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Nucleosome Remodeling Induced by RNA Polymerase II: Loss of the H2A/H2B Dimer during Transcription

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Summary

RNA polymerase II (Pol II) must transcribe genes in a chromatin environment in vivo. We examined transcription by Pol II through nucleosome cores in vitro. At physiological and lower ionic strengths, a mononucleosome imposes a strong block to elongation, which is relieved at increased ionic strength. Passage of Pol II causes a quantitative loss of one H2A/H2B dimer but does not alter the location of the nucleosome. In contrast, bacteriophage SP6 RNA polymerase (RNAP) efficiently transcribes through the same nucleosome under physiological conditions, and the histone octamer is transferred behind SP6 RNAP. Thus, the mechanisms for transcription through the nucleosome by Pol II and SP6 RNAP are clearly different. Moreover, Pol II leaves behind an imprint of disrupted chromatin structure.

Introduction

The DNA within the nucleus is packaged as chromatin, a nucleoprotein complex composed of repeating nucleosomes. Each nucleosome consists of ~150 bp of DNA wrapped in two coils around the histone octamer. The octamer is composed of two units each of histones H2A, H2B, H3, and H4 and has a tripartite organization. The H3/H4 tetramer contacts the ~90 bp central part of the nucleosomal DNA, and two H2A/H2B dimers are bound at each of the ~30 bp ends of the DNA (Luger et al., 1997). Linker histone H1 binds at the point where DNA enters and exits the nucleosome core and also to the linker DNA.

Nucleosomes are present even when genes are actively transcribed by Pol II, but nucleosome positioning is often changed, and some nucleosomes are lost when transcription is induced (for recent reviews see Clark [1995] and Orphanides and Reinberg [2000]). At the same time, even a single nucleosome can present a strong barrier for Pol II in vitro (Izban and Luse, 1991). These observations raise the following important questions. How does the nucleosomal structure accommodate passage of RNAP? What is the nature of the nucleosomal barrier to transcription? And, how does RNAP overcome the nucleosomal barrier?

Recently, several laboratories have addressed these questions using different model systems in vitro. It has been shown that nucleosomes remain associated with DNA during transcription by bacteriophage RNAPs in vitro (Clark and Felsenfeld, 1992; O'Donohue et al., 1994; Studitsky et al., 1994) and that the histone octamer is directly transferred during transcription toward the promoter-proximal portion of the DNA (Studitsky et al., 1994). The data obtained using phage RNAPs are of a special interest since it has been shown that the mechanisms of transcription through the nucleosome by eukaryotic yeast RNA polymerase III (Pol III) and SP6 RNAP are remarkably similar (Studitsky et al., 1997).

At the same time, our knowledge about transcription through the nucleosome by Pol II is limited. Nucleosomes can survive transcription by Pol II in vitro (ten Heggeler-Bordier et al., 1995), but nucleosomes present a very strong barrier for the enzyme (Izban and Luse, 1991), which cannot be relieved even when transcription is conducted in the presence of elongation factors (Chang and Luse, 1997; Izban and Luse, 1992).

In this work, Pol II transcription through the nucleosome was analyzed by assembling elongation complexes (ECs) from histidine-tagged yeast Pol II and synthetic RNA and DNA oligonucleotides (Kireeva et al., 2000b; Sidorenkov et al., 1998) and ligating the complexes to a template with a single, positioned nucleosome. In this "minimal" system, a single nucleosome imposed a strong barrier to Pol II, which could be reduced by increasing the ionic strength of the reaction. Transcription through the nucleosome by Pol II resulted in the loss of an H2A/H2B dimer, apparently without changing the position of the histones on the DNA. In contrast, transcription through the same nucleosome by bacteriophage SP6 RNAP was accompanied by efficient transfer of the complete histone octamer. Thus, eukaryotic Pol II employs a novel mechanism for transcription through the nucleosome, which results in considerable disruption of the chromatin structure.

Results

Assembly of Pol II Elongation Complexes on Mononucleosomal Templates with Positioned Cores

Elucidation of the mechanism of transcription through the nucleosome by Pol II requires an efficient method of transcription initiation. Our initial attempt to utilize a widely accepted method of initiation on an oligo-dC-tailed template (Kadesch and Chamberlin, 1982) failed, since transcription resulted in the formation of a long RNA:DNA hybrid. Therefore, an alternative approach involving assembly of Pol II ECs (Kireeva et al., 2000b) was used. The assembled ECs are characterized by efficient displacement of the 5′ end of nascent RNA by the nontemplate DNA strand (Kireeva et al., 2000a,

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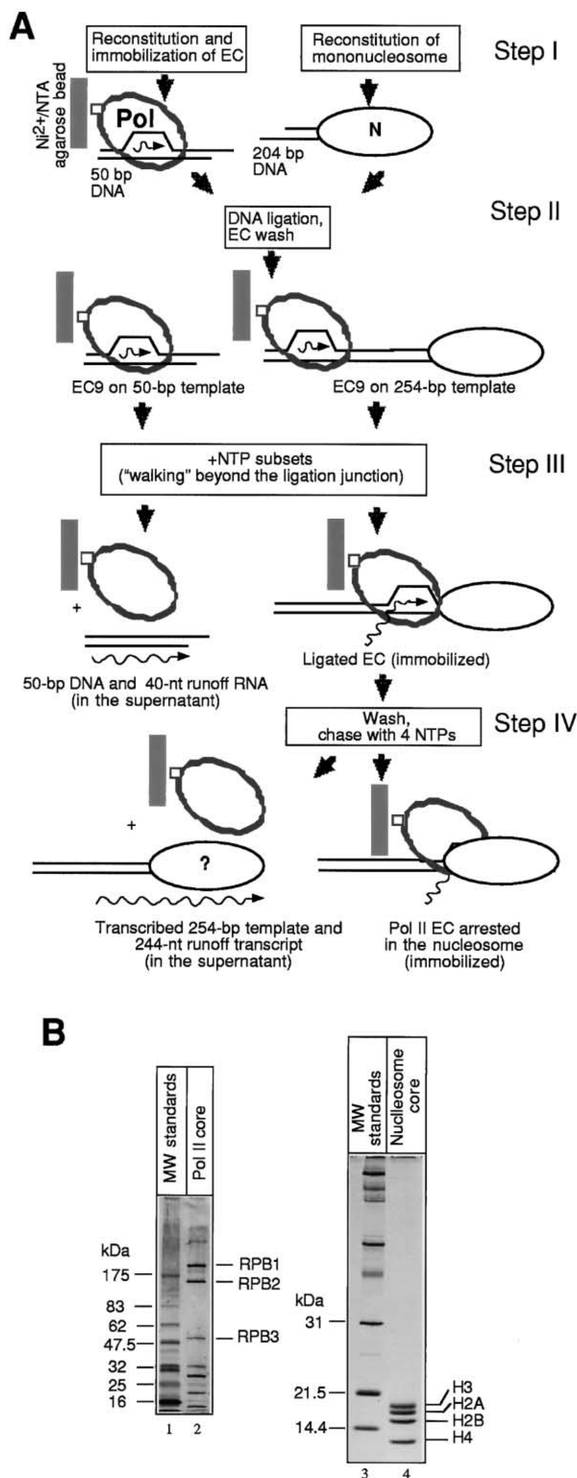

Figure 1. Experimental System for the Study of Pol II Transcription on DNA and Mononucleosomal Templates

(A) The primary experimental approach. The DNA strands are shown as bold black lines, the RNA is represented by a wavy line with an arrow at the 3′ end. The nucleosome is marked "N." The Pol II is marked "Pol" with a small square for a hexahistidine tag. The surface of the Ni^{2+}-NTA agarose bead is shown as a rectangle. See text for details.

