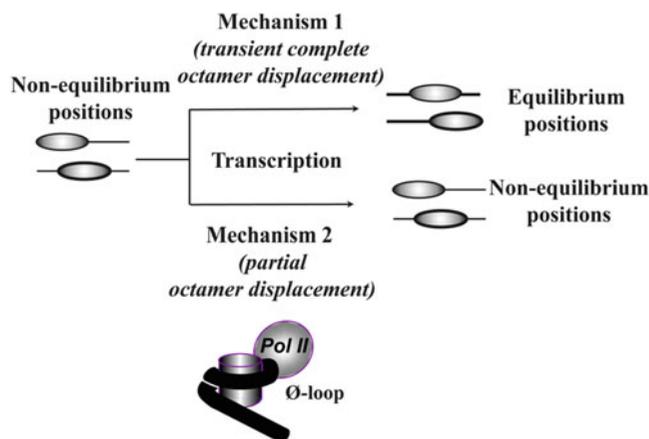
Fig. 1 Two possible mechanisms of nucleosome survival during transcription by Pol II. After Pol II transcription (or progression of other processive enzymes) through chromatin, histones often remain bound to the DNA. Such nucleosome survival could be accompanied either by complete transient displacement (mechanism 1) or only partial displacement of histone octamer from DNA (mechanism 2). According to the mechanism 1 octamer rebinds to DNA released behind the progressing enzyme; in the other case progression of the enzyme is accompanied by formation of an intranucleosomal loop (a small Ø-loop formed during Pol II transcription is shown). As a result, after the enzyme progresses through chromatin, nucleosome positions are either re-equilibrated (mechanism 1), or the original, nonequilibrium nucleosome positions are preserved (mechanism 2)

behind the progressing Pol II and nucleosome positions would be re-equilibrated during transcription (mechanism 1, Fig. 1) [34]. If the original nucleosome positions are nonequilibrium, transcription would result in a change in nucleosome positions. Alternatively, if H3/H4 never leave DNA during transcription, nucleosomes would remain at their original positions after transcription, even if these are not the preferential (equilibrated) positions on DNA before transcription (mechanism 2, Fig. 1). Since histones remain associated with DNA during progression of various processive enzymes including ATP-dependent chromatin remodelers and DNA polymerases (*see* ref. [35] for review), these approaches are likely to be useful beyond the chromatin transcription field.

Below we describe procedures for obtaining and analysis of mononucleosomes in both equilibrated and nonequilibrium positions on 200–350-bp DNA fragments.

2 Materials

2.1 Materials

1. Dialysis membranes (Spectra/Por; molecular weight cutoff of 8,000 and 12,000–14,000).

2. G-25 Quick spin columns (Boehringer Mannheim).

3. Siliconized Eppendorf tubes (PGC Scientific).

4. 3MM chromatography paper (Whatman).

5. QIAquick Gel Extraction kit (Qiagen).

6. Chicken blood (Truslow Farms).

7. CM C-25 Sephadex.

8. Centiprep-30.

2.2 Enzymes

1. T4 polynucleotide kinase (New England Biolabs, NEB).

2. Restriction enzymes (NEB).

3. Klenow fragment of *E. coli* DNA polymerase I (NEB).

4. Taq DNA polymerase (NEB).

5. T4 DNA ligase (NEB).

2.3 Reagents

1. Ethidium bromide (10 mg/ml stock).

2. Butyl alcohol (J.T. Baker).

3. Ethanol (Pharmco).

4. Equilibrated phenol (Sigma).

5. Chloroform (J.T. Baker).

6. β-Mercaptoethanol (Sigma).

7. Protease- and nuclease-free BSA (Sigma).

8. Glycerol (J.T. Baker).

9. NP-40 (Calbiochem).

10. Sodium dodecyl sulfate (GibcoBRL).

11. γ^{32}P-ATP (3,000 or 6,000 Ci/mmol; Perkin Elmer).

