

## [29] Transcription Through the Nucleosome by mRNA-Producing RNA Polymerases

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Eukaryotic genes that are actively transcribed by RNA polymerase II (Pol II) *in vivo* still retain their nucleosomal structure, indicating that even if the nucleosome is disrupted during transcription, this structure is recovered almost immediately after passage of the enzyme (Clark<sup>1</sup> and Orphanides and Reinberg<sup>2</sup> for reviews). Analysis of the outcome of this encounter resulted in the discovery that different RNA polymerases (RNAPs) use two very different mechanisms to transcribe through nucleosomes *in vitro*.<sup>3,4</sup> The first (Pol III-related) mechanism is utilized by bacteriophage SP6 and T7 RNAPs, as well as Pol III. This mechanism is characterized by a relatively low nucleosomal barrier to transcription<sup>5-7</sup> and by direct transfer of the complete histone octamer from in front of to behind the transcribing polymerase.<sup>8-12</sup> Pol II and *E. coli* RNAP use a very different mechanism for transcription through the nucleosome, which is characterized by a much stronger barrier,<sup>3,13</sup> and by the loss of an H2A/H2B dimer without changing the position of the histones on the DNA.<sup>3,4</sup>

The mechanism of transcription through chromatin and the regulation of this process are poorly studied. Until now, three types of Pol II-based experimental *in vitro* systems were available for analyzing the outcome of a Pol II-nucleosome encounter.<sup>13-15</sup> One type includes systems supporting

<sup>1</sup> D. J. Clark, in "The Nucleus." JAI Press, Greenwich, 1995.

<sup>2</sup> G. Orphanides and D. Reinberg, *Nature* **407**, 471 (2000).

<sup>3</sup> M. L. Kireeva, W. Walter, V. Tchernajenko, V. Bondarenko, M. Kashlev, and V. M. Studitsky, *Mol. Cell* **9**, 541 (2002).

<sup>4</sup> W. Walter, M. Kireeva, V. M. Studitsky, and M. Kashlev, *J. Biol. Chem.* **278**, 36148 (2003).

<sup>5</sup> V. M. Studitsky, D. J. Clark, and G. Felsenfeld, *Cell* **83**, 19 (1995).

<sup>6</sup> R. U. Protacio, K. J. Polach, and J. Widom, *J. Mol. Biol.* **274**, 708 (1997).

<sup>7</sup> J. Bednar, V. M. Studitsky, S. A. Grigoryev, G. Felsenfeld, and C. L. Woodcock, *Mol. Cell* **4**, 377 (1999).

<sup>8</sup> D. J. Clark and G. Felsenfeld, *Cell* **71**, 11 (1992).

<sup>9</sup> M. F. O'Donohue, I. Duband-Goulet, A. Hamiche, and A. Prunell, *Nucleic Acids Res.* **22**, 937 (1994).

<sup>10</sup> V. M. Studitsky, D. J. Clark, and G. Felsenfeld, *Cell* **76**, 371 (1994).

<sup>11</sup> V. M. Studitsky, *Methods Mol. Biol.* **119**, 17 (1999).

<sup>12</sup> V. M. Studitsky, G. A. Kassavetis, E. P. Geiduschek, and G. Felsenfeld, *Science* **278**, 1960 (1997).

<sup>13</sup> M. G. Izban and D. S. Luse, *Genes Dev.* **5**, 683 (1991).

promoter-dependent transcription initiation, either in crude extracts<sup>13</sup> or with highly purified proteins.<sup>15</sup> The main disadvantage of this approach is that only a small fraction of the templates is transcribed.<sup>16</sup> 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 end-initiation by Pol II *in vitro*.<sup>14</sup> However, in this system, extended DNA:RNA hybrids can be formed, and 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.<sup>17</sup> Moreover, end-initiated and promoter-initiated RNA polymerases differ in the way they progress through the nucleosome, most likely because the structures of the ECs are different.<sup>17</sup>

More recently, a novel approach for analysis of Pol II elongation complexes (ECs) was developed.<sup>18,19</sup> It employs assembly of "authentic" ECs using histidine-tagged yeast Pol II and synthetic RNA and DNA oligonucleotides. The structures and functional properties of the assembled and promoter-initiated ECs are very similar.<sup>19,20</sup> While this experimental system faithfully recapitulates many important properties of chromatin transcribed *in vivo* and allows analysis of the fate of nucleosomes during transcription,<sup>3</sup> only a single round of transcription can be conducted. Since a large fraction of assembled complexes is falling apart during transition into elongation phase of transcription,<sup>3</sup> the yield of ECs is relatively low, making their direct structural analysis very difficult.

Recently, it has been shown that *E. coli* RNAP and Pol II use very similar mechanisms for transcription through nucleosomes.<sup>4</sup> Later we describe an experimental system utilizing promoter-initiated *E. coli* RNAP that recapitulates general features of the Pol II-related mechanism of transcription through the nucleosome. This system allows analysis of multiple rounds of transcription through nucleosomes and direct structural analysis of the intermediates formed during this process. In this system, transcription through nucleosomes can be analyzed after ligation of the promoter-initiated ECs to positioned mononucleosomes that are assembled separately.<sup>4</sup>

<sup>14</sup> R. L. Dedrick and M. J. Chamberlin, *Biochemistry* **24**, 2245 (1985).

<sup>15</sup> G. Orphanides, G. LeRoy, C. H. Chang, D. S. Luse, and D. Reinberg, *Cell* **92**, 105 (1998).

