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Experimental Analysis of hFACT Action during Pol II Transcription *in vitro*

Fu-Kai Hsieh^{1,2}, Olga I. Kulaeva³, and Vasily M. Studitsky^{1,3,4}

¹Department of Pharmacology, Rutgers University-Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA

³Institute of Gene Biology, Russian Academy of Sciences, Moscow 119334, Russia

⁴Cancer Epigenetics Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

Summary

FACT (facilitates chromatin transcription) is a histone chaperone that facilitates transcription through chromatin and promotes histone recovery during transcription. Here, we describe a highly purified experimental system that recapitulates many important properties of transcribed chromatin and the key aspects of hFACT action during this process *in vitro*. We present the protocols describing how to prepare different forms of nucleosomes, including intact nucleosome, covalently conjugated nucleosome, nucleosome missing one of the two H2A/2B dimers (hexasome) and tetrasome (a nucleosome missing both H2A/2B dimers). These complexes allow analysis of various aspects of FACT's function. These approaches and other methods described below can also be applied to the study of other chromatin remodelers and chromatin-targeted factors.

Keywords

transcription; histone chaperone; histone cross-linking; chromatin assembly

1. Introduction

Nucleosomes form strong barriers to transcribing RNA polymerase II (Pol II) in eukaryotic nuclei (1). In order to efficiently transcribe genes, these nucleosome barriers need to be properly relieved. This task is mainly conducted by various chromatin remodelers which could slide, evict, transfer or exchange core histones during transcription to allow Pol II progression along the DNA template (2).

Histone chaperones represent one type of chromatin remodelers which can alter the nucleosome structure without ATP hydrolysis (3). Various histone chaperones have high binding affinity to H2A/H2B dimer, H3/H4 tetramer and/or nucleosome. Therefore, it has been suggested that histone chaperones relieve the nucleosome barriers during transcription

Corresponding author: Vasily.Studitsky@fccc.edu.

²Current address: Department of Molecular Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

by transiently disassembling/reassembling the nucleosome through their chaperone activity (4). To evaluate this hypothesis, two major questions have to be addressed: (i) whether the presence of dimer and/or tetramer in a nucleosome is critical for chaperone's activity, and (ii) whether the action of histone chaperone is indeed dependent on the disassembly/reassembly of the nucleosome. Here we describe a set of approaches to address these questions using hFACT as an exemplary histone chaperone.

FACT has histone chaperone activity, with a higher binding affinity to H2A/H2B dimer (5, 6). To study FACT's action in facilitating Pol II transcription, we have developed a methodology to systematically investigate this process (7). It includes preparation of different forms of well-positioned nucleosomes and subnucleosomes, including intact nucleosome, nucleosomes missing the promoter-distal (-D) or -proximal (-P) H2A/H2B dimer and tetrasome (nucleosome missing both H2A/H2B dimers) assembled on the same nucleosome positioning sequence (NPS) 603 (Fig. 1). These complexes allow analysis of the effect of each histone dimer and tetramer in FACT's action during transcription through chromatin. Our data indicate that the histone dimers play a major role in this process: removal of either distal or proximal dimer results in less pronounced stimulation of transcription by FACT, as compared with the intact nucleosome (7).

The second approach described here involves analysis of the role of H2A/H2B dimer dissociation during FACT-dependent Pol II transcription through chromatin. The technique we employed to tackle this problem is covalent cross-linking of the histone octamer by dimethyl suberimidate (DMS) to prevent the dissociation of dimer(s) from nucleosome. DMS does not introduce any charges to cross-linked protein and is unlikely to cause changes in the nucleosome structure. Two different ways to preparing cross-linked nucleosome were used: (i) reconstitution of the nucleosome and subsequent DMS treatment or (ii) DMS cross-linking of purified histone octamer with subsequent nucleosome assembly. The data obtained by both protocols were consistent and demonstrated that the treatment with DMS does not affect the wrapping of DNA on histone octamer, minimally affect the ability to be transcribed by Pol II, and does not interfere with FACT function during transcription. This result suggests that H2A/H2B dimer dissociation observed during transcription is dispensable for FACT action.