12. Acrylamide; *N,N'*-methylene-*bis*-acrylamide (Bis) (Bio-Rad).

13. Glycogen, 10 mg/ml solution (Boehringer Mannheim).

14. Bromophenol Blue dye (Bio-Rad).

15. Xylene Cyanol (Aldrich Chem. Co).

16. Single Stranded Salmon Testes DNA (Sigma).

*2.4 Buffers
and Solutions*

1. PBS buffer: Phosphate-buffered saline (Gibco BRL).

2. Buffer A: 15 mM Tris–HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercaptoethanol, 0.34 M sucrose, 0.1 mM PMSF.

3. Buffer B: 10 mM Tris–HCl (pH 7.5), 350 mM NaCl, 0.5 mM EDTA, 0.1 mM PMSF.

4. NLB (nuclei lysis buffer): 0.25 mM EDTA, 0.1 mM PMSF.

5. TAE buffer: 0.04 M Tris-acetate, pH 8.0, 1 mM EDTA.

6. HE buffer: 10 mM Na-HEPES, pH 8.0, 1 mM EDTA.

7. CRB 1–4 (core reconstitution buffers): All four buffers contain HE, 5 mM mM β-mercaptoethanol, 0.1 % NP-40, and NaCl at

the following concentrations: Buffer 1—1 M; 2—0.8 M; 3—0.6 M; 4—0.01 M.

8. SP6 buffer: 45 mM Na-HEPES, pH 8.0, 6 mM MgCl$_2$, 2 mM spermidine and 2 mg/ml BSA, 10 mM β-mercaptoethanol.

9. 1× TB (transcription buffer): 20 mM Tris–HCl (pH 8.0), 5 mM MgCl$_2$, 2 mM β-mercaptoethanol, and indicated concentration of KCl, mM.

10. 1× RLB (RNA loading buffer): 95 % Formamide, 10 mM EDTA, 0.1 % SDS, and 0.01 % of each brome phenol and xylene cyanol dyes.

11. 4× Chromatin loading buffer: 100 mM Tris–HCl (pH 7.5), 40 mM EDTA, 40 % sucrose, and 1 mg/ml of salmon testes DNA.

3 Methods

3.1 Protein Purification

We purified hexahistidine-tagged yeast Pol II using published protocols [7] and H2A/H2B and H3/H4 histone pairs were isolated from adult chicken blood by chromatography on hydroxyapatite [36, 37].

3.2 Preparation of Donor Chromatin from Chicken Erythrocytes

The protocol described below is a modified version of the method published earlier [6, 39] (*see* **Note 1**).

3.2.1 Red Cell Isolation

1. Collect red cells from 200 ml chicken blood by centrifugation at $1,800 \times g$ for 10 min at 4 °C.

2. Carefully remove white cells forming from the top of the pellet.

3. Resuspend cells in 48 ml PBS buffer.

4. Collect red cells by centrifugation at $3,000 \times g$ for 5 min at 4 °C.

5. Repeat **steps 3** and **4** two more times.

3.2.2 Nuclei Isolation

1. Conduct all manipulations at 4 °C with precooled buffers, unless indicated otherwise.

2. Resuspend cells in PBS buffer (half of the volume of the pellet).

3. Add 10 volumes of buffer A supplemented with 0.5 % NP-40 and mix by inversion.

4. Collect nuclei by centrifugation at $12,000 \times g$ for 10 min at 4 °C.

5. Resuspend nuclei in 200 ml of buffer A (no NP-40).

6. Collect nuclei by centrifugation at $12,000 \times g$ for 10 min at 4 °C.

7. Repeat **steps 3** and **4** several times until red color disappears.

8. Resuspend purified nuclei in a small volume (50–100 ml) of buffer A.

9. Resuspend ~1 µl of nuclei in 0.9 ml of HE buffer, add 0.1 ml of 10 % SDS, and measure A_{260}. Concentration of nuclei should be ~200–400 A_{260}/ml.

3.2.3 Chromatin Preparation

1. Adjust nuclei concentration to 100 A_{260}/ml.

2. For analytical digestion with micrococcal nuclease (MNase) warm 1 ml of nuclei to 37 °C.

3. Add $MgCl_2$ and $CaCl_2$ to 1 mM final concentration.

4. Add 1 µl of MNase to 10 U/ml final concentration and incubate at 37 °C.

5. Remove 0.1-ml aliquots after 1, 2, 4, 8, 15, 30, and 60 min and stop the digestion by adding EDTA to 10 mM and SDS to 1 % final concentrations.