<sup>16</sup> J. A. Knezetic, G. A. Jacob, and D. S. Luse, *Mol. Cell. Biol.* **8**, 3114 (1988).

<sup>17</sup> Y. V. Liu, D. J. Clark, V. Tchernajenko, M. E. Dahmus, and V. M. Studitsky, *Biopolymers* **68**, 528 (2003).

<sup>18</sup> I. Sidorenkov, N. Komissarova, and M. Kashlev, *Mol. Cell* **2**, 55 (1998).

<sup>19</sup> M. L. Kireeva, N. Komissarova, D. S. Waugh, and M. Kashlev, *J. Biol. Chem.* **275**, 6530 (2000).

<sup>20</sup> M. L. Kireeva, N. Komissarova, and M. Kashlev, *J. Mol. Biol.* **299**, 325 (2000).

## Materials and Methods

In the approaches described below, the ECs and nucleosomes are assembled separately and then ligated together (see Fig. 1). The ECs can be assembled by two ways. ECs can be formed on the strong *E. coli* A1 promoter of bacteriophage T7 (T7A1) using  $\sigma^{70}$ -containing holoenzyme. Alternatively, authentic ECs are assembled on synthetic oligonucleotides using the core enzyme.<sup>18</sup> Both approaches produce ECs having very similar properties during transcription of DNA<sup>18</sup> or nucleosomal templates.<sup>4</sup> The ECs are immobilized on Ni<sup>2+</sup>-NTA beads through a hexahistidine tag positioned on the C-terminus of  $\beta'$  subunit of RNAP. Below we only describe a protocol for promoter-dependent formation of ECs; assembly using the core enzyme has been described in detail elsewhere.<sup>21</sup>

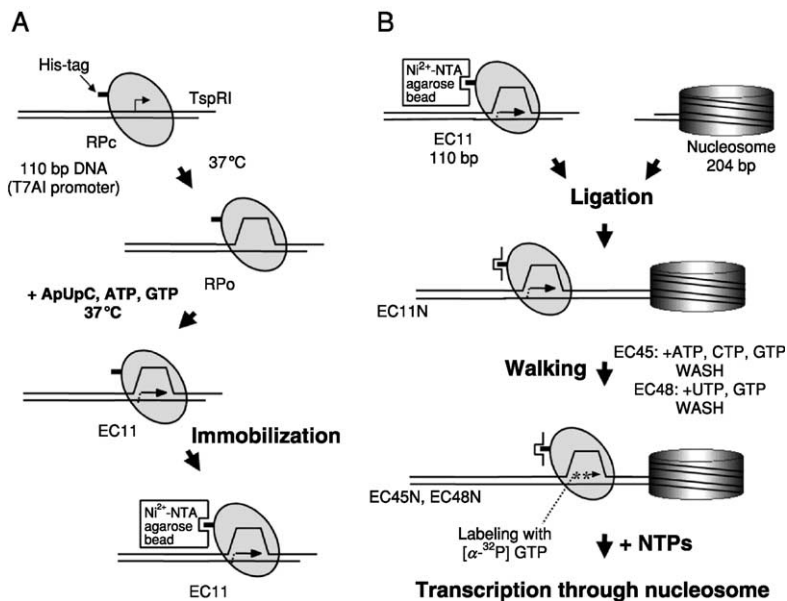


FIG. 1. Experimental system using promoter-initiated ECs to study transcription through the nucleosome by *E. coli* RNAP. (A) The formation of promoter-initiated ECs. The 110 bp TspRI-cut fragment of DNA containing the T7A1 promoter and hexahistidine-tagged RNAP are incubated at 37° to form open complexes. Transcription is initiated through the addition of ApUpC, ATP, and GTP and incubation at 37° to form EC11. EC11 is immobilized on Ni<sup>2+</sup>-NTA agarose and washed. (B) The experimental approach. Immobilized EC11 is ligated to the 204 bp DNA or nucleosomal template, and the ECs are washed. The polymerase is walked to the +45 position and in some cases the +48 position using  $\alpha$ [<sup>32</sup>P]NTPs to label the RNA. The ECs are washed after each manipulation, and transcription is resumed by addition of all four NTPs.

Nucleosomes are reconstituted on well-characterized nucleosome positioning sequences from the *Xenopus* 5S RNA,<sup>3,22</sup> and are then ligated to the ECs in the absence of NTPs. Transcript elongation is resumed in the presence of NTPs in solid phase or in solution after elution with imidazole. The result of transcription through the nucleosome is then analyzed.

*Design and Purification of the DNA Fragments Containing the T7A1 Promoter and the Nucleosome Positioning Sequence*

A 110 bp DNA fragment containing the T7A1 promoter was used for transcription initiation. This fragment contains 70 bp upstream of the transcription start site (+1) and a long sticky end starting at the +40 position created by TspRI cleavage that allows for efficient ligation to the nucleosomal template containing a complimentary TspRI end. This DNA fragment was prepared by annealing 2 long overlapping oligos (83 nt each, with a 30 bp overlap) and filling in the ends with Vent (exo-) DNA polymerase (New England Biolabs, Beverly, MA). After digestion, the promoter fragment is ligated to the piece of DNA used for nucleosome reconstitution (pVT1 template<sup>3,23</sup>), and the entire template is PCR amplified. Finally, the template is separated into its separate components (promoter fragment and nucleosome positioning sequence) *via* TspRI digestion and gel purification. The DNA for nucleosome reconstitution is moderately endlabeled so that the quality of the reconstitutes can be monitored (see later). Then nucleosomes and the elongation complexes are assembled on the isolated DNA fragments separately to avoid nucleosome formation at the promoter. Note that nonadhesive tubes (USA Scientific, Ocala, F1) were used in all experiments.