The experimental system for analysis of the mechanism of FACT action described below consists of highly purified core yeast Pol II (yPol II) and precisely positioned mononucleosomes assembled using purified DNA and *X. laevis* recombinant histones. This "minimal" experimental system faithfully recapitulates many important properties of chromatin transcribed *in vivo* (8–13), including effects of histone and Pol II mutations on transcription through chromatin (10, 14), and action of various elongation factors (including FACT) (6, 12, 15–17), ATP-dependent chromatin remodelers (18) and multiple complexes of Pol II (11) during this process.

In summary, the protocols described here present some useful strategies for preparation of various nucleosomes/subnucleosomes for analysis of chromatin structure/dynamics during Pol II transcription and other chromatin remodeling processes *in vitro*.

2. Materials

2.1. DNA template preparation

1. Plasmid pGEM-3Z-603, a single copy of 603 sequence introduced into pGEM-3Z vector.
2. Oligonucleotides:
Uplong1:
5'TCTCAAATTTTATGGCACTGGGCGAGACATACACGAA
TATGGCGTTTTCTAGTACAAATCACCC-3';
603F tsp dim: 5'-AAGCGACAGCTTTGGCACT
GGGTCACTCGGGCTTCTAAGTACG-3';
603R: 5'-ACCCCAGGGACTTGAAGTAATAAG-3'
and 603 hexR: 5'-CTTTCAACATCGATGCACG GTG -3'.
3. γ -[³²P] ATP (6000 Ci/mmol).
4. T4 polynucleotide kinase.
5. *TspR1* enzyme (New England Biolabs, NEB).
6. Phenol and Chloroform (Sigma-Aldrich).
7. Gel Extraction kit (QIAquick, Qiagen, or comparable).
8. TB buffer: 20 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2 mM β -mercaptoethanol, and various concentrations of KCl. For instance, TB40 contains 40 mM KCl.
9. TE buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

2.2. Nucleosome reconstitution

1. Core Reconstitution Buffers (CRB) 1–6: all six 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.
2. Purified histones and histone octamer (*see reference (19) for detail*).
3. Salmon sperm dsDNA.

2.3. Histone cross-linking

1. Dimethyl suberimidate dihydrochloride, DMS.
2. Cross-linking Buffer: 0.1 M sodium borate (pH 10), 0.1 M NaCl, 0.2 mM EDTA.
3. Stop Buffer: 25 mM Tris-HCl (pH 6.9), 1 mM EDTA.

4. Storage Buffer: 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.1% NP40, 0.2 mM EDTA, 5 mM β -mercaptoethanol.
5. Neutralization Buffer: 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM β -mercaptoethanol and 50 mM NaCl.

2.4. Transcription assays

1. Low adhesion microcentrifuge tube.
2. ssDNA oligonucleotide 50 T: 5'-GGTGTGCTGCTTGGGTTGGCTTTTTCGGGCTGTCCCTCTCGATGGCTGTAAGT-3'.
3. 9-nt RNA primer: 5'-AUCGAGAGG-3'.
4. Non-template DNA strand 59NT: 5'-ACTTACAGCCATCGAGAGGGACACGGCGAAAAGCCAACCCAAGCGACACCGGCACTGGG-3'.
5. Purified yeast Polymerase II (*see* reference (20) for detail).
6. Purified FACT (*see* reference (6) for detail).
7. Ni²⁺-NTA agarose (Qiagen) or comparable His affinity resin.
8. Acetylated BSA.
9. Imidazole.
10. T4 DNA ligase.
11. Polyethylene glycol (PEG)-8000.
12. α -[³²P] GTP (6000 Ci/mmol).
13. Ultrapure NTPs.
14. Ethylenediaminetetraacetic acid (EDTA).
15. RNA loading buffer (RLB): 95% formamide, 10 mM EDTA, 0.1% SDS, and 0.01% bromophenol blue and xylene cyanol dyes.

2.5. Gel electrophoresis and quantification

1. 40% (w/v) acrylamide/bis-acrylamide solution (39:1).
2. 40% (w/v) acrylamide/bis-acrylamide solution (19:1).
3. 30% (w/v) acrylamide/bis-acrylamide solution (29:1).
4. Urea.
5. 10% SDS.
6. 1.5 M Tris-HCl (pH8.8) and 0.5 M Tris-HCl (pH6.8)
7. 10% (w/v) ammonium persulfate.

8. N,N,N',N'-Tetramethylethylenediamine, TEMED.
9. 5× TBE stock solution in 1 L of H₂O: 54 g of Tris base, 27.5 g of boric acid and caution 20 mL of 0.5 M EDTA (pH 8.0).