6. Extract the samples once with one volume of 1:1 (v/v) phenol:chloroform.

7. Precipitate DNA with 3 volumes of ethanol, wash with 70 % (v/v) ethanol, dry, and dissolve in HE buffer.

8. Analyze DNA in 1 % agarose gel and identify the digestion point where the sizes of DNA fragments are 3–20 kb (Fig. 2, 15-min digestion point was selected).

9. For preparative digestion with MNase warm the nuclei in a 500 ml conical glass flask to 37 °C.
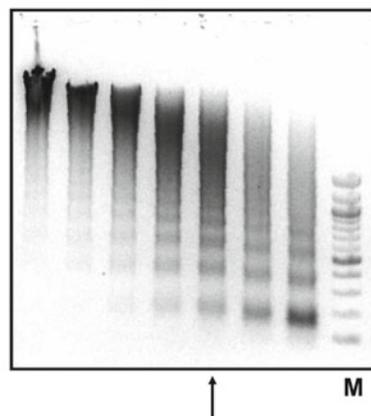
Fig. 2 Time course of digestion of chicken erythrocyte nuclei with micrococcal nuclease (MNase). Nuclei were digested for various time intervals (1, 2, 4, 8, 15, 30, or 60 min), DNA purified, and analyzed in 1 % agarose gel (ethidium bromide stain). 15-min digestion point was selected for preparative digestion of the nuclei (indicated by *arrow*). M—100-bp DNA ladder (New England Biolabs)

10. Add $MgCl_2$ and $CaCl_2$ to 1 mM final concentration, and MNase to 10 U/ml final concentration, and incubate at 37 °C for the required time.

11. Stop the digestion by adding EDTA to 10 mM final concentration.

12. Collect nuclei by centrifugation at $12,000 \times g$ for 10 min at 4 °C.

13. Estimate the volume of the pellet and resuspend the nuclei in equal volume of NLB buffer (nuclei should become semitransparent).

14. Collect nuclear debris by centrifugation at $12,000 \times g$ for 10 min at 4 °C.

15. Remove the supernatant containing soluble chromatin (it should be slightly opaque and gray). Discard the pellet.

16. Resuspend an aliquot of chromatin in 0.9 ml of HE buffer, add 0.1 ml of 10 % SDS, and measure A_{260}. Concentration of chromatin should be ~50–80 A_{260}/ml.

3.2.4 Removal of Linker Histones H1/H5 from Chromatin

1. Adjust concentration of chromatin to 50–100 A_{260}/ml.

2. Presoak CM C-25 Sephadex (Pharmacia, 36 mg of resin per mg of chromatin) in buffer B for 1 h.

3. Slowly add 2 M NaCl to chromatin to 0.35 M final concentration (*see* **Note 2**).

4. Add 1/3 of presoaked CM C-25 Sephadex to chromatin and slowly stir for 2 h at 4 °C.

5. Collect the resin by centrifugation at $12,000 \times g$ for 10 min at 4 °C. Remove supernatant containing soluble chromatin.

6. Repeat **steps 4** and **5** two more times.

7. Analyze protein composition of –H1/5 chromatin by SDS-PAGE (Fig. 3). Dissolve ~10 μg of histones in 10–60 μl of Laemmli loading buffer. Electrophorese histones in an 18 % (acrylamide:bis = (30:0.15)) Laemmli gel ($17 \times 17 \times 0.15$ cm) for 5–6 h at 32 mA. Stain the gel with Coomassie Blue.

8. Concentrate –H1 chromatin to ~100 A_{260}/ml (5 mg/ml) on Centriprep-30 by centrifugation at $1,300 \times g$ for required time at 4 °C.

9. Dialyze –H1/5 chromatin overnight against buffer B and store at –70 °C (*see* **Note 3**).

3.3 Preparation of DNA Fragment for Reconstitution

The protocol described below is a modified version of the method published earlier [7].