1. The upper oligo (5' AAAGGATCCAGATCCCGAAAATTTATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGACTACTTACAGCCATCGAGAGGG 3') and lower oligo (5' GTTTCCTGTGTGTGCCAGTGCCGGTGTCTCGCTTGGGTTGGCTTTTCGCCGTGTCCCTCTCGATGGCTGTAAGTATCCTATAGG 3', Invitrogen Corporation, Carlsbad, CA) were mixed in equimolar quantities (192 pmole each) in 1× annealing buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM EDTA) at a final volume of 50 μl.

<sup>21</sup> N. Komissarova, M. L. Kireeva, I. Sidorenkov, and M. Kashlev, *Methods Enzymol.*, in press.

<sup>22</sup> J. J. Hayes, D. J. Clark, and A. P. Wolffe, *Proc. Natl. Acad. Sci. USA* **88**, 6829 (1991).

<sup>23</sup> W. Walter, M. L. Kireeva, V. Tchernajenko, M. Kashlev, and V. M. Studitsky, *Methods Enzymol.*, in press.

2. The oligos were annealed by heating them to 85° in a H<sub>2</sub>O bath for 10 min and allowing them to cool to RT slowly.
3. The annealed oligos were filled in by incubating at 72° for 20 min in the presence of 1× Vent DNA polymerase buffer [10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100], 200 μM dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ), 0.1 mg/ml BSA, and 1.5 U of Vent (exo-) DNA polymerase (New England Biolabs, Beverly, MA). 10 μl of annealed oligo was used per 50 μl of fill in reaction.
4. The duplex was ethanol precipitated and digested with TspRI (New England Biolabs, Beverly, MA) overnight at 65°.
5. The TspRI-cut 113 bp band was PAGE purified, extracted, and ethanol precipitated.
6. The 113 bp TspRI-cut T7A1 DNA fragment was ligated to the 204 bp TspRI-cut pVT1 DNA fragment.<sup>3,23</sup> The 317 bp fragment was gel purified, extracted, and ethanol precipitated.
7. The DNA was PCR amplified in preparative amounts using the primers (Invitrogen Corporation, Carlsbad, CA): upper-5' GGATC-CAGATCCCGAAAATTTATC 3' and lower-5' CCTTCC AAG-TACTAACCAGGCC 3'. Some of the lower primer is radiolabeled with γ[<sup>32</sup>P]ATP (7000 Ci/mmol, ICN Biomedicals, Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, as per NEB recommendations) prior to the PCR.
8. The resulting 314 bp product is purified by ethanol precipitation and digested with TspRI as described above.
9. The sample is loaded onto an 8% (19:1) polyacrylamide gel containing 1× TAE and 4 M urea (to prevent reassociation of the 9 nt, GC-rich sticky ends of the TspRI-digested fragments). The 110 and 204 bp TspRI-cut fragments are cut out of the gel, the gel slices are crushed, and the DNA is extracted overnight at 4° in 3–5 volumes of TE buffer, ethanol precipitated, and resuspended in dH<sub>2</sub>O.
10. The template DNA is further “cleaned up” using QIAquick gel extraction kit columns (Qiagen, Chatsworth, CA, as per kit protocol).

### *Transcription Buffers*

TB0 contains 20 mM Tris-HCl (pH 7.9), 5 mM MgCl<sub>2</sub>, and 1–2 mM β-mercaptoethanol. TB40, TB150, TB300, and TB1000 contain 40 mM, 150 mM, 300 mM, and 1 M KCl, respectively. Acetylated BSA was purchased from Sigma (St. Louis, MO).

### *Formation of ECs by Promoter Initiation and Immobilization on Ni<sup>2+</sup>-NTA Agarose*

In this protocol, the ECs are formed separately in solution, immobilized on Ni<sup>2+</sup>-NTA agarose (Qiagen, Chatsworth, CA) and washed, and then ligated to the nucleosomal template (see Fig. 1). If the templates were to be analyzed after transcription, the promoter fragment was 5' end labeled with [ $\gamma$ -<sup>32</sup>P]ATP (7000 Ci/mmol, ICN Biomedicals, Irvine, CA) and T4 polynucleotide kinase. It should be noted that each wash step involves adding the wash buffer to the resin, microcentrifuging for 10 s, and then collecting the supernatant. Large orifice pipet tips (USA Scientific, Ocala, FL) were used for pipetting the resin. The scheme for promoter initiation is illustrated in Fig. 1A.

1. To form open complexes, a 9  $\mu$ l reaction containing 1 pmol (0.5  $\mu$ g) of hexahistidine-tagged RNAP holoenzyme and 0.7 pmol of 110 bp T7A1 promoter fragment in TB40 was incubated at 37° for 5–10 min.
2. To form EC11 (numerical index indicates the length of the RNA in the stalled EC), 1  $\mu$ l of 10 $\times$  start mix (200  $\mu$ M ApUpC [*Oligos, Etc.*, Wilsonville, OR], 200  $\mu$ M ATP, and 100  $\mu$ M GTP [Amersham Pharmacia Biotech, Piscataway, NJ]) was added, and the reaction was incubated at 37° for 5 min.
3. Twenty microliters of Ni<sup>2+</sup>-NTA agarose (50% suspension, Qiagen, Chatsworth, CA) was washed 3 times with 0.5 ml of TB40, incubated in the presence of 0.5 mg/ml of acetylated BSA (Sigma, St. Louis, MO) for 10 min, and washed 2 times with 0.5 ml TB40. The volume was adjusted to 25  $\mu$ l.
4. EC11 was immobilized on the resin by constant shaking for 15 min at RT.
5. The ECs were washed 6 times with 0.25 ml of TB40.