(B) Analysis of the Pol II and nucleosome core particles by SDS polyacrylamide gel electrophoresis. The mobilities of the three largest Pol II subunits and all histone proteins are indicated on the right side of each gel. The sizes of the molecular weight markers are shown on the left of each gel.

2000b). In this work, we have developed a novel in vitro system where immobilized ECs are ligated to templates containing positioned nucleosome cores, as outlined in Figure 1A.

Briefly, EC reconstitution was performed as follows: (i) the 9 nt RNA was annealed to a 50 nt template DNA strand (TDS50) followed by binding of hexahistidine-tagged Pol II core enzyme (Figure 1B, lane 2); (ii) the complementary nontemplate DNA strand (NDS59) was then hybridized to the template DNA strand in the complex, completing the formation of the transcription bubble. This created a 9 nt 3′ end overhang on the downstream edge of EC9 (the numerical index indicates the length of the RNA in the complex), which was later used to ligate the EC to the mononucleosome; and (iii) the assembled EC9 complex was immobilized on Ni^{2+}-NTA agarose beads (see Figure 1A, step I, left).

In a separate reaction, mononucleosomes were reconstituted by histone octamer transfer from long −H1 chromatin on a 204 bp DNA fragment containing the *Xenopus borealis* somatic *5S* RNA gene nucleosome positioning sequence (Hayes et al., 1991). The purity and stoichiometry of the core histones were verified by SDS-PAGE (Figure 1B, lane 4). The nucleosome (Figure 1A, step I, right) was ligated to the immobilized EC9, and any nonligated 204 bp template was washed away (step II). The resulting immobilized ECs existed on either nonligated 50 bp templates or ligated 254 bp templates.

Immobilization of Pol II makes it possible to obtain ECs stalled at any position on the template through the addition of NTP subsets ("walking," step III). The DNA sequence located between the 3′ end of the RNA oligonucleotide (+9 position; the +1 position indicates the 5′ end of the RNA primer), and the ligation junction has no adenosine residues in the template strand until position +46. Therefore, in a transcription reaction lacking UTP, Pol II runs off the unligated 50 bp template, and on the full-length template, it transcribes across the ligation junction to form EC45.

Upon addition of all four NTPs (step IV), Pol II could either complete transcription, releasing the run-off transcript and the fully transcribed template, or become arrested before reaching the end of template with the nascent RNA and the template remaining bound to the immobilized polymerase. The third possible scenario, dissociation of the EC and the release of templates that were not transcribed to completion, could be avoided (as demonstrated in Figure 4) and is not depicted in the cartoon. Thus, immobilization of Pol II in solid phase provided a convenient tool for separation and analysis of the nucleosomal templates that were completely transcribed.

Analysis of Nucleosome Positioning Before and After Ligation to the EC

Nucleosome positioning on the 204 bp template was analyzed by restriction enzyme mapping and native gel electrophoresis (Figure 2A). Nucleosomes positioned

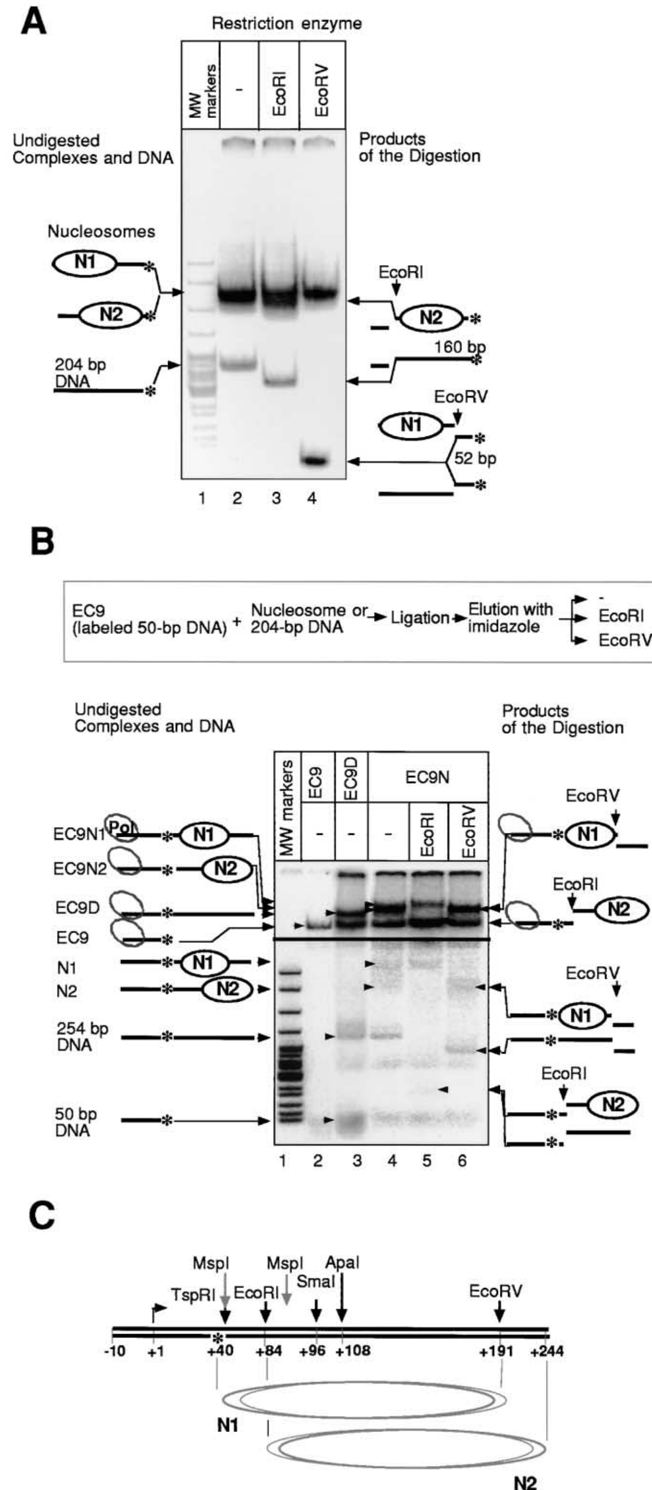

Figure 2. Nucleosome Positioning on the DNA

(A) The 204 bp TspRI-StuI mononucleosomes are a mixed population of two differently positioned nucleosome cores. The 204 bp nucleosomal template (labeled at the StuI end, marked by an asterisk) was analyzed in a native gel before or after digestion with EcoRI or EcoRV. The mobilities of the nucleosomes, 204 bp DNA, and the products of their digestion by EcoRI and EcoRV are indicated. The same molecular weight marker (an MspI digestion of pBR322, lane 1) was used in all native gels.

(B) Nucleosome positioning does not change after ligation to the Pol II EC. EC9 was assembled with a labeled TDS50 (marked by an asterisk) and ligated to the DNA or nucleosomal template. Imidazole-eluted complexes were analyzed before and after digestion with EcoRI and EcoRV. The part of the gel shown below the horizontal line has been exposed for a longer time than the upper part. The EC9 ligated to the N1 and N2 nucleosomes (EC9N1 and ECN2), EC9 ligated to naked DNA (EC9D), unligated EC9 (EC9), and the products of EC dissociation (free nucleosomes, 254 bp DNA and unligated, 50 bp DNA) are indicated.

