

It should be useful to apply the structural and biochemical studies described here to other HAT families. Additionally, it is likely that some of the themes uncovered by the GCN5/PCAF and Esa1 studies may apply to other nuclear HAT families. The structurally conserved CoA-binding domain for both of these HAT families suggests that other nuclear HAT families may interact with the cofactor comparably. Similar to Esa1, steady-state kinetic analysis suggests that p300 participates in a ping-pong mechanism, which raises the possibility that the Esa1 structure has some similarity with the p300/CBP HAT domain. However, the complete lack of sequence homology between Esa1 and p300/CBP suggests that there may be important differences. Further mechanistic insights into the chemistry of this fascinating HAT superfamily will require additional structural and biochemical studies on members of other nuclear HAT families, as well as biologically relevant multi-protein *in vivo* HAT complexes.

⁶⁶ F. Dyda, D. C. Klein, and A. B. Hickman, *Annu. Rev. Biophys. Biomol. Struct.* **29**, 81–103 (2000).

[42] Assay of the Fate of the Nucleosome During Transcription by RNA Polymerase II

By W. WALTER, M. L. KIREEVA, V. TCHERNAJENKO, M. KASHLEV, and V. M. STUDITSKY

Eukaryotic genes transcribed by RNA polymerase II (Pol II) retain nucleosomal structure¹. Thus, Pol II encounters nucleosomes every ~200 bp during transcript elongation *in vivo*. Until now, only two types of Pol II-based experimental systems *in vitro* were available for analyzing the outcome of a Pol II-nucleosome encounter.^{2–4} One type includes systems supporting promoter-dependent transcription initiation, either in crude extracts³ or with highly purified proteins.⁴ The main disadvantage of this approach is that only ~1 % of the templates are transcribed.⁵ This low efficiency of template utilization makes analysis of the fate of nucleosomes after transcription nearly impossible. In contrast, DNA templates containing a single-stranded, 3'-extending oligo dC "tail" support efficient

¹ G. Orphanides and D. Reinberg, *Nature* **407**, 471 (2000).

² R. L. Dedrick and M. J. Chamberlin, *Biochemistry* **24**, 2245 (1985).

³ M. G. Izban and D. S. Luse, *Genes Dev.* **5**, 683 (1991).

⁴ G. Orphanides, G. LeRoy, C. H. Chang, D. S. Luse, and D. Reinberg, *Cell* **92**, 105 (1998).

end-initiation by Pol II *in vitro*.² However, in this system, determination of the fate of the nucleosome during transcription is complicated by the formation of extremely stable DNA-Pol II complexes at the end of DNA.⁶ Moreover, it is likely that end-initiated and promoter-initiated RNA polymerases differ in the way they progress through the nucleosome.⁶

A recent novel approach for analysis of Pol II elongation complexes (ECs) employs assembly of "authentic" ECs using histidine-tagged yeast Pol II and synthetic RNA and DNA oligonucleotides.^{7,8} The structure and functional properties of the assembled and promoter-initiated ECs are very similar.^{8,9} In this system, the fate of nucleosomes during transcription can be analyzed after ligation of the ECs to positioned mononucleosomes that are assembled separately.¹⁰ Nucleosomes form an absolute block to Pol II, but this barrier can be reduced by increasing the ionic strength of the reaction, thus allowing more templates to be transcribed. Transcription through nucleosomes by Pol II induces the loss of an H2A/H2B dimer without changing the position of the histones on the DNA.¹⁰

Use of this experimental system for analysis of the fate of the nucleosome during transcription is described below. This "minimal" model system can be easily adopted for analysis of polynucleosomal templates and for analysis of the role of different elongation factors during transcription through chromatin. Similar approaches can be applied for analysis of a variety of biological processes (such as chromatin remodeling, DNA replication, recombination, and repair) that may involve changes in nucleosome positioning or the histone content of nucleosomes.

2.0. Materials and Methods

Template Preparation

Purification of core histones and donor chromatin

The detailed protocols for purification of core histones on hydroxyapatite^{11,12} and purification of donor chromatin^{13,14} are described elsewhere.

⁵ J. A. Knezetic, G. A. Jacob, and D. S. Luse, *Mol. Cell Biol.* **8**, 3114 (1988).

⁶ Y. V. Liu, D. J. Clark, V. Tchernajenko, M. E. Dahmus, and V. M. Studitsky, *Biopolymers*, **68**, 528 (2003).