### *Reconstitution and Analysis of Mononucleosomes for Transcription*

It is highly advantageous to find an efficient method of making nucleosomal templates where the amount of free DNA is <15%, and the nucleosomes do not have to be further purified after reconstitution. The most efficient protocol that we have found for reconstitution of mononucleosomes on the 204 bp pVT1 template involves the use of donor chromatin as a source of histone octamers for exchange onto the template DNA.<sup>24,25</sup> The detailed protocol for preparation of donor chromatin has been

<sup>24</sup> G. Meersseman, S. Pennings, and E. M. Bradbury, *EMBO J.* **11**, 2951 (1992).

<sup>25</sup> S. Pennings, G. Meersseman, and E. M. Bradbury, *Proc. Natl. Acad. Sci. USA* **91**, 10275 (1994).

published<sup>23</sup> and will not be discussed here. Nucleosomes prepared this way contain excess donor chromatin, but it can be removed by washing after the template is ligated to immobilized ECs. The method described below is for templates that are about 150–250 bp in size and, thus, allow for only one nucleosome per molecule of DNA. The quality of nucleosome preparations is determined by the amount of free DNA and subnucleosomal particles that contaminate the sample and how well the nucleosomes formed in the proper location on the template. The position of a nucleosome can be determined with about 10 bp resolution based on its mobility during native PAGE.<sup>26</sup> However, this method cannot discriminate between two symmetrically positioned nucleosomes or differently positioned nucleosomes formed on DNA ~200 bp or less.<sup>27</sup> The exact position can be further narrowed down by restriction enzyme digestion or micrococcal nuclease mapping (not discussed here<sup>11</sup>). See Fig. 2 for an example of restriction enzyme mapping on the ligated, 314 bp template. The map of the nucleosomes on the template is illustrated in Fig. 2A. Although only one nucleosome can be formed on each individual template, a mixture of different nucleosome positions is obtained. Two regions of the template were preferred locations for nucleosome formation (N1 and N2), and each of these regions had two local heterogeneous positions (a and b), giving rise to four different nucleosome positions (N1a, N1b, N2a, and N2b). The N1 nucleosomes are resistant to cleavage with EcoRI, and N1a is sensitive to cleavage with EcoRV. N1b is only slightly sensitive to EcoRV because this restriction enzyme site is right on the nucleosomal border. The N2 nucleosomes are resistant to cleavage by EcoRV and sensitive to cleavage by EcoRI. The ligated nucleosomes are sensitive to MspI digestion, showing, in combination with the EcoRI and EcoRV digestion data, that ligation does not alter the nucleosome positioning (see Fig. 2B).<sup>3,4</sup>

1. 5' end-labeled 204 bp TspRI-cut pVT1 DNA is prepared as described above. One to five micrograms of the DNA is mixed with long –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).
2. The sample is dialyzed overnight at 4° 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.

<sup>26</sup> S. Pennings, G. Meersseman, and E. M. Bradbury, *J. Mol. Biol.* **220**, 101 (1991).

<sup>27</sup> S. Pennings, G. Meersseman, and E. M. Bradbury, *Nucleic Acids Res.* **20**, 6667 (1992).