(C) Positions of the nucleosomes on the ligated template. The position of the radioactive label is shown by asterisk. The numbers below the DNA show the position on the template relative to the 5′ end of the RNA (+1). The positions of TspRI, EcoRI, EcoRV, SmaI, MspI, and ApaI cleavage sites are indicated by arrows. The two positions of the nucleosomes N1 and N2, and the microheterogeneity revealed by micrococcal nuclease mapping are shown by ovals.

differently on DNA fragments shorter than 200 bp have the same mobilities in a native gel (Pennings et al., 1991), but different positions can be discriminated using restriction enzymes because nucleosomal DNA is highly protected from digestion (Studitsky et al., 1994). EcoRI and EcoRV, each having a unique recognition site at the opposite flanks of the 204 bp DNA, were used for the analysis. Unexpectedly, only ~50% of the nucleosomal DNA was sensitive to EcoRI (lane 3), indicating that only about half of the nucleosomes occupied the 5S nucleosome positioning sequence (N2). The remaining (N1) nucleosomes were sensitive to EcoRV treatment. The distance from the left DNA end (TspRI site) to the EcoRV site was 152 bp, and the distance from the right DNA end to the EcoRI site was 160 bp. These distances are close to the minimal 146 bp DNA length occupied by

the histone octamer (Luger et al., 1997). Thus, EcoRV marked the right border of the N1 nucleosome, and EcoRI marked the left border of the N2 nucleosome. Nucleosome positioning was further confirmed by mapping with micrococcal nuclease (data not shown).

Nucleosome positioning was also analyzed after ligation to the EC. Immobilized EC9 with labeled TDS50 was ligated to the unlabeled 204 bp DNA or nucleosomal templates, and the products were analyzed by native PAGE after elution from the resin with imidazole (Figure 2B). As expected, the EC9 mobility (lane 2) progressively decreased after its ligation to the 204 bp naked DNA (EC9D, lane 3) and to the nucleosome (EC9N, lane 4). The efficiency of ligation was about 50% for both the naked DNA and the nucleosome. Approximately half of the DNA in the EC9N was resistant to EcoRI (lane 5), and the other half was resistant to EcoRV (lane 6). In addition, a small fraction of free nucleosomes (5%–10% of the total radioactivity in the lane), derived from spontaneous dissociation of the EC9N during imidazole elution (lanes 4–6), contained the N1 and N2 nucleosomes at the same 50/50 ratio as before ligation (Figure 2A). Thus, the presence of the nucleosome in either position did not affect the efficiency of the ligation, and the positions of the nucleosomes did not change after ligation. A detailed map of the ligated 254 bp template is shown in Figure 2C.

The Nucleosome Is a Strong Barrier for Pol II

The nature of the nucleosomal barrier to Pol II was investigated by comparing the efficiency of transcription on the naked DNA and nucleosomal templates (Figure 3). For selective analysis of only the ligated complexes, Pol II was walked from EC9 to form EC45 and from EC45 to EC49, and the RNA was labeled at positions +51, +53, and +55 by incubation with α-[^{32}P]ATP and unlabeled CTP during the formation of EC55. The resulting ECs consisted of a mixture of EC55 and EC64 (Figure 3A, lane 2), which is most likely due to contamination of the α-[^{32}P]ATP with GTP. The amount of the labeled RNA was consistently similar for the naked DNA and nucleosomal templates (lanes 2 and 8, and data not shown), suggesting that the presence of the nucleosome in either position did not significantly affect transcription upstream of position +55.

Transcription was resumed from EC55 at different concentrations of KCl with the addition of all four NTPs (Figure 3A). On free DNA, the polymerase was able to complete the synthesis of the 244 nt run-off transcript with negligible pausing or arrest at all KCl concentrations tested (lanes 3–7). In contrast, the nucleosome presents an almost absolute block to transcription at or below physiological ionic strength (40 and 150 mM KCl, lanes 9 and 10), halting the polymerase in a wide range of positions within the +65 to +150 region. The majority of the run-off transcript produced, which constitutes less than 10% of the total radioactivity in the lane under these conditions, can probably be attributed to transcription of the small amount of free DNA contamination in the nucleosome preparation. In 300 mM KCl, where the nucleosome is not disrupted (Walter and Studitsky, 2001; Yager and van Holde, 1984), about 30% of the Pol II could pass through the nucleosomal barrier (lane 11).

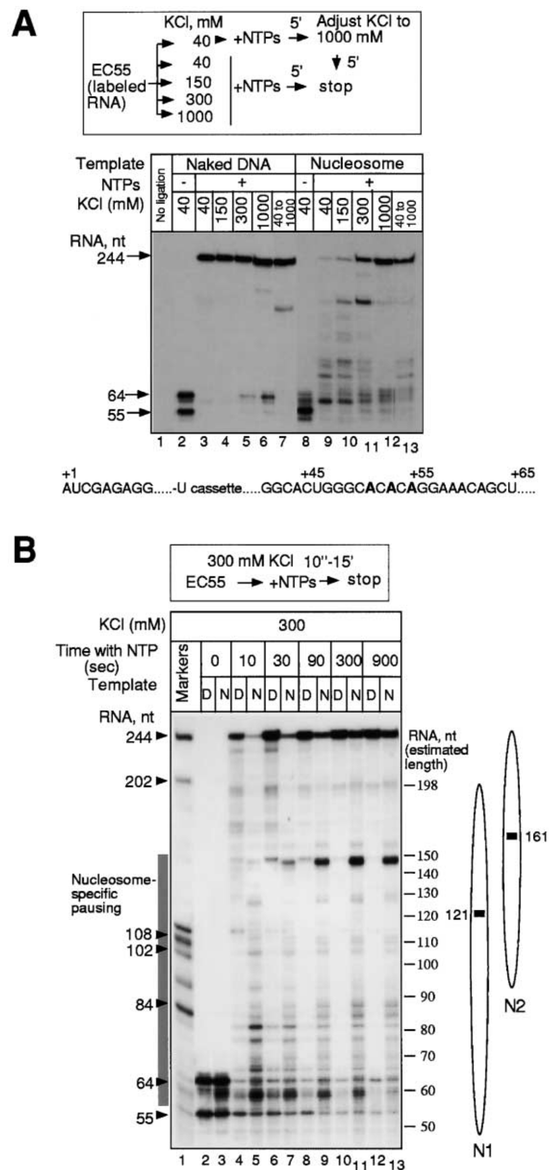

Figure 3. The Nucleosome Prevents Efficient Transcript Elongation by Pol II

(A) The strong nucleosomal block to Pol II elongation is relieved at elevated ionic strength. EC55 contained RNA labeled at +51, +53, and +55 (shown in bold in the sequence below the gel). The sample shown in lane 1 was not ligated. Arrows on the left of the gel indicate the initial 55 nt and read-through 64 nt RNA products and the 244 nt run-off transcript. The samples shown in lanes 3–6 and 9–12 were incubated with NTPs at the indicated concentration of KCl. Lanes 7 and 13 are the EC55 incubation with NTPs in TB40 for 5 min, subsequent increase of the KCl concentration to 1 M, and incubation of EC with NTPs for an additional 5 min.

(B) Time courses of transcription on the naked DNA and nucleosomal templates in 300 mM KCl. To obtain markers (lane 1), EC55 ligated to naked DNA was digested with EcoRI, ApaI, or Sau3A and chased with NTPs. A shaded bar on the left outlines the region of nucleosome-specific pausing. RNA lengths and positions of nucleosomes N1 and N2 are indicated on the right.