1. The 204-bp DNA fragment was obtained by PCR amplification using pVT1 5S-containing plasmid as was described previously [38].
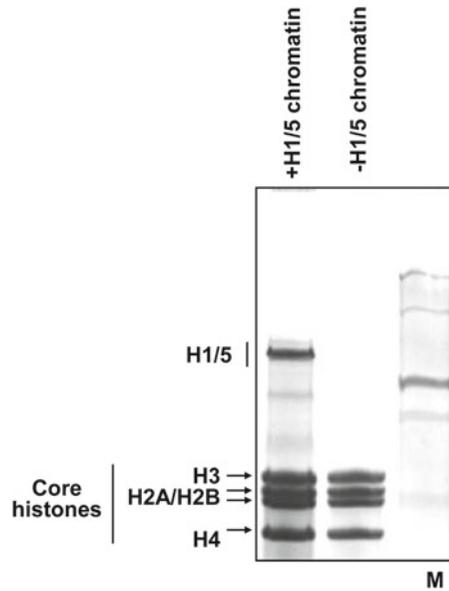
Fig. 3 Analysis of protein composition of histone H1/5-containing and H1/5-depleted chicken erythrocyte chromatin. Histones were resolved by 18 % Laemmli SDS-PAGE. Positions of histones on the gel are indicated. M—10–250 kDa protein markers (BioRad)

2. 5′-End-label the PCR primers with γ [^{32}P] ATP (6,000 Ci/mmol) using T4 polynucleotide kinase according to the manufacturer's protocol.

3. PCR-amplify DNA fragments in 500 μl volume (5 × 100 ml reactions) using Taq DNA polymerase [7].

4. Resolve the obtained DNA fragments in a 1.5 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and TAE buffer at 4–6 V/cm for 1.5–3 h, depending on the resolution required for clear band separation.

5. Using a long-wavelength UV lamp (to reduce nicking of DNA), identify and excise the required band(s).

6. Purify the fragment using the QIAquick Gel Extraction kit.

7. Extract the samples once with one volume of 1:1 (v/v) phenol:chloroform.

8. Precipitate DNA with 3 volumes of ethanol, wash with 70 % (v/v) ethanol, dry, and dissolve in 100 μl of HE buffer.

9. Determine DNA concentration by measuring the A$_{260}$ (using A$_{260}$ = 20 for 1 mg/ml DNA) and store at –20 °C.

10. 204-bp PCR fragment was digested with *Tsp*R1 restriction enzyme for 3 h at 65 °C.

11. Resolve the obtained DNA fragments in a 1.5 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide, 4 M urea, and TAE buffer at 4–6 V/cm for 1.5–3 h.

12. Using a long-wavelength UV lamp (to reduce nicking of DNA), identify and excise the required band(s).

13. Purify the fragment using QIAquick Gel Extraction kit (Qiagen).

14. Extract the sample once with one volume of 1:1 (v/v) phenol:chloroform.

15. Precipitate DNA with 3 volumes of ethanol, wash with 70 % (v/v) ethanol, dry, and dissolve in 100 µl of HE buffer.

16. Use the 204-bp fragment for reconstitution or ligation with 50-bp promoter fragment for 3 h at 15 °C using T4 DNA ligase to obtain the 254-bp DNA fragment (Fig. 4) according to the manufacturer's protocol.