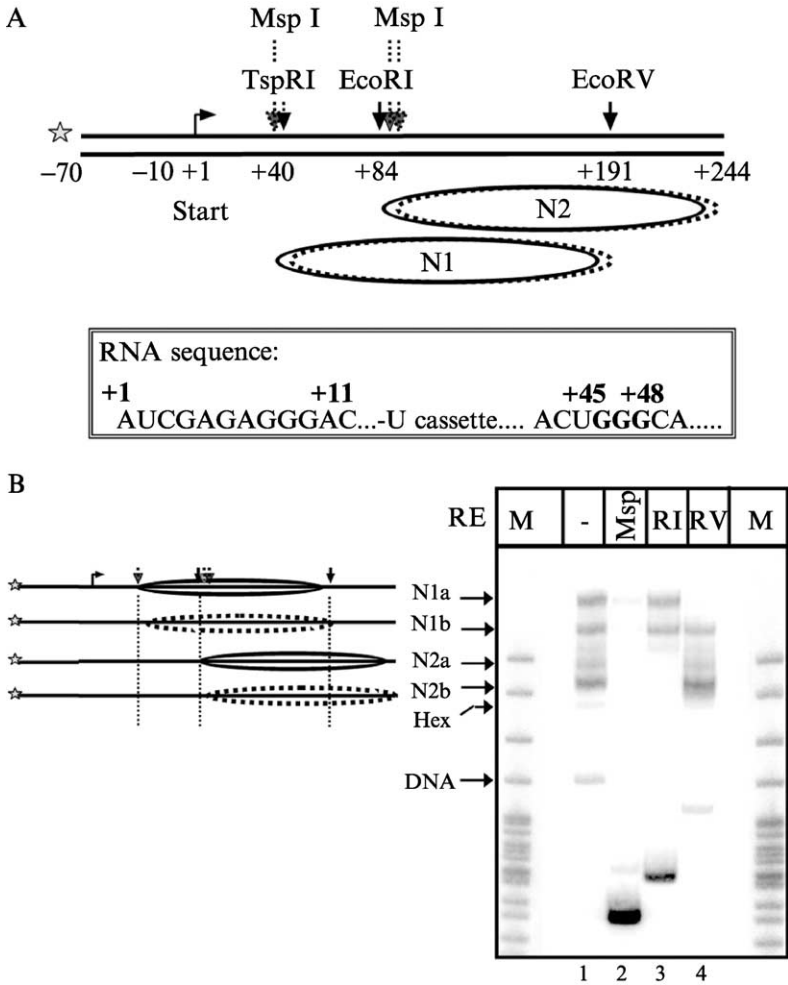


FIG. 2. Restriction enzyme mapping of the 314 bp nucleosomal template. (A) Nucleosome positioning on the ligated 314 bp template. The numerical labeling on the template represents the positions along the DNA relative to the transcription start site (+1). Locations of the TspRI, MspI, EcoRI, and EcoRV restriction sites are indicated. The template was labeled at the 5' end (indicated by a star). The nucleosome positions, N1a, N1b, N2a, and N2b are shown as ovals (a is a solid oval, b is a dashed oval) below the template. N2 occupies the 5S positioning sequence. The transcribed sequence is shown in the bottom panel, and important RNAP "walking" steps are indicated. Three hundred and fourteen base pair nucleosomes were obtained by ligating 204 bp nucleosomes to 5' end-labeled 110 bp T7A1 promoter fragment, and the templates were analyzed by native PAGE before and after restriction enzyme digestion. (B) Analysis of nucleosomes in a native gel. The mobilities of the nucleosomes (N1a, N1b, N2a, and N2b), hexasomes, and free DNA are indicated. Positions of

3. The concentration of the reconstituted nucleosomes is determined by the specific activity of the DNA.
4. Reconstitutes (10 ng aliquots) are supplemented with buffer providing 20 mM Na-HEPES (pH 7.8), 5 mM MgCl<sub>2</sub>, 2 mM spermidine (Sigma, St. Louis, MO), and 0.5 mg/ml BSA.
5. One sample is not digested, while appropriate restriction enzymes (10 U) are added to the others, and digestion is performed at room temperature (RT) for 0.5–1 h.
6. Buffer is added providing a final concentration of 10 mM EDTA, 10% sucrose, and 50 μg/ml sheared herring testes DNA (Intergen, Purchase, NY).
7. The templates are 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 2.5–4 h (depending on the size of the DNA fragment and the degree of resolution desired) as described.<sup>23</sup>
8. Here, and in all other experiments described, quantitation is performed using a Cyclone Storage Phosphor System (Packard, Meriden, CT).

#### *Ligation of the ECs to the 204 bp DNA or Nucleosomal Template*

The scheme for ligation of the ECs to the nucleosomes is illustrated in Fig. 1B.

1. Washed EC11 (with the volume adjusted so that the final volume of the ligation reaction would be 25 μl) was incubated in the presence of 100 ng of TspRI-cut 204 bp template (nucleosome or DNA), 100 μM ATP, 1% PEG-8000, 0.5 mg/ml Acetylated-BSA, and 50 U T4 DNA ligase at 12° for 1–2 h.
2. The ligated EC was washed 3 times with 0.5 ml TB300, incubated in TB300 for 10 min, and washed 3 times with 0.5 ml TB40.

#### *Transcription and Analysis of RNA*

The scheme for transcription through the nucleosome is illustrated in Fig. 1B. The early transcribed sequence for this template is shown in Fig. 2A. To analyze RNAP pausing during transcription through the nucleosome, the RNA can be labeled in several different ways. For instance, the RNA can be labeled via incorporation of α[<sup>32</sup>P]ATP, CTP, or GTP

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the restriction enzyme sites along the DNA are shown at the top. The location of DNA labeling is indicated by a star. Schematics illustrating the individual nucleosome positions and their corresponding restriction enzyme sensitivities are shown on the left.

during the formation of EC11. Essentially, the RNA can be labeled at any position by walking the RNAP along the template in the presence of different combinations of NTPs.<sup>28</sup> In the protocol below, the RNA is labeled from +46 to +48 by incorporation of  $\alpha$ [<sup>32</sup>P]GTP (see Fig. 2A). Walking RNAP past +40 moves the polymerase across the ligation junction (TspRI site). Thus, the RNAP runs off of the unligated template, and only the RNA being transcribed on ligated templates is labeled and analyzed. An example of transcription through the nucleosome at different salt concentrations is shown in Fig. 3.