When the ionic strength was increased to 1 M KCl, thus destabilizing the nucleosome (Yager and van Holde, 1984) but leaving the EC intact (Kireeva et al., 2000b),

Pol II was able to complete transcription (lane 12). Furthermore, when a transcription reaction conducted at 40 mM KCl was subsequently continued at 1 M KCl, more than 50% of the polymerase molecules that were initially arrested in the nucleosome at the lower ionic strength were able to finish transcription (compare lanes 9 and 13). Thus, the majority of the ECs trapped in the nucleosome at the lower ionic strength were intact, but arrested, and the nucleosome-specific barrier was at least partially reversible.

Time courses of transcription on the DNA and nucleosomal templates were analyzed under conditions where ∼30% of the ECs were able to complete transcription (300 mM KCl, Figure 3B). After 30 s, transcription of the histone-free DNA was nearly complete (lane 6) while only a small fraction of the nucleosomal templates were transcribed to completion (lane 7). Thus, even at an elevated ionic strength, the rate of the run-off transcript accumulation on the nucleosomal template was more than ten times slower than that on the naked DNA.

Transcript elongation on naked DNA is marked by several transient pauses (estimated positions +57, 58, 75, 80, 105, 113, 115, 129, 138, 140, 147, 155, 170, 175, 185, 195, and 235), which are completely chased after a 15 min incubation with NTPs (lane 12). Several products (positions +55, +64, +65, and 200) result from EC arrest and do not completely disappear upon prolonged incubation with NTPs.

Unlike most of the ECs paused on the naked DNA, the majority of paused intermediates on the nucleosomal template (positions +57, 58, 65, 69, 75, 80, 84, 87, 92, 105, 109, 113, 126, 145, 155, and 200) tend to become arrested and are not completely chased upon the prolonged incubation with NTPs. About one half of the nucleosome-specific sites of pausing and arrest coincide with the sites of transient pausing on naked DNA (positions +57, 58, 65, 75, 80, 105, 113, and 155), in agreement with results reported by Izban and Luse (1991). However, the most prominent arrest site at position +145, along with weaker sites at +69, 84, 87, 92, and 126, do not coincide with even minor pausing on the naked DNA. Thus, the nucleosome has multiple effects on RNA chain elongation: (i) it dramatically enhances the sequence-specific pause sites; (ii) it increases the amount of ECs converted into the arrested state; and (iii) it induces new pause and/or arrest sites.

Transcription by Pol II Removes an H2A/H2B Dimer from the Nucleosome

To analyze the fate of the nucleosome after transcription by Pol II, EC9 was assembled using labeled TDS50, and ligated to the nucleosomal and naked DNA templates. The ECs were incubated in the presence of ATP, CTP, and GTP to allow the polymerase to form EC45 on the ligated template or run off the unligated 50 bp template. The washed, immobilized EC45D and EC45N were eluted from the beads with imidazole and analyzed by native PAGE (Figure 4A) after the addition of ATP, CTP, and GTP (mock-transcription control, lanes 2 and 4) or all four NTPs (chase, lanes 3, 5, and 6). The intact EC formed on the naked DNA template migrated as three distinct bands in the native gel (lane 2). The upper band was quantitatively converted to free 254 bp DNA after incubation with all four NTPs (compare lanes 2 and 3), indicating that it contained transcriptionally active EC45D. On the contrary, the two lower bands were not affected by the addition of NTPs, indicating that these were arrested ECs containing ligated 254 bp DNA (upper band) or unligated 50 bp DNA (lower band). This arrest of ECs with 15–50 nt RNAs is a common property of Pol II transcription in vivo and in vitro (Kireeva et al., 2000a; Pal et al., 2001; reviewed by Lis, 1998). The increased mobility of the arrested ECs has been observed before (Zaychikov et al., 1999).

EC45N also migrated as three bands in the native gel (lane 4), and only the upper band was affected by incubation with all four NTPs (lanes 5 and 6). As a result of NTP addition in both 300 and 150 mM KCl (lanes 5 and 6), the labeled DNA from the active EC45N band was distributed between slower migrating complexes (presumably ECs arrested at different points within the nucleosome) and two products dissociated from Pol II: a novel nucleoprotein band that migrated faster than the N2 nucleosome (compare with Figure 2B, lanes 1 and 4) and free 254 bp DNA. Despite the fact that the polymerase cannot overcome the nucleosomal barrier at 150 mM KCl as readily (see Figure 3A), the novel complex is also formed during transcription under these conditions (Figure 4A, lane 6), demonstrating that it is not an artifact of elevated ionic strength.

The products dissociated from Pol II in an NTP-dependent manner could be, in principle, released either as a result of run-off transcription (and represent fully transcribed nucleosomal templates) or as a result of dissociation of ECs arrested within the nucleosome. We took advantage of the immobilized system to isolate the templates dissociated from Pol II and to confirm that the templates released into the supernatant after NTP addition were the result of run-off transcription. Two parallel experiments were performed. In one case, the EC was assembled with labeled RNA9, and the transcripts in the supernatant and pellet fractions were analyzed by denaturing PAGE (Figure 4B, lanes 1–4). In the other case, the EC was assembled using labeled TDS50, and the template released to the supernatant was analyzed by native PAGE (Figure 4B, lanes 6–9).

The 244 nt run-off RNA (Figure 4B, lane 3) as well as the novel nucleoprotein complex and the 254 bp DNA (lane 8) are released from Pol II only as a result of EC45 incubation with all four NTPs. All of the ECs arrested in the nucleosome (lane 4) were stable and did not release transcripts into the supernatant (lane 3), indicating that only fully transcribed nucleosomal templates are dissociated from Pol II in an NTP-dependent manner. Only trace amounts of RNA shorter than 45 nt, along with a small amount of N1 and N2 nucleosomes and 50 bp DNA, are found in the supernatant of both the mock-transcribed (lanes 1 and 6) and transcribed (lanes 3 and 8) samples.

Transcription in physiological conditions (150 mM KCl) also resulted in the appearance of the novel nucleoprotein complex (Figure 4B, lane 7), but in a lesser amount as compared to 300 mM KCl (lane 8). This is in agreement with the amount of run-off transcripts produced at each of the salt concentrations (Figure 3A). Notably, the amount of histone-free DNA released at 150 mM KCl (Figure 4B, lane 7) and 300 mM KCl (lane