3. Methods

Removal of dimer(s) from an intact, well-positioned mononucleosome was done by truncating corresponding DNA sequence (*e.g.*, nucleosome positioning sequence NPS-603). The truncated DNA templates were generated by PCR amplification with specific primers (Fig. 1). Different truncated forms of DNA template were prepared and incubated with different amounts of various combinations of purified histones to obtain the nucleosomes, hexasomes or tetrasomes. Alternatively, cross-linking of all core histones in the nucleosome was induced by DMS; the cross-linking was conducted either before or after nucleosome reconstitution.

To obtain the nucleosomes for transcription by γ Pol II, DNA fragments containing NPS-603 and bearing a *TspRI* restriction site upstream of the NPS were PCR amplified. After amplification and digestion with *TspRI* enzyme, the fragment was purified, the nucleosome was reconstituted and ligated to the preassembled Pol II elongation complex (EC) through the *TspRI* sticky end. In order to evaluate the quality of nucleosomes, the promoter-distal end of nucleosomal DNA was weakly labeled with γ -[³²P]-ATP. This weak labeling minimally interferes with subsequent analysis of more intensely pause-labeled RNA.

3.1. Preparation of DNA templates for assembly of intact, distal (–D) or proximal (–P) dimer-truncated nucleosomes/subnucleosomes

1. Reverse primers 603 hexR or 603R were labeled with 0.1–0.3 μ Ci γ -[³²P] ATP using T4 polynucleotide kinase in the TB40 buffer for 40 min at 37°C with subsequent enzyme inactivation for 20 min at 65°C.
2. Intact and –D DNA templates were PCR-amplified from pGEM-3Z-603 plasmid with the universal unlabeled forward primer Uplong1 (containing *TspRI* site) and labeled reverse primers 603R and 603 hexR, respectively. –P DNA template was PCR amplified from pGEM-3Z-603 plasmid with unlabeled forward primer 603F *tsp dim* (containing *TspRI* site) and labeled reverse primer 603R.
3. The PCR product was extracted with one volume of 1:1 (v/v) phenol:chloroform, precipitated with ethanol, washed with 70% ethanol, resuspended in 1× Buffer 4 (NEB), and digested with *TspRI* at 65 °C for 4 h or overnight.
4. The digested 231-bp product of intact fragment, 195-bp product of –D fragment or 118-bp product of –P fragment were resolved in 2% agarose gel containing 4 M urea. The DNA fragments were excised on the trans-illuminator using long UV wavelength (*see Note 1*).

5. The DNA fragment was further purified using QIAquick Gel Extraction kit according to the manufacturer instruction and eluted with 30–50 µl of TE buffer.
6. DNA concentrations were determined by measuring the A260; purified DNA fragments were stored at 20°C.

3.2. Reconstitution of nucleosomes/subnucleosomes

1. 250 ml of each of CRB1-6 buffers was cooled to 4°C.
2. 0.5–3 µg of single end-labeled full length, –P or –D DNA fragments were dissolved in the CRB1 buffer for assembly with purified histones. Intact nucleosome can also be assembled by histone core octamer transfer (*see* Note 2 and Section 3.3.1).
3. In case of intact nucleosome assembly from purified histones, H3 and H4 histones were added in 2-fold molar excess to DNA, while H2A and H2B were added in 3-fold molar excess; in case of –D and –P hexasome assembly, H3 and H4 histones were added in 2-fold and H2A and H2B were added in 1.5-fold molar excess; in case of tetrasome (assembly with –D DNA template), H3 and H4 histones were added in 2-fold molar excess to DNA and no H2A and H2B were added.
4. For intact nucleosome, –D or –P hexasome preparations, salmon sperm dsDNA was added to achieve 3-fold weight excess over the amount of the DNA fragments; for tetrasome, salmon sperm dsDNA was added to achieve 6-fold weight excess (*see* Note 3).
5. The reaction volume in all cases was adjusted to 40–100 µl to achieve 10–100 ng/ml total concentration of DNA.
6. The samples were subsequently dialyzed against CRB1, CRB2, CRB3, and CRB4, each for 1 h, CRB5 for 2.5 h, and CRB6 overnight at 4°C.
7. The resulting nucleosomes were stored at 4°C.
8. The quality of nucleosome reconstitution was evaluated by analysis of the samples by 4.5% (39:1 acrylamide:bis) native PAGE in 0.5× TBE buffer for 3 h at 110 V.
9. The gel was transferred to Whatman 3MM paper, covered with a plastic wrap, vacuum dried, and exposed to a PhosphorImager screen for 2–3 h or overnight. Assembled nucleosomes migrate as distinct bands and have a

¹After digestion with *TspR1*, the fragments have 9-nt long sticky ends which could still attach to each other *via* base-pairing and may not completely separate in regular agarose gel. The presence of urea in the gel helps to prevent this non-covalent attachment and improves the separation of the digested fragments.