1. EC45 was formed by incubating EC11 in the presence of 0.5 mg/ml Acetylated-BSA and 10  $\mu$ M each of ATP, CTP, and GTP in TB40 for 10 min at RT. The final volume of the reaction was  $\sim$ 50  $\mu$ l.
2. EC45 was washed 6 times with 0.5 ml TB40.
3. EC48 was formed by incubating EC45 in the presence of 0.4  $\mu$ M  $\alpha$ [<sup>32</sup>P]GTP (3000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) and 5  $\mu$ M cold UTP for 5 min at RT. The final volume of the reaction was 30  $\mu$ l.
4. EC48 was washed 6 times with 0.5 ml TB40.
5. The samples were supplemented with 0.5 mg/ml Acetylated-BSA, aliquoted, and the KCl concentration of each aliquot was adjusted as desired (40 mM, 150 mM, 300 mM, and 1 M KCl). The volume of each aliquot depends on the number of reactions (see # 6 below).
6. Transcription was performed at RT for the desired time point in the presence of 200  $\mu$ M NTPs. The typical transcription reaction volume is about 10  $\mu$ l, and this protocol provides enough material for 24–36 reactions.
7. The reaction was stopped with an equal volume of loading buffer containing 10 M urea and 25 mM EDTA.
8. The sample was boiled and 3  $\mu$ l was loaded on a 10% (19:1) denaturing uria polyacrylamide gel.

### *Transcription in Solid Phase and Analysis of Released Templates*

To analyze the fate of the nucleosomes during transcription, EC11 containing labeled DNA is obtained and ligated to nucleosomes as described previously. The templates are transcribed and analyzed in a native gel. The scheme for transcription through the nucleosome is illustrated in Fig. 1B. An example of the results one should obtain using this protocol is shown in Fig. 4 (lanes 1–3). It should be noted that during early elongation

<sup>28</sup> M. Kashlev, E. Nudler, K. Severinov, S. Borukhov, N. Komissarova, and A. Goldfarb, *Methods Enzymol.* **274**, 326 (1996).

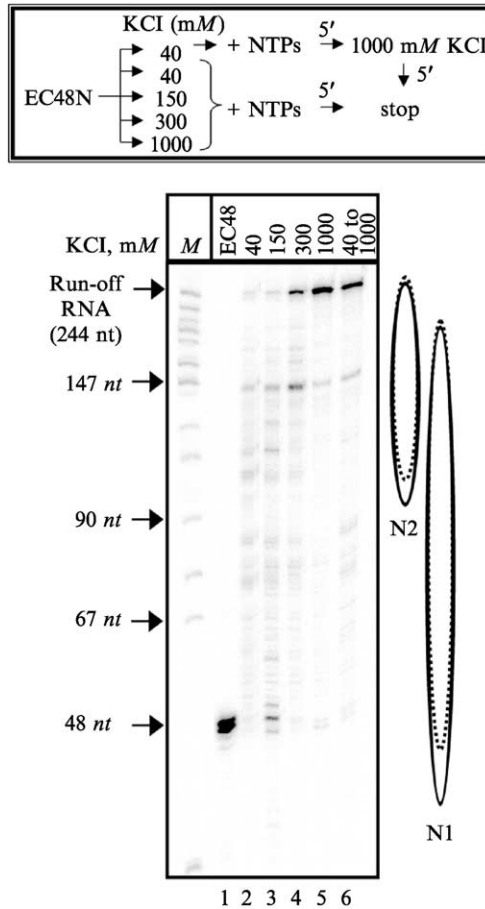


FIG. 3. The nucleosomal barrier is reduced by increasing the ionic strength of the transcription reaction. Markers are an *Msp*I digest of pBR322. DNA marker lengths are indicated at the left in italics. A schematic for the experiment is at the top. RNA was labeled from positions +45 to +48 as described in the text. ECs were washed and the salt concentration was adjusted as indicated above the lane. Transcription was resumed for 5 min at RT with the addition of NTPs. The 48-mer starting material and 244 nt run-off RNA are indicated with arrows on the left. Nucleosome positions (N1 and N2) on the template are depicted on the right.

(before formation of EC45), some of the ECs are unstable and release non-transcribed templates into solution (see Fig. 4, lane 1). Thus, by washing EC45, any templates that were released by RNAP and not transcribed to completion were removed from the analysis.<sup>3,4</sup> A second incubation of