⁷ I. Sidorenkov, N. Komissarova, and M. Kashlev, *Mol. Cell* **2**, 55 (1998).

⁸ M. L. Kireeva, N. Komissarova, D. S. Waugh, and M. Kashlev, *J. Biol. Chem.* **275**, 6530 (2000).

⁹ M. L. Kireeva, N. Komissarova, and M. Kashlev, *J. Mol. Biol.* **299**, 325 (2000).

¹⁰ M. L. Kireeva, W. Walter, V. Tchernajenko, V. Bondarenko, M. Kashlev, and V. M. Studitsky, *Mol. Cell* **9**, 541 (2002).

Template Design and Purification of DNA

Careful consideration should go into the design of the template to be used for analyzing transcription through the nucleosome by Pol II. First, the ECs and nucleosomes are assembled separately and then ligated together. Thus for efficient ligation, a long, asymmetric sticky end, such as that generated by TspRI cleavage, is preferable. Second, a defined template with a positioned nucleosome(s) is desirable for mechanistic studies. For this, strong and well-characterized nucleosome positioning sequences, such as that of the *Xenopus* 5S RNA gene, can be employed.^{10,15} Finally, to map nucleosome positioning before and after the reaction, there should be several restriction enzyme sites along the entire length of the template for restriction endonuclease protection assays.

The pVT1 Template¹⁰

The template DNA is PCR amplified from pVT1 plasmid by using the primers (Invitrogen Corporation, Carlsbad, CA) pVT1-431-454-up (5' GAC ACT ATA GAA TTA ATG GGG ATC 3') and pVT1-737-716-low (5' CCT TCC AAG TAC TAA CCA GGC C 3'). Some of the lower primer is radiolabeled with γ [³²P]ATP (7000 Ci/mmol ICN Biomedicals, Inc., Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) prior to the PCR. The resulting 306 bp product is digested with TspRI, and the sample is loaded onto an 8% (19:1) polyacrylamide gel containing 1X TAE and 4 M urea (to prevent re-association of the 9 nt, GC-rich sticky ends of the TspRI-digested fragments). The 204 bp TspRI-StuI fragment is cut out of the gel, the gel slice is crushed, and the DNA is extracted overnight at 4°C in 3 to 5 volumes of TE buffer. The ethanol is then precipitated and resuspended in dH₂O. The template DNA is further "cleaned up" using QIA quick gel extraction kit columns (Qiagen, Chatsworth, CA).

Reconstitution, characterization, and purification of mononucleosomes and hexasomes

It is desirable to find efficient reconstitution conditions where the amount of free DNA is minimal (less than 10 to 15%) so the nucleosomes do not have to be further purified. The methods described below are for

¹¹ R. H. Simon and G. Felsenfeld, *Nucleic Acids Res.* **6**, 689 (1979).

¹² C. von Holt, W. F. Brandt, H. J. Greyling, G. G. Lindsey, J. D. Retief, J. D. Rodrigues, S. Schwager, and B. T. Sewell, *Methods Enzymol.* **170**, 431 (1989).

¹³ J. J. Hayes and K. M. Lee, *Methods* **12**, 2 (1997).

¹⁴ R. T. Utley, T. A. Owen-Hughes, L. J. Juan, J. Cote, C. C. Adams, and J. L. Workman, *Methods Enzymol.* **274**, 276 (1996).

¹⁵ J. J. Hayes, D. J. Clark, and A. P. Wolffe, *Proc. Natl. Acad. Sci. USA* **88**, 6829 (1991).

templates that are about 150 to 250 bp in size and, thus, allow for only one nucleosome per molecule of DNA.

Reconstitution of mononucleosomes via octamer exchange from donor chromatin

One method of making nucleosomal templates is to use donor chromatin as a source of histone octamers for exchange¹⁴ onto a template of interest. This method was chosen to reconstitute nucleosomes for transcription because it is generally very efficient (the amount of free DNA is less than 15%), and nucleosomes do not have to be purified. Nucleosomes prepared this way contain excess donor chromatin, but it can be removed by washing after the template is ligated to immobilized ECs.

DNA (1 to 5 μg) is mixed with long $-\text{H1}$ donor chromatin at a ratio of 1:60 (wt:wt), respectively, (sample volume is determined by donor chromatin concentration) in buffer containing 1 M NaCl and 0.1% Igepal CA-630 (Sigma, St. Louis, MO). The sample is dialyzed overnight at 4°C against a gradient (~ 1 L) starting at 1 M NaCl and ending with no NaCl in buffer containing 10 mM Tris-HCl, pH, 7.5, 0.2 mM EDTA, and 0.1% NP-40.