²Purified histone core octamer is intact at high salt condition (2 M NaCl) without the DNA. Therefore, like donor chromatin (22), purified histone core histone octamer can also be used for nucleosome reconstitution.

³The nucleosomes and hexasomes are typically obtained with 90–95% yield; however, the tetrasome yield is only ~60–70% due to competitive binding of multiple histone tetramers to the DNA fragment.

lower mobility in the gel as compared with histone-free DNA (Fig. 2 and *see* Note 4).

3.3. Preparation of cross-linked nucleosomes

(1) Cross-linking of nucleosomes

1. Intact nucleosomes were reconstituted as described above using purified histone core octamer in 4-fold molar excess relative to DNA (*see* Note 5).
2. Reconstituted nucleosome was dialyzed against the cross-linking buffer for 90 min at 4°C.
3. Freshly prepared DMS (in the cross-linking buffer) was added to nucleosomes at final concentration 0.5 mg/ml and incubate for 60 min at room temperature (RT) (*see* Note 6).
4. Sample was dialyzed against stop buffer for 90 min at RT to stop the cross-linking reaction.
5. Cross-linked nucleosomes were dialyzed against the storage buffer for 90 min at RT and then stored at 4°C.
6. Successful cross-linked nucleosome was analyzed by 8% SDS-PAGE with Coomassie blue staining. The cross-linked nucleosome appears as a slow-mobility complex on the top of gel (not shown).
7. The quality of nucleosomes was evaluated as described in Section 3.2.8-9 (Fig. 3A).

(2) Cross-linking of histone octamer before reconstitution

1. Incubate 10–15 µg of purified histone core octamer with freshly prepared DMS to final concentration of 0.5 mg/ml in the cross-linking buffer containing 2 M NaCl for 60 min at RT.
2. Add 0.25 M Tris-HCl (pH 6.9) to 50 mM final concentration to stop the cross-linking reaction.
3. Dialyze the sample against the neutralization buffer for 2 hours at RT.
4. Check cross-linked histone octamer by 8% SDS-PAGE, stain with Coomassie blue. The cross-linked histone octamer appears as a slow-mobility complex on the top of gel.
5. Nucleosome reconstitution was performed as described above (*see* Section 3.2) with cross-linked histone octamer in 5-fold molar excess relative to DNA.

⁴The formation of the dimer form of the nucleosome minimally affects the outcome of transcription as the dimers are not transcribed.

⁵The purified histone octamer was used because accidental histone-histone cross-linking was observed when purified histones were used.

⁶DMS is an imidoester cross-linker which reacts with primary amines to form amidine bonds. To ensure specificity for primary amines, imidoester reactions are best done in amine-free, alkaline conditions (pH 10), such as in the borate-containing buffer.