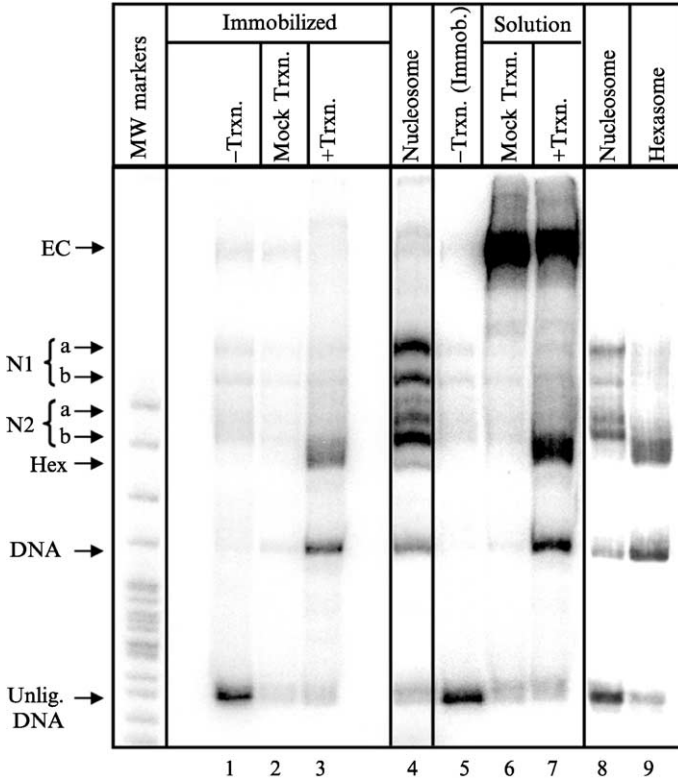


FIG. 4. Transcription through the nucleosome by promoter-initiated *E. coli* RNAP results in the loss of an H2A/H2B dimer. Markers are an MspI digest of pBR322. Labeled 314 bp templates were analyzed in a native gel before transcription (“-Trxn.”; EC11 + ACG, templates released as a result of dissociation of early ECs during transcription of initial 44 nt of the template), after mock transcription (washed EC45 + ACG, templates released due to residual falling apart of the early ECs), and after transcription (“+Trxn.”; washed EC45 + NTPs, templates released due to transcription). Lanes 1-3 are from transcription with immobilized RNAP. Lane 5 (and lane 1) is the supernatant from walking the immobilized RNAP to form EC45. EC45 is washed and eluted with imidazole to transcribe in solution (lanes 6 and 7). The nucleosome and hexasome mobility controls (lanes 4, 8, and 9) were created by ligating 204 bp reconstituted nucleosome and hexasome to 5' end-labeled 110 bp T7A1 promoter fragment. The mobilities of the ECs, nucleosomes (N1a, N1b, N2a, and N2b), hexasomes (not resolved by position), free DNA, and unligated DNA are indicated.

EC45 with ATP, CTP, and GTP served as a control for nontranscribed templates released due to residual falling apart of the early ECs (lane 2). After the addition of all 4 NTPs, fully transcribed templates were released into the supernatant (lane 3). The release of full length transcript into solution after

incubation of EC45 with all 4 NTPs can be confirmed in a control experiment with labeled transcripts rather than labeled templates.<sup>4</sup> Transcription through the nucleosome by *E. coli* RNAP resulted in the appearance of a faster migrating band (lane 3) as compared to the mobility of the original nucleosomes (lanes 1 and 4). This band had the same mobility in a native gel as the reconstituted hexasome control (lane 9). The identity of hexasome can also be confirmed using other approaches described previously.<sup>23</sup>

1. EC45 was formed by incubating EC11 in the presence of 0.5 mg/ml Acetylated-BSA, 20  $\mu\text{g/ml}$  Rifampicin (Sigma, St. Louis, MO), and 100  $\mu\text{M}$  ATP, CTP, and GTP in TB300 for 10 min at RT. The final volume of the reaction was 34  $\mu\text{l}$ . Rifampicin was added to limit transcription to a single round. The reaction was mixed, centrifuged for 10 s, and 8  $\mu\text{l}$  of supernatant was collected (-transcription control: EC11 + ACG, contains templates released as a result of EC instability during transcription of the initial 40 bp of the template rather than templates that were transcribed to completion).
2. EC45 was washed 3 times with 0.5 ml TB300 containing 20  $\mu\text{g/ml}$  of Rifampicin (TB300 + Rif), incubated in 0.5 ml TB300 + Rif for 15 min at RT, and washed 3 times with 0.5 ml TB300 + Rif.
3. The sample was supplemented with 0.5 mg/ml Acetylated-BSA in a 70  $\mu\text{l}$  volume. Thirty microliters was aliquoted into 2 tubes.
4. Transcription was performed in a final volume of 15  $\mu\text{l}$  for 15 min at RT by the addition of NTPs.
  - A. Mock Transcription control (EC45 + ACG, contains templates released as a result of dissociation of ECs during transcription of the initial 40 bp of the template rather than templates that were transcribed to completion): The 15  $\mu\text{l}$  reaction contained 200  $\mu\text{M}$  ATP, CTP, and GTP.
  - B. Transcription (EC45 + NTPs, contains fully transcribed templates): The 15  $\mu\text{l}$  reaction contained 200  $\mu\text{M}$  of all 4 NTPs.
5. The sample was mixed, centrifuged for 10 s, and 8  $\mu\text{l}$  of supernatant was collected from each sample.
6. The three supernatant species collected (EC11 + ACG, EC45 + ACG, and EC45 + NTPs) can be aliquoted for further analysis (such as restriction enzyme mapping). Each sample is enough for 1–6 aliquots.
7. Buffer is added providing a final concentration of 10 mM EDTA, 10% sucrose, 50  $\mu\text{g/ml}$  sheared herring testes DNA (Intergen, Purchase, NY), and 350 ng of donor chromatin (to block nonspecific interactions with the wells of the gel). The donor chromatin was obtained as described.<sup>29</sup>

8. The templates are resolved by native gel electrophoresis (4.5% acrylamide [39:1], 5% glycerol, 20 mM Na-HEPES [pH 8.0], 0.1 mM EDTA) at 100 V for 2.5–4 h (depending on the size of the DNA fragment and the degree of resolution desired) as described.<sup>23</sup>

### *Transcription in Solution*

Transcription in solution is an important control experiment to evaluate whether immobilization of RNAP changes the pathway of transcription through nucleosomes. Transcription in solution can be performed very similarly to what was described above. It is recommended to start with the reaction in the immobilized state so that excess promoter fragments and unligated nucleosomes can be removed from the reaction. Immobilized RNAP is then eluted from the resin using imidazole. In fact, if the imidazole elution is performed after the formation of EC45, the nontranscribed templates released as a result of EC dissociation during early transcription can also be removed from the reaction. See Fig. 4 (lanes 5–7) for an example of the results one should obtain using this procedure.