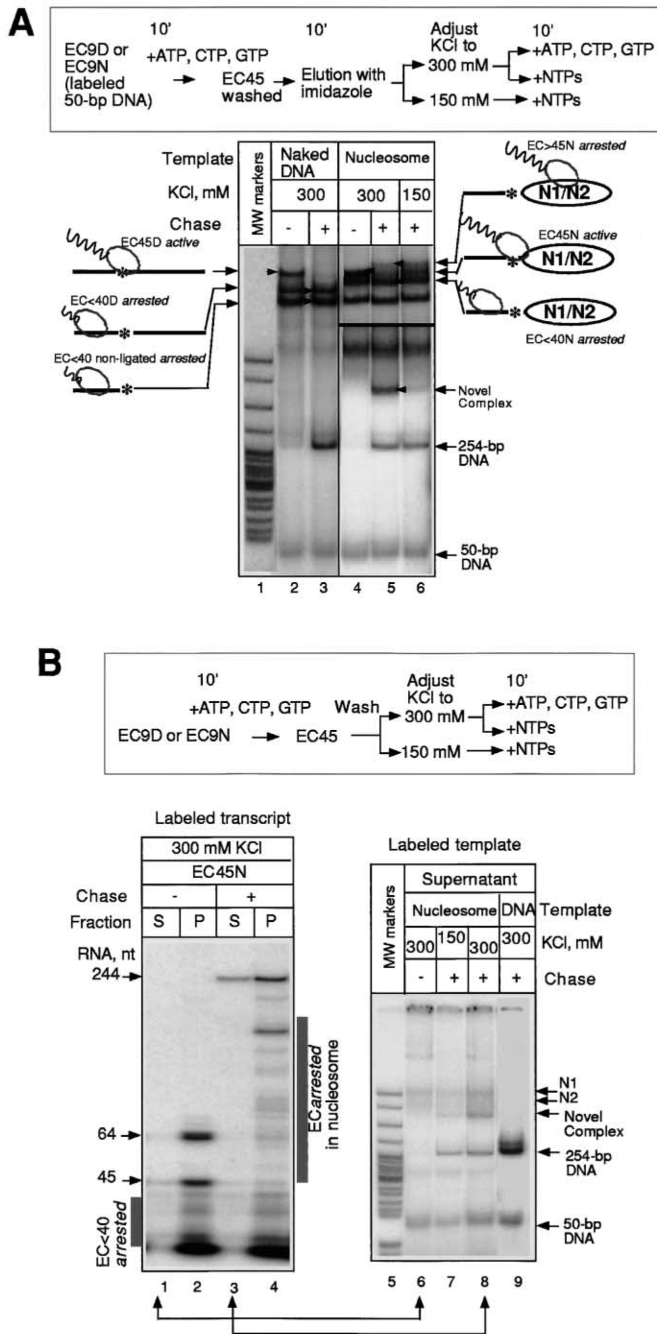

Figure 4. Transcription by Pol II Generates a Novel Nucleoprotein Complex

(A) Chase of Pol II ECs results in the formation of a novel nucleoprotein complex. The part of lanes 4–6 below the horizontal black line has been exposed for a longer time than the upper part of the gel. The positions of the active EC45 (EC45D *active*) and arrested ECs with RNA shorter than 45 nt (EC < 45D *arrested*) on the naked 254 bp DNA are indicated on the left along with arrested ECs on the nonligated 50 bp DNA (EC < 45 *arrested* nonligated). The positions of active EC45 (EC45N *active*) and ECs arrested upstream of the ligation junction (EC < 45N *arrested*) on the nucleosomal template and ECs arrested in the nucleosome (EC > 45N *arrested*) are illustrated on the right. The wavy line depicts the RNA in the EC. Mobilities of ECs containing N1 and N2 nucleosomes are indistinguishable on this gel.

(B) The novel nucleoprotein complex and free DNA dissociate from Pol II as a result of run-off transcript formation. ECs containing 5′ end-labeled RNA (lanes 1–4) or labeled TDS50 (lanes 5–9) were treated as outlined on the top. One half of the supernatant (S) from the EC with labeled RNA was withdrawn and loaded to a 6% denaturing gel along with the remaining supernatant and pellet (P). Arrows at the left indicate the positions of the 45 nt RNA and the 64 nt read-through product synthesized on the 254 bp template in the presence of ATP, CTP, and GTP and the 244 nt run-off transcript. The positions of RNAs from the arrested ECs are shown by shaded bars. Supernatant fractions collected from the ECs with labeled templates were analyzed in a native gel. The arrows below the gel point to the lanes showing identical fractions. The positions of the nucleosomes, novel complex, and DNA are indicated.

8) is similar, suggesting that this is the result of transcription of the small amount of free DNA contamination present in the nucleosome preparation. Therefore, the release of the free DNA is not likely to be the result of histones being stripped from the nucleosomal template during transcription. As expected, transcription of the naked DNA template leads to the release of only the 254 bp free DNA (Figure 4B, lane 9). The nucleosomes are quantitatively converted into the novel complex: the small amount of nucleosomes N1 and N2 in the transcribed fraction (Figure 4B, lanes 7 and 8) is the same as their amount in the mock-transcribed fraction (Figure 4B, lane 6).

The possibility that a new nucleosome position was generated as a result of transcription was highly unlikely because nucleosomes located at the end of a template (such as N2) have the fastest mobility in a native gel (Pennings et al., 1991). Therefore, the data suggested that the passage of Pol II resulted in the formation of a subnucleosomal complex. To identify the novel complex, histone octamer, hexamer (lacking one H2A/H2B dimer), and H3/H4 tetramer were reconstituted on the 204 bp DNA (Figure 5A), purified from the native gel, and ligated to the TDS50:NDS59 DNA duplex to create the 254 bp template.

The comparison of the mobility of the reconstituted

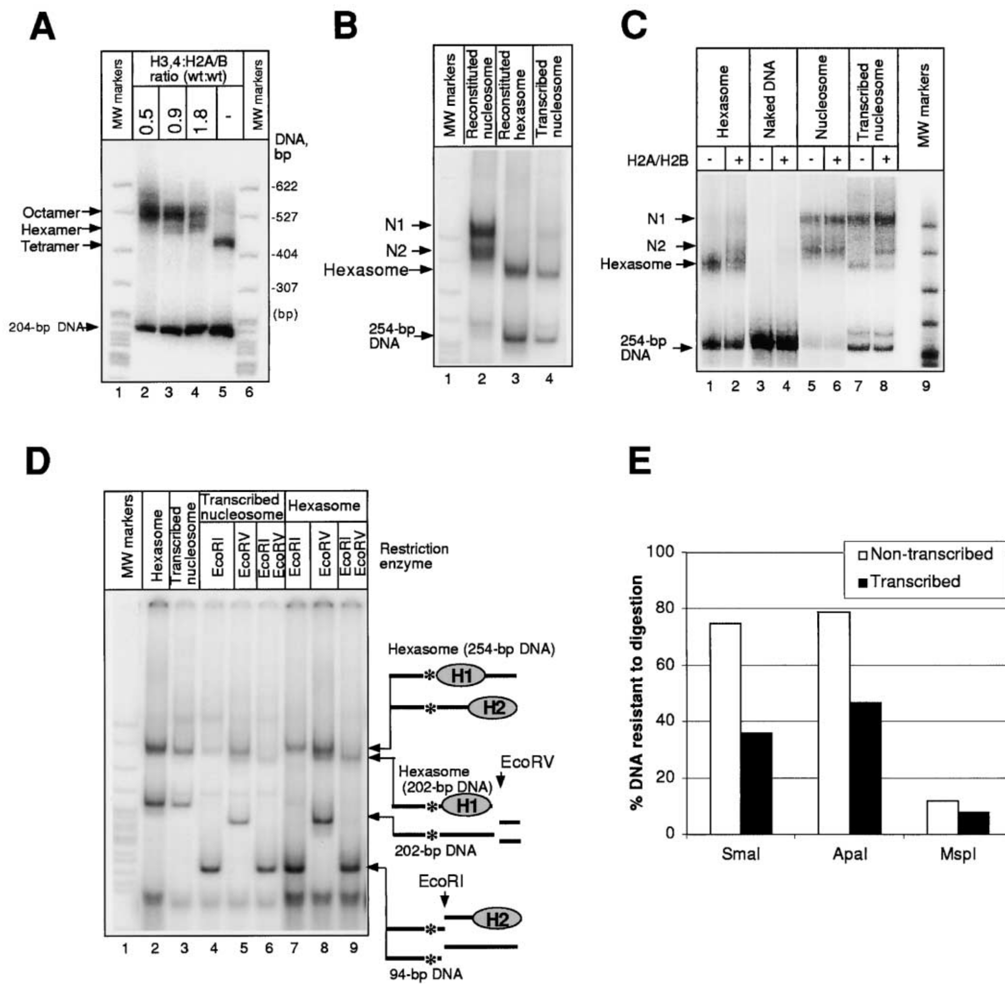

Figure 5. Transcription by Pol II Removes the H2A/H2B Dimer from the Nucleosome

(A) Reconstitution of nucleosomes and subnucleosomal complexes on the 204 bp TspRI-StuI fragment. The DNA was labeled at the StuI end. The approximate ratios of H3/H4 to H2A/H2B are indicated. Mobilities of the histone octamer, hexamer, and tetramer bound to DNA are indicated. Sizes of the markers are indicated at the right in base pairs.