6. The quality of nucleosomes was evaluated as described in Section 3.2.8-9 (Fig. 3B).

3.4. Transcription in the presence of FACT

1. The low adhesion microcentrifuge tubes are used in all steps.
2. 200 pmol of template ssDNA oligonucleotide 50T was phosphorylated with 1 mM ATP using T4 polynucleotide kinase in TB40 buffer for 20 min at 37°C with subsequent enzyme inactivation for 20 min at 65°C (*see Note 7*).
3. RNA:DNA duplex was obtained by addition of 200 pmol of 9-nt RNA primer to 200 pmol of phosphorylated 50 T DNA oligonucleotide in 20 µl of TB40. Reaction was continued for 10 min at 45°C; then the temperature was decreased by 2°C every 2 min to the RT.
4. The RNA:DNA duplex was incubated with 3-fold molar excess of yeast core Pol II (purified as described in (21)) to form active EC for 10 min at RT.
5. Formation of the EC was completed by adding 3-fold molar excess of unlabeled non-template DNA strand 59NT for 10 min at RT.
6. 20 µl of Ni²⁺-NTA agarose was washed three times with 0.5 ml of TB40, incubated in the presence of 0.5 mg/ml of acetylated BSA for 10 min, and washed two times with 0.5 ml TB40. The volume was adjusted to 25 µl (*see Note 8*).
7. Pol II EC was incubated with 25 µl of Ni²⁺-NTA suspension for 15 min at RT.
8. Immobilized EC was washed once with TB40, twice with TB300, and twice with TB40. The volume was adjusted to 25 µl.
9. EC was eluted from Ni²⁺-NTA beads in the presence of 100 mM imidazole after incubating for 5 min at RT.
10. The eluted EC was ligated with 100–200 ng of the nucleosomal template in the presence of 100 µM ATP, 1% PEG-8000, and 50 units of T4 DNA ligase in a volume of 50 µl at 16°C for 1–2 h.
11. RNA transcript was pulse-labeled and EC-5 (with active center of Pol II located 5-bp upstream of promoter-proximal nucleosomal boundary) was formed in the presence of 1 µM α-[³²P] GTP and unlabeled 2 µM CTP and 10 µM ATP for 10 min at RT.
12. Complete formation of EC-5 was achieved by adding nonlabeled GTP to final concentration of 10 µM for 5min at RT.

⁷The efficient phosphorylation of 50T oligonucleotide is critical for the subsequent ligation step.

⁸The treatment of acetylated BSA with Ni²⁺-NTA minimizes non-specific binding of the beads to microcentrifuge tube.

13. Transcription was continued (EC-5 was chased) in the presence of 400 μM NTPs for 10 min at RT.
14. To evaluate the effect of FACT, 0.1 μM of FACT was added during the chase.
15. To characterize pulse-labeled RNA, the reaction was terminated by adding EDTA to final concentration of 20 mM. Pulse-labeled RNA was extracted with one volume of 1:1 (v/v) phenol:chloroform, precipitated with ethanol, washed with 70% ethanol, dried, and dissolved in 5 μl of RLB (RNA loading buffer).
16. Samples were boiled for 1 min and analyzed in 8% polyacrylamide gel (19:1 acrylamide:bis) containing 8M urea after electrophoresis in 0.5x TBE buffer for 1–1.5 h at 2000 V.
17. Gel was dried on Whatman 3MM paper and exposed to a PhosphorImager screen at RT overnight (Fig. 4).

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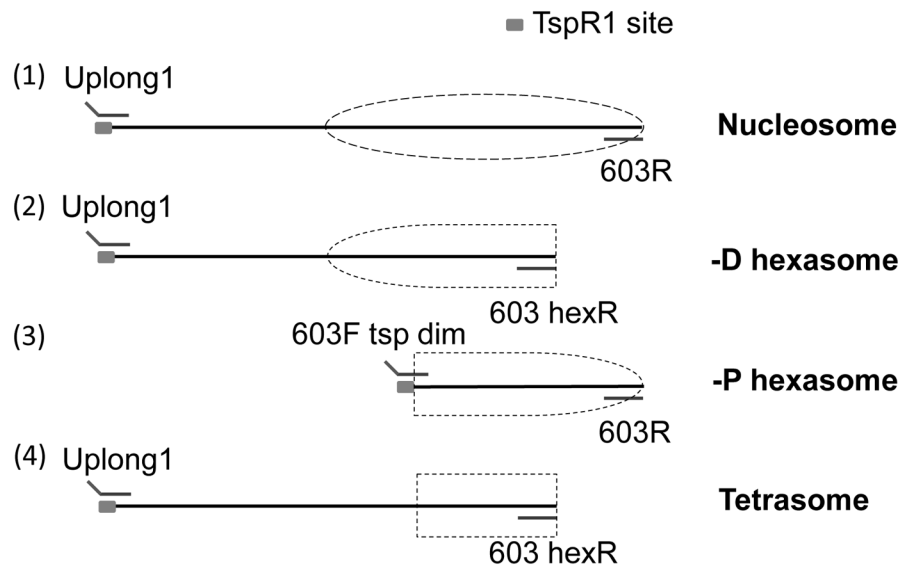


Fig. 1. Schematic diagrams of the templates for analysis of the mechanism of FACT action (1) intact nucleosome, (2) -D hexasome, (3) -P hexasome and (4) tetrasome. Primers used for PCR amplification of each template are shown. *TspR1* site is indicated as solid gray rectangle. Position of nucleosomes/subnucleosomes on DNA are indicated by dashed lines.