Reconstitution of mononucleosomes and subnucleosomal particles from purified histones

Nucleosomes can also be reconstituted from purified histones. The protocol described below is a slightly modified version of the method used by the Bradbury laboratory.^{16,17} This procedure can also be employed to create subnucleosomal particles by varying the amount of H2A/H2B used.

To make nucleosomes, 5 μg of DNA is mixed with 1.23 μg of H3/H4 and 2.70 μg of H2A/H2B (ratio of H3/H4:H2A/H2B = 0.455) at a volume of 100 μl in buffer containing 2 M NaCl, 0.2 mM EDTA, 10 mM Tris-HCl, pH 7.4, and 0.1% Igepal CA-630 (Sigma, St. Louis, MO). To make hexasome, the amount of H3/H4 is kept constant while the amount of H2A/H2B is reduced to 1.35 and 0.67 μg (with ratios of H3/H4:H2A/H2B = 0.91 and 1.82, respectively). Tetrasome is reconstituted by using the same amount of H3/H4 but eliminating the H2A/H2B. Dialysis is performed at 4°C against the same buffer but with decreasing NaCl concentration (2 M, 1.5 M, 1 M, 0.75 M, 0.5 M, and 10 mM NaCl) for 1 hour at each step.

Characterization and purification of nucleosomes and chromatin subparticles

Nucleosome and hexasome preparations must be analyzed for the amount of free DNA, hexasome, and nucleosome present in each preparation, as well as the location of histones on the template. The position of a

¹⁶ G. Meersseman, S. Pennings, and E. M. Bradbury, *EMBO J.* **11**, 2951 (1992).

¹⁷ S. Pennings, G. Meersseman, and E. M. Bradbury, *Proc. Natl. Acad. Sci. USA* **91**, 10275 (1994).

nucleosome can be determined with about 10 bp resolution based on its mobility during native PAGE.¹⁷ However, this method cannot discriminate between two symmetrically positioned nucleosomes or differently positioned nucleosomes formed on DNA ~200 bp or less.¹⁷ The exact position can be further narrowed down by restriction enzyme digestion or micrococcal nuclease mapping (described elsewhere¹⁸).

For analysis, the reconstitutes (10 ng aliquots) are supplemented with buffer providing 20 mM Na-HEPES, pH 7.8, 5 mM MgCl₂, 2 mM spermidine (Sigma, St. Louis, MO), and 0.5 mg/ml BSA. One sample is not digested, while appropriate restriction enzymes (10 U) are added to the others. Digestion is performed at room temperature (RT) for 0.5 to 1 hour. Buffer is added, providing a final concentration of 10 mM EDTA, 10% sucrose, and 250 μg/ml sheared herring testes DNA (Intergen, Purchase, NY). The templates are then resolved by native gel electrophoresis (4.5% acrylamide (39:1), 5% glycerol, 20 mM Na-HEPES (pH 8), 0.1 mM EDTA) at 100 V for 2.5 to 4 hrs (depending on the size of the DNA fragment and the degree of resolution desired) as described.¹⁸ Quantitation is performed using a Cyclone Storage Phosphor System (Packard, Meriden, CT). If gel purification is required,¹⁸ the samples are loaded without carrier DNA. The appropriate band is cut out of the gel, the gel is crushed, and the DNA, nucleosome, or hexasome are extracted overnight at 4 °C in 1 to 2 volumes of 10 mM Na-HEPES, pH 8.0, 0.1 mM EDTA, and 0.5 mg/ml BSA. The supernatant is collected, and the concentration of the sample is determined by the specific activity of the DNA.

Analysis of the pVT1 Reconstitutes¹⁰

Nucleosomes formed via octamer exchange

Nucleosomes are reconstituted very efficiently on the 204 bp (TspRI-StuI) pVT1 template by octamer exchange (Fig. 1, lane 1). There is one nucleosomal band, but the templates are too short to be resolved based on nucleosome position. Digestion with EcoRI (lane 2), EcoRV (lane 3), and EcoRI+EcoRV (not shown) reveals that about half of the nucleosomes are resistant to EcoRI and sensitive to EcoRV (N1), while half are resistant to EcoRV and sensitive to EcoRI (N2). The pVT1 DNA contains the strong 5S nucleosomal positioning sequence, so one nucleosome position (N2) was expected. However, the TspRI end of the DNA is an equally good site for nucleosome formation (N1).