(B) The novel subnucleosomal complex has the same mobility as the reconstituted hexasome. Transcribed nucleosome was obtained as in Figure 4B, lane 8. The 204 bp reconstituted nucleosome and hexasome were ligated to the labeled TDS50:NDS59 duplex.

(C) Addition of histones H2A/H2B to the hexasome restores the nucleosome. The 254 bp DNA, reconstituted nucleosomes, hexasomes, or the nucleosomal template transcribed by Pol II were incubated with H2A/H2B in TB300 and analyzed by native PAGE. The mobilities of the nucleosomes (N1 and N2), hexasomes, and the DNA template are indicated.

(D) Transcription-derived and reconstituted hexasomes have the same pattern of sensitivity to restriction enzymes. Hexasomes were obtained as in (B). The products of digestion of the hexasome are shown on the right.

(E) Hexasomes are not transferred during transcription by Pol II. Transcribed and nontranscribed nucleosomes were obtained as in (B) and resolved by a 6% denaturing PAGE before and after digestion with restriction enzymes. The gel was scanned, and the amount of radioactive label in the 254 bp DNA band was quantified. The results are shown as a graph (undigested sample was taken as 100%).

histone hexamer bound to the 254 bp DNA (hexasome) and the novel nucleoprotein complex are shown in Figure 5B. The transcription-derived novel complex has the same mobility in the native gel as the reconstituted hexasome (lanes 2 and 3), which strongly suggests that Pol II passage through the nucleosome results in the loss of an H2A/H2B dimer. This conclusion was further verified by adding histones H2A and H2B to the reconstituted hexasome and the transcribed template (Figure 5C). Upon addition of H2A/H2B, the amount of hexasome decreased while the amount of N1 and N2 nucleosomes increased (lanes 1 and 2). Incubation of the naked DNA template with the histones led to the formation of a very small amount of complexes with hexasome, N1, and N2 mobilities (lanes 3 and 4), presumably due to minor contamination of the H2A/H2B histones with histones H3/H4. As expected, H2A/H2B did not interact with the nucleosomal template (lanes 5 and 6). Finally, when the transcribed nucleosome was incubated with H2A/H2B, the amount of the product with the hexasome mobility decreased while the amount of nucleosomes N1 and N2 substantially increased (lanes 7 and 8). Thus, the product of transcription through the nucleosome by Pol II is a hexasome.

The conversion of the reconstituted hexasome to the original N1 and N2 nucleosomes upon incubation with

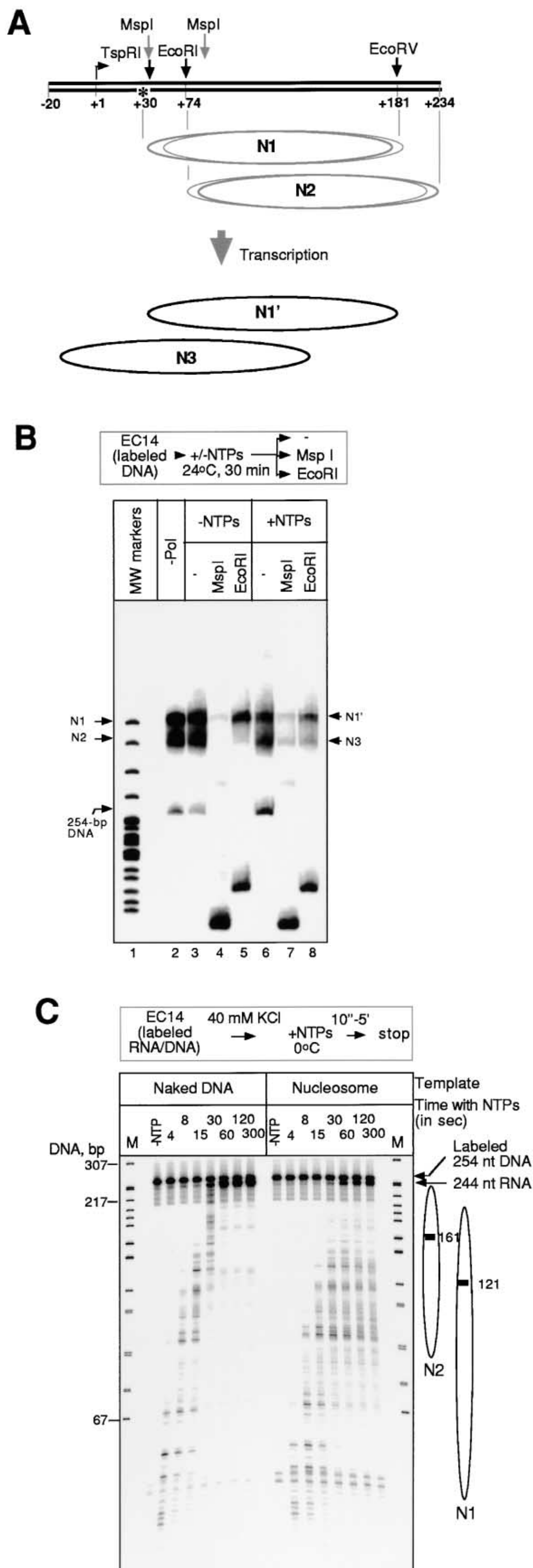

Figure 6. Efficient Transcription through the Nucleosome by SP6 RNAP Is Accompanied by Octamer Transfer

(A) The 254 bp SP6 RNAP template and the fate of nucleosomes during transcription. The first 14 bp to be transcribed constitute a −CTP cassette. The symbols are the same as in Figure 2C. The black ovals indicate the positions of the nucleosomes after transcription.

(B) The octamer is transferred during transcription with SP6 RNAP. Labeled nucleosomal templates were analyzed before and after transcription by their mobility in native PAGE and restriction enzyme mapping. The mobilities of the nucleosomes (N1, N1′, N2, and N3) and free DNA are indicated.

(C) Kinetics of transcription with SP6 RNAP on the 254 bp DNA and nucleosomal templates. DNA and nucleosomal templates were transcribed for indicated times at 0°C after the formation of EC14 (with the RNA pulse labeled during the formation of the 14-mer). Mobilities of the full-length RNA and labeled DNA (used for normalization) are indicated by arrows.

H2A and H2B suggests that the hexasomes occupy the same positions on the template as the corresponding nucleosomes. In agreement with this, the digestion patterns of the transcription-derived and reconstituted hexasomes are similar, with ∼50% of the complexes being sensitive to EcoRV and the others sensitive to EcoRI (Figure 5D). This conclusion was confirmed by mapping using other restriction enzymes. SmaI and ApaI sites, located in the DNA region shared by both nucleosomes (see Figure 2C), were highly protected in the nontranscribed nucleosomes (Figure 5E). A fraction of transcribed templates is sensitive to SmaI and ApaI (Figure 5E), which is consistent with the enrichment of this fraction by free DNA (see Figure 4B, lane 8). Most importantly, the MspI site located upstream of both N1 and N2 was fully accessible before and after transcription (Figure 5E), indicating that the nucleosomes were not transferred to the promoter-proximal end of the DNA. Thus, the histones remained in their original positions on the DNA after transcription.