17. The ligated products were gel-purified as a template for reconstitution with donor –H1 chromatin.

3.4 Reconstitution of Mononucleosome Cores Using Donor – H1 Chromatin Before or After Ligation

Reconstitution was performed using donor –H1 chromatin or purified histones (*see* ref. [6] for detail). Nucleosome assembly was performed on the 204-bp DNA fragment and then ligated to the 50-bp promoter-containing fragment, or on the 254-bp DNA fragment that already contains the promoter (Fig. 4 [34]).
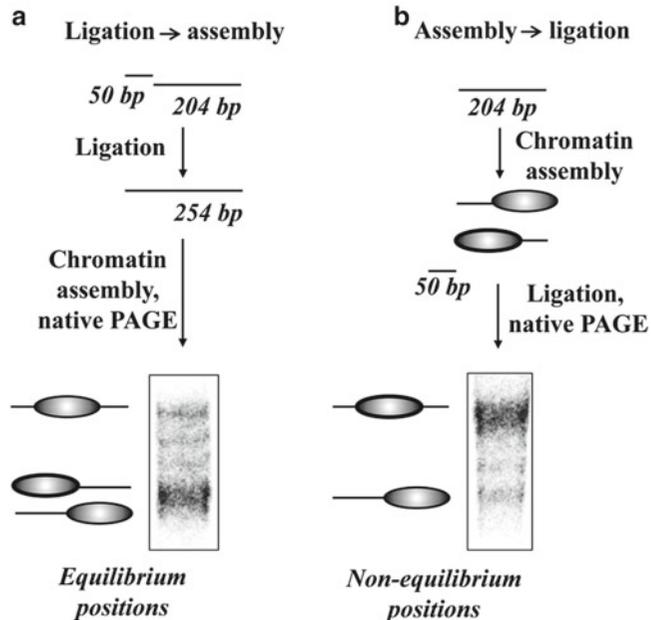
Fig. 4 Experimental approach for obtaining equilibrated and nonequilibrium sets of mononucleosomes. Nucleosome assembly on the 204-bp DNA fragment was conducted either after (**a**) or before (**b**) ligation to the promoter-containing 50-bp fragment. Positions of nucleosomes are equilibrated during but not after assembly. Therefore in both cases nucleosome positions are equilibrated during the process of assembly, but in (**b**) ligation to the promoter-containing DNA fragment to the assembled nucleosomes produces templates containing nucleosomes occupying nonequilibrium positions before transcription by Pol II

1. Cool 500 ml each of CRB1 to CRB4 buffers to 4 °C.

2. Mix one to three micrograms of the DNA fragment with long –H1/H5 donor chromatin at a ratio of 1:60 (w:w) in 0.04–0.1 ml of CRB1 buffer (*see* **Note 4**).

3. Dialyze successively against CRB2 and CRB3, each for 2 h at 4 °C. Then dialyze the sample against CRB4 for 3 h or overnight.

4. Transfer the reconstitute to a siliconized Eppendorf tube and store at 4 °C (do not freeze).

5. Ligate nucleosomes assembled on 204-bp fragment with 50-bp promoter fragment [17] for 3 h at 15 °C using T4 DNA ligase according to the manufacturer's protocol.

6. Check the samples by analysis by native PAGE (*see* below).

3.5 Mapping the Positions of Nucleosome Cores by Native PAGE

1. Preparation of the sample to load to native gel: the concentration of the reconstituted nucleosomes is determined by the specific activity of the DNA.

2. Loading buffer is added providing a final concentration of 10 % sucrose and 50 lg/ml sheared herring testes DNA (Intergen, Purchase, NY).

3. The templates are resolved by native gel electrophoresis (4.5 % acrylamide (39:1), 5 % glycerol, 20 mM Na–HEPES, pH 8, and 0.1 mM EDTA) at 100 V for 4 h at 4 °C as described [38].

4. Quantitation is performed using a Cyclone Phosphor System (Packard, Meriden, CT).

Nucleosome mobility in the gel is dictated by nucleosome positioning on the 254-bp DNA fragment. Nucleosomes occupy different positions when assembly was conducted before or after DNA ligation (Fig. 4), suggesting that nucleosome occupied non-equilibrium positions when the assembly was conducted before ligation [34]. Since these nonequilibrium nucleosome positions are preserved after Pol II transcription [29], the data suggest that histones never leave DNA during this process.

4   Notes

1. Donor chromatin isolated from chicken erythrocytes contains mostly unmodified core histones [39]. The main advantage of using this source of histones is the minimal presence of proteases in chicken erythrocytes.

2. Chromatin becomes insoluble at 150 mM NaCl and then becomes soluble again when the concentration of NaCl reaches 350 mM.

3. Donor –H1 chromatin could be further purified by chromatography on Sephacryl S-400HR (Pharmacia) [40].

4. The ratio of DNA to donor chromatin may need to be adjusted because different chromatin preparations produce slightly different results.

Acknowledgements

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