Nucleosomes and hexasomes reconstituted from purified histones

Nucleosomes are not reconstituted as efficiently from purified histones as they are with exchange (compare Fig. 2A, lane 1 and Fig. 1, lane 1).

¹⁸ V. M. Studitsky, *Methods Mol. Biol.* **119**, 17 (1999).

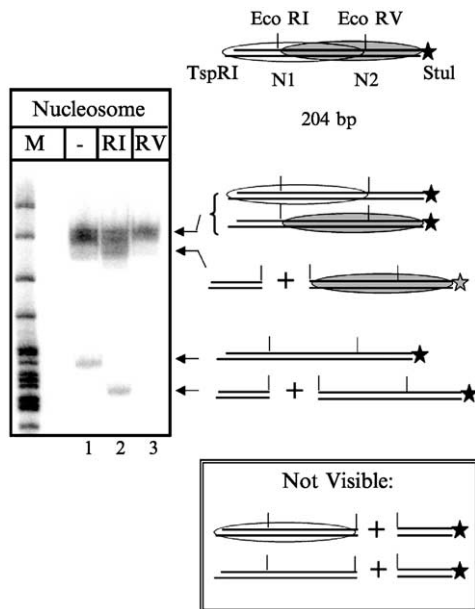


FIG. 1. Analysis of nucleosomes reconstituted by octamer exchange. Nucleosomes formed on 204 bp DNA (labeled at the *StuI* end, indicated by a star) were loaded onto a native gel before and after digestion with *EcoRI* and *EcoRV*. Positions of the *EcoRI* and *EcoRV* sites on the template are labeled. Mobilities of the nucleosomes (N1 and N2), free DNA, and the products of their digestion are indicated at the right. Molecular weight markers are an *MspI* digest of pBR322 DNA.

Restriction enzyme digestion with *EcoRI* and *EcoRV* reveal that nucleosomes formed by this method are indistinguishable from those formed by exchange. As the amount of H2A/H2B is decreased, less nucleosome is formed, and more hexasome is present in the sample (Fig. 2A).

Due to the large amount of free DNA in the samples, the nucleosomes and hexasomes have to be gel purified (Fig. 2B). The yield for this procedure is extremely low. Therefore, this was not the protocol of choice for preparing nucleosomes used for transcription.

Transcription of Defined Nucleosomal Templates

Transcription Buffers

TB0 contains 20 mM Tris-HCl, pH, 7.9, 5 mM MgCl₂, and 1 to 2 mM β -mercaptoethanol (2-ME). TB40, TB150, TB300, TB700, and TB1000 contain 40 mM, 150 mM, 300 mM, 700 mM, and 1 M KCl, respectively. Acetylated BSA was purchased from Sigma (St. Louis, MO).

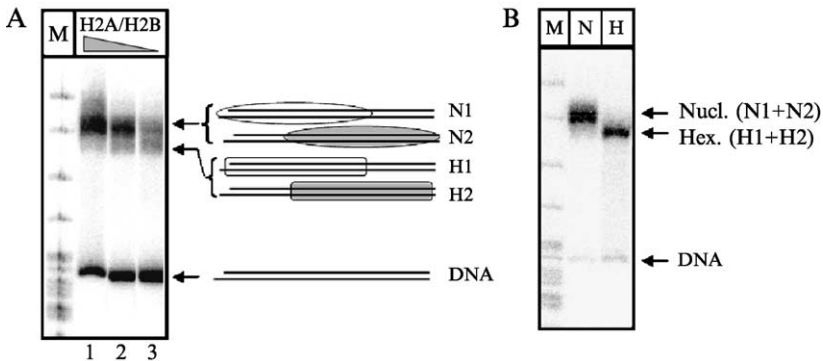


FIG. 2. Analysis of nucleosomes and hexasomes reconstituted from purified histones. (A) Nucleosomes and hexasomes formed on 204 bp pVT1 DNA. Mobilities of the nucleosomes (N1 and N2) and hexasomes (H1 and H2), as well as the free DNA are indicated at the right. (B) Gel-purified nucleosomes (N) and hexasomes (H).