In summary, our results demonstrate that transcription through the nucleosome by Pol II results in the dissociation of one of the H2A/H2B dimers and the formation of a hexasome. There is no indication that the nucleosome or hexasome is transferred upstream of the transcribing Pol II.

Intact Nucleosomes N1 and N2 Are Translocated during Transcription by SP6 RNAP

The experiments described above suggest that the mechanism of transcription through the nucleosome by Pol II is considerably different from the one used by SP6 RNAP, which completes transcription on at least 70% of the mononucleosomal templates at a low ionic strength and transfers the complete histone octamer to the promoter-proximal part of the template (Studitsky et al., 1995). To eliminate the possibility that the differences in the mechanisms were due to the use of different nucleosome positioning sequences, the nucleosomes used for transcription by SP6 RNAP were assembled on the same 204 bp DNA fragment that was used for transcription by Pol II and then ligated to a 50 bp DNA fragment containing a promoter for SP6 RNAP (Figure 6A).

To analyze the fate of the nucleosomes during transcription, the templates were resolved in a native gel

before and after transcription at low ionic strength. As expected, the mobilities of the nontranscribed nucleosomes (Figure 6B, lanes 2 and 3) were the same as the mobilities of the Pol II template nucleosomes N1 and N2 (Figures 2C and 4A). N2 is completely sensitive to EcoRI and MspI (Figure 6B, lanes 4 and 5), indicating that no nucleosomes are positioned on the promoter-proximal end of the 254 bp template. There is slight resistance of the N1 nucleosome to MspI due to heterogeneity in positioning (lane 4).

After transcription, ~10% of the histones dissociated from the template, resulting in an increased amount of histone-free DNA (Studitsky et al., 1994). In strict contrast to the data obtained with Pol II (Figure 5), no hexasome was detected after transcription. A fraction of the nucleosomes became resistant to MspI (Figure 6B, lane 7), suggesting that some of the histone octamers were transferred to the promoter-proximal end of the template (N3). The transfer was further confirmed by treatment with EcoRI: approximately the same fraction of N2 became resistant to EcoRI after transcription (lane 8).

Time courses of transcription on the DNA and nucleosomal templates are shown in Figure 6C. The rate of run-off transcript accumulation on the nucleosomal template is three to five times slower than on the naked DNA template, in agreement with previous data (Studitsky et al., 1995). Both the localization and the extended length of the pausing region are the expected results if the two individual nucleosomal pausing patterns were superimposed. As expected, the nucleosomal barrier is not as strong for SP6 RNAP as it is for Pol II.

Thus, we have shown that the lack of histone octamer transfer during transcription with Pol II is not an artifact of using different nucleosome positioning sequences, but rather, the result of different mechanisms employed by Pol II and SP6 RNAPs to transcribe through nucleosomes.

Discussion

In this work, a special experimental strategy combining the use of positioned nucleosome cores and Pol II ECs assembled in vitro was developed (Figure 1A). This approach revealed that at or below physiological ionic strength (40–150 mM KCl), a single nucleosome constitutes a very strong barrier for transcribing Pol II (Figure 3A). These observations are in agreement with the data obtained with promoter-initiated Pol II (Chang and Luse, 1997; Izban and Luse, 1991, 1992). Increasing the ionic strength allows transcription to continue. Surprisingly, when Pol II transcribes through the nucleosome, the majority of the nucleosomes are converted into hexasomes, which remain bound at or near the original positions of the nucleosome (Figures 4 and 5). Transcription of the same nucleosomes by SP6 RNAP gives a dramatically different result: the barrier is much lower, and complete histone octamers are transferred upstream of the polymerase (Figure 6).

Pol III and SP6 RNAP use very similar mechanisms for transcription through the nucleosome, including efficient transfer of the histone octamer (Studitsky et al., 1997). The loss of the H2A/H2B dimer was never observed with these model systems (Studitsky et al., 1994,

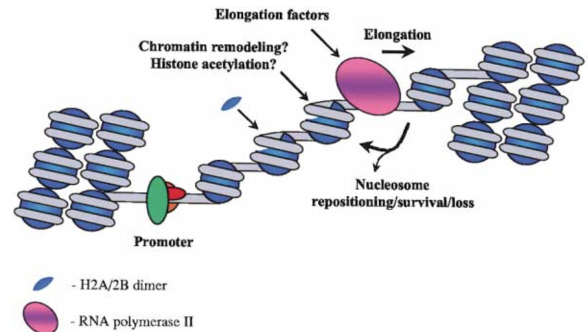

Figure 7. Hypothetic Mechanism of Transcription through Chromatin by Pol II In Vivo

Pol II converts nucleosomes in its path to DNA-bound histone hexamers. Facilitation of this process could be achieved with the aid of nucleosome-specific elongation factors such as FACT. Transcription may also result in other outcomes, which were previously observed on actively transcribed genes, such as nucleosome redistribution and partial nucleosome depletion. The loss of the H2A/H2B dimer from the nucleosome could lead to higher-order chromatin disruption. A window of opportunity might be provided for chromatin remodeling factors or DNA binding proteins by the transiently destabilized nucleosome structure. Unless the disrupted state is stabilized by chromatin remodeling machinery, complete nucleosomes are eventually restored.

1997) as it was with Pol II, even at higher ionic strength (Walter and Studitsky, 2001) or when SP6 ECs were halted in the nucleosome so that only 80 bp remained associated with the octamer (Bednar et al., 1999). Thus, displacement of one H2A/H2B dimer during transcription by Pol II is in strict contrast to the survival of the complete octamer even after very dramatic unfolding of nucleosomal DNA from the histone octamer. This suggests that transcription through the nucleosome by Pol II may involve the direct disruption of histone-histone interactions between the H2A/H2B dimers and the H3/H4 tetramer (Luger et al., 1997). Pol II and Pol III have similar sizes, suggesting that this property is not determined by the size of RNAP, either.

Our data on the displacement of one H2A/H2B dimer by Pol II in vitro is consistent with multiple lines of evidence on the correlation between Pol II-mediated transcription and H2A/H2B dimer dissociation from the nucleosome in vivo (reviewed by van Holde et al. 1992). First, transcription-dependent exchange of histones H2A and H2B (but not H3 and H4) on a small fraction of chromatin has been observed in vivo (Jackson, 1990; Kimura and Cook, 2001). Second, it was reported that H2A/H2B histones are partially depleted from the intensely transcribed *Drosophila hsp 70* gene in vivo (Nacheva et al., 1989). Interestingly, loss of H2A/H2B was not detected on the same gene when it was transcribed at slower rate and, therefore, contained a lower number of ECs (Studitsky et al., 1988). This suggests that in vivo when the distance between transcribing polymerases is large, the complete nucleosomal structure could eventually be reassembled after the initial loss of H2A/H2B. The reassembled nucleosomes might still be less stable than nontranscribed ones because nucleosome cores enriched in transcribed DNA sequences tend to lose

one H2A/H2B dimer upon binding to Pol II (Baer and Rhodes, 1983). Third, a transcription-dependent nucleosome disruption attributed to possible loss of H2A/H2B has been detected in a fraction of transcriptionally active nucleosomes isolated using mercury-affinity chromatography (Bazett-Jones et al., 1996). Furthermore, nucleosome disruption in vivo was observed during transcription with Pol II but not with bacteriophage T7 RNAP (Sathyanarayana et al., 1999), consistent with our observations in vitro.