M DNA Hex Tet

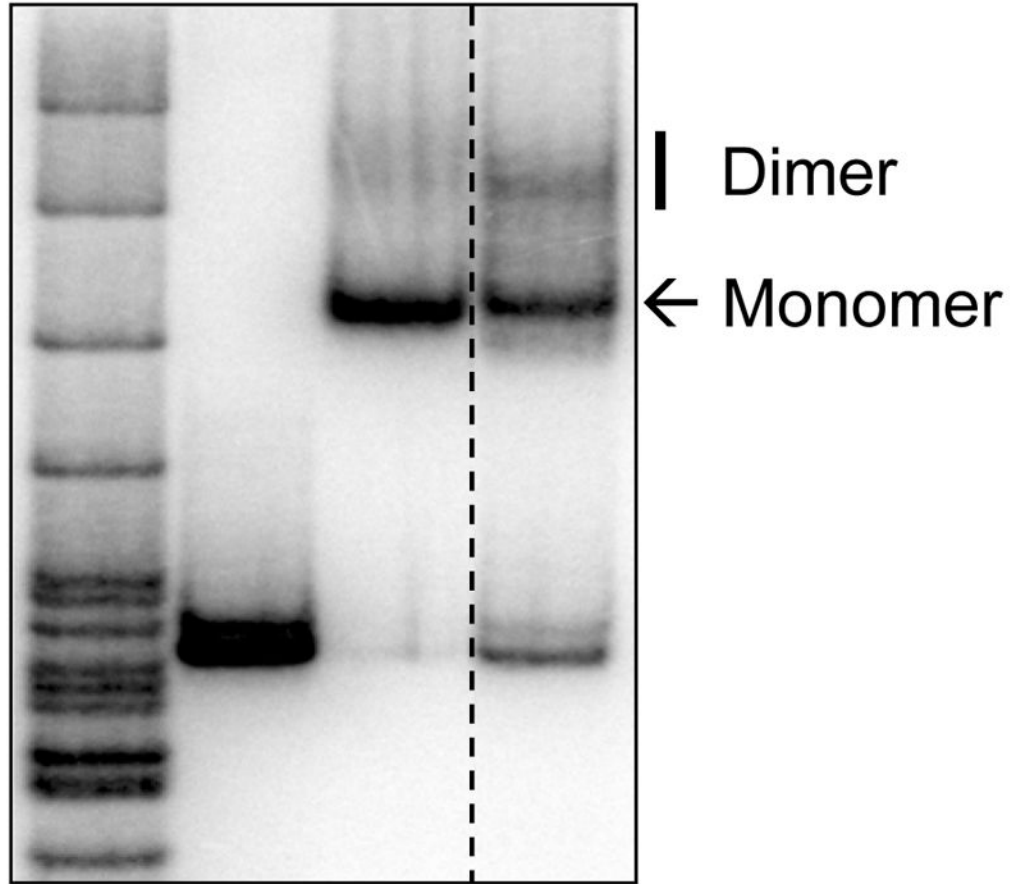


Fig. 2. Analysis of reconstituted -D hexasome and tetrasome by native PAGE
Monomer and dimer forms of nucleosomes/subnucleosomes are indicated. M: pBR322
DNA-*MspI* digest.