EC assembly and ligation to DNA or nucleosomal templates

Yeast Pol II ECs are assembled from purified components as described in Komissarova *et al.*¹⁹ using the following oligonucleotides (*Oligos, Etc. Inc.*, Wilsonville, OR): TDS50 (5' GGTGTCGCTTGGGTTGGCTTTTC GGGCTGTCCC- TCTCGATGGCTGTAAGT 3'), RNA9 (5' AUCGAGAGG 3'), and NDS59 (5' ACTTACAGCCATCGAGAGGGACACG GCG AAAAGCCAACCAAGCGACACCGGCACTGGG 3'). The TDS50 oligo is labeled with γ [³²P]ATP (7000Ci/mmol, ICN Biomedicals, Inc., Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) as described.¹⁹ The Ni²⁺/NTA agarose (Qiagen, Chatsworth, CA) is pre-treated with 0.2 mg/ml acetylated BSA for 10 min and washed 2X with 1 ml of TB40 prior to immobilization of Pol II.

ECs assembled with \sim 2 to 3 pmol of Pol II (immobilized on 50 μ l of 50% resin suspension, enough for \sim 10 to 15 reactions) are incubated in the presence of 100 to 200 ng of template, 100 μ M ATP, 1% PEG-8000, and 50 units of T4 DNA ligase (New England Biolabs, Beverly, MA) in a volume of \sim 100 μ l at 12°C for 1 to 2 hours. The ECs are washed with TB40, incubated for 10 min with TB700, and washed twice with TB40. The ligation efficiency achieved by using the 9 nt 3' overhang generated by TspRI cleavage (5' CCCAGTGCC 3') is at least 50%.

¹⁹ N. Komissarova, M. L. Kireeva, I. Sidorenkov, and M. Kashlev, *Methods Enzymol.* in press. (2002).

Analysis of ECs and nucleosome mapping after ligation

ECs (assembled with ~ 1 to 1.5 pmol of Pol II, ligated to nucleosomes and washed) are eluted in a volume of $\sim 50 \mu\text{l}$ in TB40 containing 0.5 mg/ml acetylated BSA and 100 mM imidazole (Sigma, St. Louis, MO). The samples are incubated at RT for 10 minutes, diluted 2-fold, mixed, and microcentrifuged for 10 sec. $50 \mu\text{l}$ of the supernatant is withdrawn and diluted 5-fold with buffer providing 20 mM Na-HEPES, pH 7.6, 5 mM MgCl_2 , 2 mM spermidine (Sigma, St. Louis, MO), 0.5 mg/ml acetylated BSA, and 1 mM β -mercaptoethanol. The sample is aliquoted ($10 \mu\text{l}$ each), restriction enzyme digestions (with 10 U of enzymes) are performed for 30 to 60 minutes at RT, and the products are analyzed in a native gel as described previously.

Analysis of ECs after ligation to the pVT1 nucleosomal template and mapping of the ligated nucleosomes

Nucleosome positioning on the pVT1 template was analyzed after ligation to labeled EC9 (EC9 denotes an elongation complex with RNA that is 9 nt long) and elution with imidazole. Note the faster mobility of the unligated EC9 as compared to the EC9 ligated to the nucleosomal template (EC9N, Fig. 3B, lane 1). Some of the ECs (5 to 10%) spontaneously dissociate during elution and electrophoresis. The free nucleosomes (N1 and N2) are resolved based on their positions along the DNA. As expected, digestion with EcoRI and EcoRV (lanes 2 and 3) reveals that about half of the templates (Pol II-bound or not) are sensitive to EcoRI, and half are sensitive to EcoRV. A detailed map of the templates used for transcription is shown in Fig. 3A.

Transcript elongation

Immobilized Pol II can be walked to any point along the DNA by simply adding a subset of NTPs to the reaction, washing the resin, adding a different subset of NTPs, and so on. The concentration of NTPs added and the length of incubation vary based on the template, the location of the EC on the DNA, and the NTPs used.²⁰ The conditions described below are specific for the pVT1 template.

The nucleosome is an extremely strong barrier for Pol II under physiological conditions. This makes analysis of the transcribed templates difficult as most of the polymerase is stuck in the nucleosome, and the templates are not released. However, at 300 mM KCl, $\sim 30\%$ of the polymerase can

²⁰ M. Kashlev, E. Nudler, K. Severinov, S. Borukhov, N. Komissarova, and A. Goldfarb, *Methods Enzymol.* **274**, 326 (1996).