1. Washed EC45 was eluted with 100 mM imidazole (pH 7.5) in TB300 + Rif containing 0.5 mg/ml Acetylated-BSA for 10 min at RT. The final volume of the elution mix was 45  $\mu$ l.
2. The sample was mixed, centrifuged for 10 s, and 22  $\mu$ l of supernatant was collected. Ten microliters of the sample was aliquoted into 2 tubes. The sample was diluted 2-fold with TB300 + Rif containing 0.5 mg/ml Acetylated-BSA.
3. Transcription is performed in the presence of 200  $\mu$ M NTPs, and a mock transcription control is performed in the presence of 200  $\mu$ M ATP, CTP, and GTP for 15 min at RT.
4. The reactions are stopped by the addition of transcription stop/gel loading buffer providing 10 mM EDTA, 10% sucrose, 50  $\mu$ g/ml sheared herring testes DNA, and ~350 ng of donor chromatin.
5. Analysis of templates by native PAGE was performed as described earlier.

### *Reconstitution and Purification of Hexasomes from Purified Histones*

Transcription through the nucleosome by Pol II and *E. coli* RNAP results in the loss of an H2A/H2B dimer and the formation of a subnucleosomal particle, the hexasome.<sup>3,4</sup> Thus, hexasomes are reconstituted on the 204 bp pVT1 template, gel purified, and ligated to the T7A1 promoter

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<sup>29</sup> J. Ausio, F. Dong, and K. E. van Holde, *J. Mol. Biol.* **206**, 451 (1989).

fragment for use as a mobility control in native gel electrophoresis. Hexasomes are reconstituted from purified histones.<sup>3,23</sup> The reconstitution protocol described below is a slightly modified version of the method used by the Bradbury laboratory,<sup>24,25</sup> and is described in more detail in Walter and Kireeva.<sup>23</sup>

1. Five micrograms of DNA is mixed with 1.23  $\mu\text{g}$  of H3/H4 and 0.67  $\mu\text{g}$  of H2A/H2B (ratio of H3/H4:H2A/H2B = 1.82).
2. Dialysis is performed at 4° 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 h at each step.
3. Reconstitutes are collected in low adhesion microcentrifuge tubes (USA Scientific, Ocala, FL) and supplemented with buffer providing a final concentration of 10% sucrose.
4. The templates are resolved by native gel electrophoresis as described above.
5. The appropriate band is cut out of the gel, the gel is crushed, and the hexasomes are extracted overnight at 4° in 1–2 volumes of 10 mM Na-HEPES (pH 8.0), 0.1 mM EDTA, and 0.5 mg/ml BSA. Note that the hexasome preparation always contains nucleosomes, so nucleosomes can also be purified at this stage.
6. The supernatant is collected and the concentration of the sample is determined by the specific activity of the DNA.

*Ligation of the 204 bp Nucleosomes or Hexasomes to the T7A1 Promoter Fragment for Use as Mobility Controls in Native PAGE*

It is suggested that a nontranscribed nucleosome control for mobility during native PAGE be used. Then, the starting material can be directly compared to the transcribed template (see Fig. 4, lanes 4 and 8). This is also a useful tool for mapping of the nucleosomes after ligation to the promoter fragment (see Fig. 2). Also, since transcription leads to the formation of a hexasome, a reconstituted hexasome mobility control is usually provided in the analysis of the transcribed template (see Fig. 4, lane 9).

1. Gel-purified 204 bp hexasomes or nucleosomes from gel-purification or donor chromatin exchange are ligated to 5' <sup>32</sup>P-labeled TspRI-cut T7A1 DNA as mobility controls for the 314 bp hexasomes or 314 bp nontranscribed nucleosomes. Equimolar amounts of the promoter fragment and the 204 bp hexasomes or nucleosomes are incubated in the presence of 100  $\mu\text{M}$  ATP, 1% PEG-8000, and 50 units of T4 DNA ligase (New England Biolabs, Beverly, MA) at 12° for 1–2 h.

2. The samples are aliquoted and prepared for digestion or electrophoresis as described for the mononucleosome preparation above.

### Concluding Remarks

It is a tremendous advantage that *E. coli* RNAP and Pol II use the same mechanism for transcription through the nucleosome.<sup>4</sup> The EC assembly protocol used for transcription with Pol II<sup>19</sup> and *E. coli* RNAP,<sup>21</sup> though highly useful, has its limitations as well. Assembled ECs tend to be more susceptible to dissociation during transcription of the early region of the template, whereas promoter-initiated ECs do not fall apart as readily.<sup>3,4,23</sup> Moreover, reactions with promoter initiated RNAP can be scaled up by transcribing in solution rather than being limited by the capacity of the Ni<sup>2+</sup>-NTA agarose resin.

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## [30] Reconstitution and Transcriptional Analysis of Chromatin *In Vitro*

By WOJIN AN and ROBERT G. ROEDER

The DNA in eukaryotic cells is packaged by histones and nonhistone proteins to form a highly repressive structure known as chromatin. The basic unit of chromatin is the nucleosome, which consists of 146 bp of negatively supercoiled DNA wrapped around an H3/H4 tetramer and a pair of H2A/H2B dimers.<sup>1,2</sup> It has become increasingly evident that covalent modifications of histones and ATP-dependent remodeling of chromatin structure play major roles in the regulation of a variety of nuclear processes involving DNA, but the underlying mechanisms are not well characterized.<sup>3-7</sup> This

<sup>1</sup> K. E. van Holde, "Chromatin." Springer, New York, 1988.

<sup>2</sup> K. Luger, A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond, *Nature* **389**, 251 (1997a).