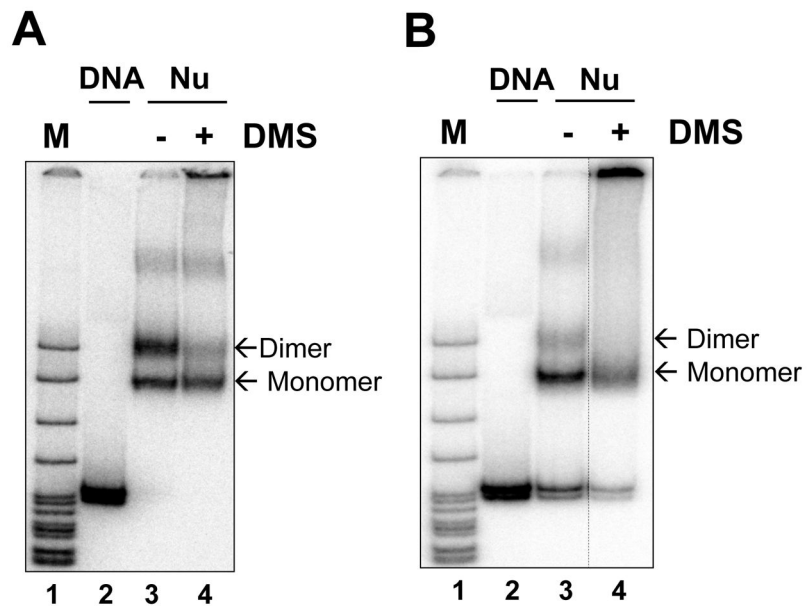


Fig. 3. Analysis of 603 DNA intact and DMS-cross-linked nucleosomes (Nu) by native PAGE ((17), reproduced from PNAS with permission)
 Cross-linking was conducted before (A) or after (B) nucleosome assembly. The positions of mononucleosomes and dinucleosomes are indicated.

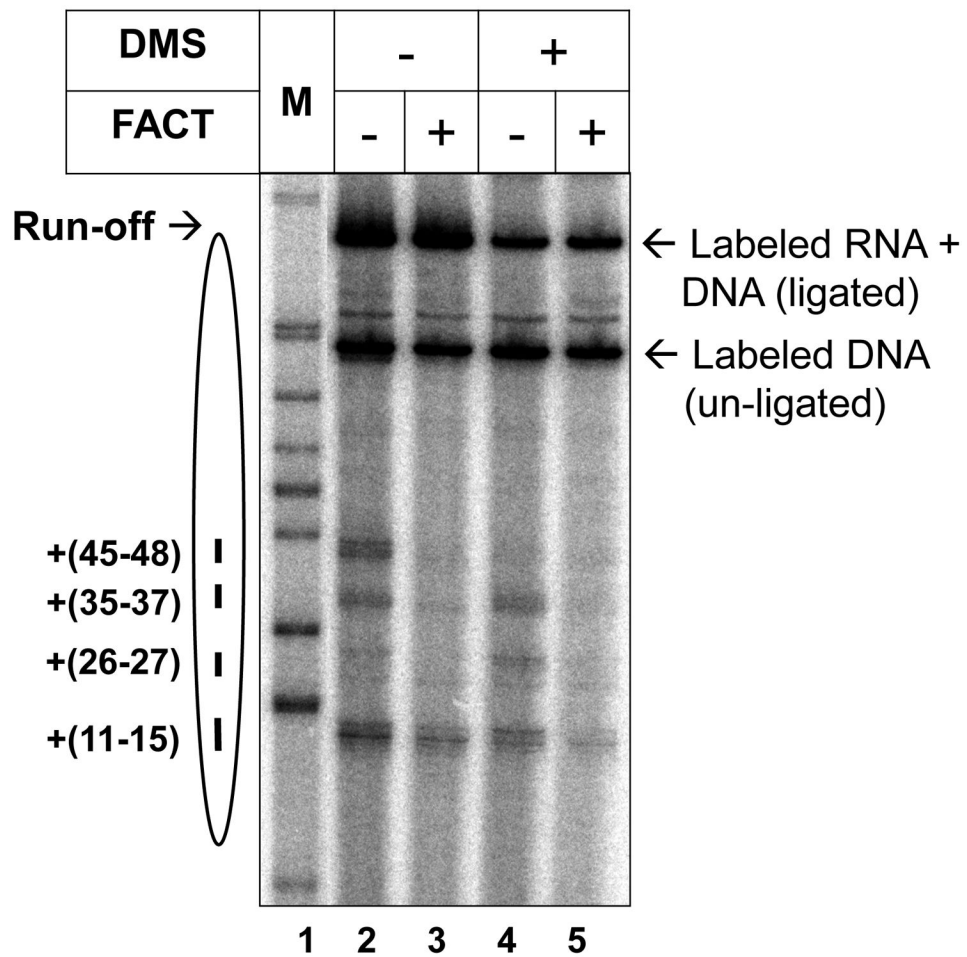


Fig. 4. Effect of cross-linking of histone octamers on transcription through the nucleosomes in the presence or absence of hFACT ((17), reproduced from PNAS with permission)
 603 nucleosomes were transcribed by Pol II at 150 mM KCl before and after cross-linking of the octamer by DMS and subsequent nucleosome assembly, followed by analysis of pulse-labeled RNA by denaturing PAGE.