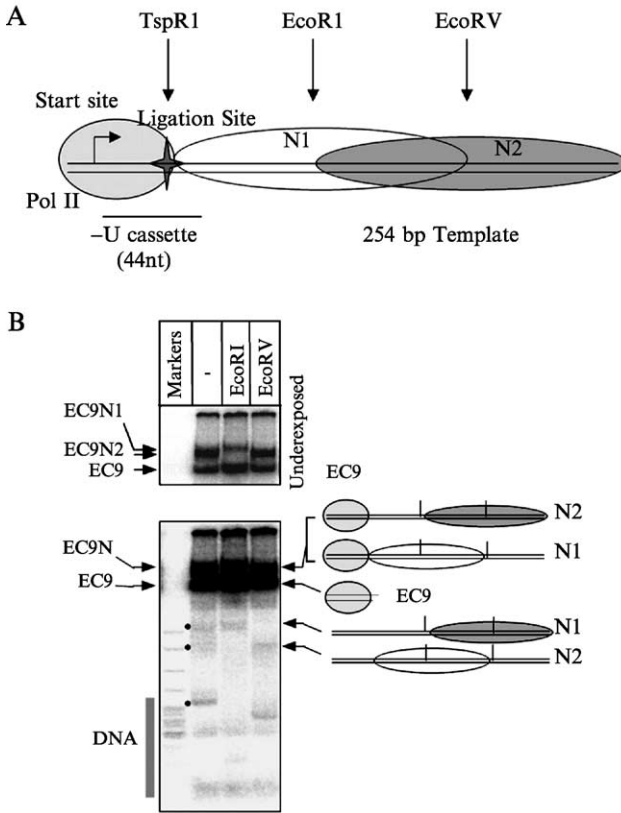


FIG. 3. Analysis of Pol II ECs and nucleosomes after ligation. (A) A map of the pVT1 template. ECs are labeled at the TspRI site (star) and ligated to nucleosomes. The Pol II start site is indicated with an arrow. The first 45 nt to be transcribed by Pol II lack UTP. Positions of nucleosomes (N1 and N2) and restriction enzyme sites used for mapping are indicated. (B) Imidazole-eluted ECs were analyzed in a native gel before and after EcoRI or EcoRV digestion. The upper panel is a shorter exposure of the gel. Mobilities of unligated EC (EC9) and EC ligated to the nucleosomes (EC9N1 and EC9N2) are indicated. The lower panel is a longer exposure of the gel and reveals the free nucleosomes (N1 and N2) and free DNA fragments (marked by dots).

overcome the barrier and finish transcription through the nucleosome.¹⁰ Therefore, to analyze the fate of the nucleosome after transcription, the reaction is carried out at 300 mM KCl so that enough templates are transcribed and released for analysis.

It is important to analyze only those templates that are fully transcribed and released by the polymerase. However, it is common for ECs to become arrested during transcription of the first 15 to 50 nt. Many of these ECs

dissociate, and this results in contamination of the supernatant with non-transcribed templates. For this reason, Pol II is walked to the +45 position on the pVT1 template, and the ECs are washed extensively to remove any non-transcribed templates.

Walking Pol II on the pVT1 template to form EC45

The first 45 nt of the pVT1 template consist of a -UTP cassette (see Fig. 3A). Thus, in the absence of UTP, Pol II can be walked to the +45 position (EC45). The volume of the EC9N (assembled with ~2 to 3 pmol of RNAP) is adjusted to 100 μ l (enough for 10 to 10 μ l reactions) in TB40 containing 0.2 mg/ml acetylated BSA. The ECs are incubated in the presence of 200 μ M ATP, CTP, and GTP for 10 minutes at RT. The sample is mixed, microcentrifuged for about 10 seconds and 50 μ l of the supernatant (S1) is withdrawn and divided into 5 μ l aliquots. The S1 supernatant contains free templates as a result of early EC dissociation and is representative of non-transcribed templates. The pellet is washed 2 times with 1 ml of TB300, incubated in 1 ml of TB300 for 15 minutes and washed 2 more times with 1 ml of TB300. This procedure is repeated to remove any remaining non-transcribed templates.

Immobilized transcription of the pVT1 template

Many controls are done to make sure that the templates analyzed are actually released as a result of transcription and not present due to further EC dissociation. The volume of the washed pellet (EC45) is adjusted to 100 μ l in TB300 containing 0.5 mg/ml acetylated BSA. The sample is aliquoted (10 μ l per experimental point) and incubated for 5 minutes at RT with (I) no NTPs (S2), (II) 100 μ M ATP, CTP, and GTP (S3), or (III) 100 μ M NTPs (S4). The samples are mixed, briefly centrifuged, and the supernatants (S2-4, 5 μ l per experimental point) are withdrawn for analysis. Supernatant S2 is a control for EC dissociation without NTP addition, S3 is a mock-transcription control for EC dissociation in the presence of NTPs, and S4 contains the transcribed templates.

Transcription of the pVT1 template in solution

For transcription in solution, the volume of the washed EC45 is adjusted to 100 μ l in TB300 containing 0.5 mg/ml acetylated BSA and 100 mM imidazole (Sigma, St. Louis, MO). The ECs are eluted for 10 minutes at RT. The sample is mixed, centrifuged, and 50 μ l of the supernatant is withdrawn. The supernatant is diluted 5-fold with TB300 containing 0.5 mg/ml acetylated BSA. The EC45 is aliquoted (10 to 20 μ l per experimental point) and incubated in the presence of (I) no NTPs (E2), (II) 100 μ M ATP, CTP, and GTP (E3), or (III) 100 μ M NTPs (E4) for 5 minutes at RT.

Analysis of the transcribed templates

For analysis, the samples (S1-4 or E1-4) are supplemented with buffer providing 20 mM Na-HEPES, pH 7.8, 5 mM MgCl₂, 2 mM spermidine (Sigma, St. Louis, MO), and 0.5 mg/ml BSA and aliquoted into several tubes. One sample of each fraction is not digested, while appropriate restriction enzymes (10 U) are added to the others, and digestion is performed at RT for 0.5 to 1 hour. Buffer is added, providing a final concentration of 10 mM EDTA, 10% sucrose, 250 μg/ml sheared herring testes DNA (Intergen, Purchase, NY), and 10 ng of carrier nucleosomes (to block non-specific interactions with the wells of the gel, donor chromatin works well). The templates are resolved by native gel electrophoresis as described previously. Quantitation is performed using a Cyclone Storage Phosphor System (Packard, Meriden, CT).

Reconstituted and gel-purified 204 bp hexasome is ligated to annealed NDS59 and ³²P-labeled TDS50 to make a mobility control for the 254 bp hexasome. Ligation is performed by incubating equimolar amounts of the annealed TDS50 and NDS59, as well as the 204 bp hexasome in the presence of 100 μM ATP, 1% PEG-8000, and 50 units of T4 DNA ligase (New England Biolabs, Beverly, MA) at 12°C for 1 to 2 hours. The sample is aliquoted, digested, and prepared for electrophoresis as described previously.

Results from analysis of transcription of the pVT1 nucleosome, both with immobilized Pol II and in solution, are shown in [Figures 4A and 4B](#), respectively. The transcribed template has the same mobility in a native gel as the hexasome control. Furthermore, the restriction enzyme digestion profile of the transcribed template and the reconstituted hexasome are very similar. Moreover, the digestion patterns (half sensitive to EcoRI and half sensitive to EcoRV) are similar between the templates before and after transcription. Thus, transcription by Pol II results in the loss of an H2A/H2B dimer, but the remaining histones stay in their original positions on the DNA.

Remarks

This section briefly describes some additional controls that are used for this procedure.

the hexasome, free DNA, and non-ligated promoter fragment are indicated. S1 is non-transcribed templates, S3 is the mock-transcribed control, and S4 is transcribed templates. (B) Templates transcribed in solution. Samples were analyzed by their mobility in a native gel beside the 254 bp reconstituted hexasome control. Mobilities of the ligated EC45, non-ligated EC45, free nucleosomes (N1 and N2), hexasome, DNA, and non-ligated promoter DNA are indicated. An underexposure of the top of the same gel is shown in the upper panel. Active and arrested ECs are indicated.

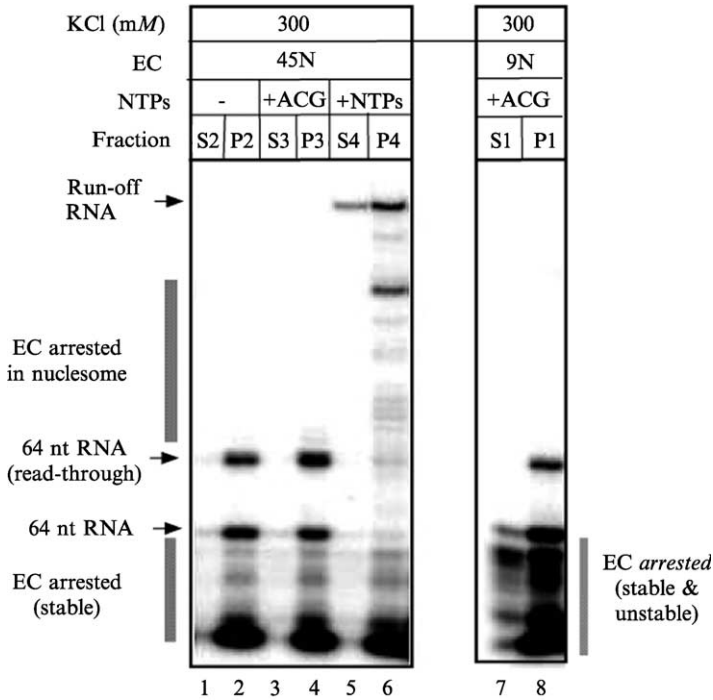


FIG. 5. Analysis of EC stability and NTP-dependent release of RNA/DNA.²¹ ECs with labeled RNA9 were ligated to nucleosomes, and EC45N was formed. Supernatant (S1-4) and pellet (P1-4) fractions were collected and loaded onto a 6% denaturing gel. The mobilities of the 45 nt RNA and the run-off product are indicated by arrows. Transcripts from arrested ECs are marked by shaded bars.

Analysis of RNA released after transcription

It can be demonstrated that only the fully transcribed templates are released into the S4 supernatant by analyzing the transcripts that are released into the supernatant. A small amount of smaller transcripts may be present (from EC dissociation), so the controls (S2 and especially S3) are important for determining the background amount of EC dissociation.

EC9 is assembled with γ -[³²P]ATP-labeled RNA9 and phosphorylated TDS50 and immobilized on acetylated BSA-treated Ni²⁺/NTA agarose as described.¹⁹ EC9 (from ~1.5 pmol Pol II) is ligated to the nucleosomal template as described above, and the volume is adjusted to 50 μ l, 10 μ l of resin is aliquoted into 1 tube, while 40 μ l of resin is aliquoted into another tube. EC45 is formed in each tube by the addition of 200 μ M ATP, CTP, and GTP for 10 minutes at RT in TB40 and 0.2 mg/ml acetylated BSA.

For tube #1, 5 μ l of the supernatant (S1) is withdrawn, and the pellet fraction (P1, also 5 μ l) is kept for analysis. For tubes #2 to 4, the resin is washed with 1 ml of TB300 twice, incubated with 1 ml of TB300 for 15 minutes, and washed with 1 ml of TB300 twice again. This procedure is repeated, and the resin is adjusted to a volume of 40 μ l in TB300 containing 0.5 mg/ml acetylated BSA. 10 μ l of the resin is aliquoted into each of the 3 tubes. 5 μ l of the supernatants are withdrawn after a 5-minute incubation with: (I) no additions (S2), (II) 100 μ M each ATP, CTP, and GTP (S3), and (III) 100 μ M NTPs (S4). The corresponding pellet fractions (P2-4, also 5 μ l each) are also kept for analysis. An equal volume (5 μ L) of gel-loading buffer containing 50 mM EDTA and 8 M urea is added, the samples are boiled for 5 to 10 minutes at 95°C, and the RNA is analyzed in a 6% (19:1) denaturing polyacrylamide gel. Quantitative analysis is performed with a Cyclone Storage Phosphor System (Packard, Meriden, CT). [Figure 5](#) is an example of the results obtained with this procedure on the pVT1 nucleosomal template.

Analysis of templates released after transcription of free DNA

It is also important to transcribe the free DNA template exactly like the nucleosomal templates are transcribed. The procedures for ligation, transcription, and analysis of the DNA template are the same as those described above for the nucleosomal template.

Acknowledgment

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[43] Probing Chromatin Immunoprecipitates with CpG-Island Microarrays to Identify Genomic Sites Occupied by DNA-Binding Proteins

By MATTHEW J. OBERLEY and PEGGY J. FARNHAM

Many methods have been developed to identify genomic targets of DNA-binding factors. We outline and discuss our adaptation of the chromatin immunoprecipitation (ChIP) assay to a high-throughput microarray-based method for discovering genomic regions occupied by human DNA-binding proteins. Others, primarily using yeast model systems, have also explored the use of coupled-chromatin immunoprecipitation and microarrays to identify large sets of binding sites of DNA-binding proteins.