The mechanism used by Pol II to transcribe through nucleosomes suggests several possible roles that chromatin may play in the regulation of gene expression. The efficiency of transcription through the nucleosome by Pol II is much *lower* compared with other RNAPs. A likely explanation for this paradoxical property of Pol II is that the intense nucleosomal barrier is actually used for regulation at the level of transcript elongation. Indeed, nucleosomes positioned at the early-transcribed region of the human *hsp 70* gene form an extremely strong barrier, which can be relieved in the presence of an activator (Brown et al., 1996, 1998). The transiently disrupted chromatin left behind transcribing Pol II may serve as a "window of opportunity" for the binding of factors that further destabilize nucleosome structure. Indeed, depletion of H2A/H2B facilitates binding of transcription factors to nucleosome-covered promoters in vitro (Hayes and Wolffe, 1992). Furthermore, loss of H2A/H2B dimers decreases the ability of polynucleosomes to fold into higher-order structures (Hansen and Wolffe, 1994), and this could also facilitate interaction of protein complexes with DNA.

The potency of the nucleosomal barrier to transcription necessitates the involvement of elongation factors such as SWI/SNF or FACT that stimulate transcription through the nucleosome by Pol II in vitro (Brown et al., 1996; Orphanides et al., 1998). While the mechanism of stimulation by the SWI/SNF complex is unknown, it has been shown that FACT specifically interacts with histones H2A/H2B in vitro and that covalent cross-linking of the core histones in the nucleosome abrogates FACT activity (Orphanides et al., 1999). In combination with the data on obligatory displacement of the H2A/H2B dimer during Pol II transcription, this suggests that the disruption of the histone octamer by Pol II could constitute a rate-limiting step in transcription and that FACT could facilitate Pol II-induced disruption of the nucleosome via direct interaction with the H2A/H2B dimer.

The considerations above suggest the following scenario for transcription through chromatin in vivo (Figure 7). As Pol II progresses along a gene, it transiently converts nucleosomes on its way to hexasomes. This process could be greatly facilitated by nucleosome-specific elongation factors, such as FACT, and could be accompanied by the disruption of higher-order chromatin structure, repositioning of nucleosomes, and partial nucleosome depletion, events which were previously observed on actively transcribed genes (Clark, 1995; Orphanides and Reinberg, 2000). The considerably destabilized structure could provide a window of opportunity for chromatin remodeling factors or DNA binding proteins. The complete nucleosomal structure is eventually restored behind the transcribing Pol II.

Experimental Procedures

Nucleosome Reconstitution

Plasmid pVT1 was constructed by PCR amplifying the nucleosome positioning sequence from the pXP10 plasmid (Hayes et al., 1991) with an upper primer that introduces BglII and TspRI sites and a lower primer that introduces StuI and NcoI sites. The sequences of primers are available upon request. The 232 bp product was digested with BglII and NcoI, and the resulting 218 bp DNA was cloned into pB22 plasmid (Studitsky et al., 1994). The template DNA was PCR amplified from pVT1, and the 204 bp TspRI-StuI fragment was gel purified. Nucleosomal templates were prepared by octamer exchange (Utley et al., 1996). The restriction enzyme digestions were performed as described (Studitsky et al., 1994).

Reconstitution of the histone octamer was conducted as described (Studitsky et al., 1994). Purification of the DNA-bound octamer, hexamer, and tetramer was performed as described (Studitsky et al., 1995). In the add-back experiment, approximately 1 ng of template in TB300 with 0.5 mg/ml BSA was incubated alone or with 5.4 ng/μl H2A and H2B for 1 hr at room temperature.

The nucleoprotein complexes were resolved by native gel electrophoresis as described (Studitsky et al., 1995). Quantitation was performed using a Cyclone Storage Phosphor System (Packard Instrument Company, Meriden, CT).

Pol II EC Assembly, and Ligation to the DNA or Nucleosome

Pol II was purified according to (Kireeva et al., 2000b). 1.3 μM RNA9 (5′ AUCGAGAGG 3′) or TDS50 (5′ GGTGTCGCTTGGGTTGGCTTT TCGGGCTGTCCCTCTCGATGGCTGTAAGT 3′) were labeled at the 5′ end as described (Sidorenkov et al., 1998). If RNA was to be labeled, TDS50 was phosphorylated with ATP. TDS50 and RNA9 were annealed and assembled with Pol II, and NDS59 (5′ACTTA CAGCCATCGAGAGGGACACGGCGAAAAGCCAACCCAAGCGACA CCGGCACTGGG3′) was incorporated as described previously (Kireeva et al., 2000b; Sidorenkov et al., 1998). The Ni^{2+}-NTA agarose was washed with TB40 (TB contains 20 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, and 1 mM β-mercaptoethanol; the numerical index indicates KCl concentration in mM), incubated with 0.5 mg/ml BSA for 30 min, and washed with TB40 prior to EC immobilization. EC9 was immobilized, washed three times with 1 ml TB40, incubated for 10 min in TB1000, and washed twice with TB40.

Immobilized EC9 was incubated with 0.1–0.2 μg of the 204 bp StuI-TspRI DNA (with or without nucleosome), 100 μM ATP, 1% PEG 8000, and 50 U of T4 DNA ligase (New England Biolabs, Beverly, MA) at 12°C for 1 hr. The ligated EC was washed with TB40, incubated for 10 min with TB700, and washed twice with TB40. ECs were eluted with 100 mM imidazole in TB containing 0.5 mg/ml BSA.

Pol II Transcription

EC45 was obtained from EC9 ligated to the StuI-TspRI DNA or nucleosome by 15 min incubation with 200 μM ATP, 200 μM CTP, and 200 μM GTP in TB40. EC45 was washed three times with 1 ml of TB40, incubated for 10 min with TB700, and washed twice with 1 ml of TB40. EC55 was derived by EC45 incubation for 5 min with 10 μM GTP and 10 μM UTP to form EC49, which was labeled by a 20–30 min incubation with 0.1–0.2 μM of α-[^{32}P]ATP and 5 μM CTP. The EC55 was washed three times with 1 ml of TB40, incubated for 5 min with TB700, and washed twice with 1 ml of TB40.

ECs were chased with 500 μM NTPs in TB containing the indicated concentration of KCl and 0.5 mg/ml BSA. The reactions were stopped by adding gel-loading buffer. The products were analyzed by 6% denaturing PAGE and quantified using Typhoon instrument (Molecular Dynamics, Sunnyvale, CA).

Analysis of RNA and DNA Released as a Result of Transcription

Immobilized EC45 was washed with TB300 twice, incubated with 1 ml of TB300 for 15 min, and washed with TB300 twice again. This procedure was repeated, and the supernatant was collected after a 5 min incubation of the resulting EC in TB150 or TB300 containing 0.5 mg/ml BSA and 100 μM ATP, 100 μM CTP, and 100 μM GTP, or 100 μM NTPs. Transcribed templates were obtained by collecting supernatant from EC45 containing labeled TDS50. For transcription

in solution, EC45 was eluted with imidazole and incubated with 100 μM ATP, 100 μM CTP, and 100 μM GTP, or 100 μM NTPs .

SP6 RNA Polymerase Transcription

SP6-TDS50 DNA oligo (5′ GGT GTC GCT TTC GTG GAT CCC CAT TAA TTC TAT AGT GTC ACC TAA ATC GT 3′) was 5′ end labeled and annealed to the SP6-NDS59 DNA oligo (5′ ACG ATT TAG GTG ACA CTA TAG AAT TAA TGG GGA TCC ACG AAA GCG ACA CCG GCA CTG GG 3′). The resulting fragment contains the SP6 promoter 20 bp from the 5′ end and has the same size and sticky end as the DNA in the assembled Pol II EC.

Nucleosomes reconstituted from purified histones were ligated with the annealed oligos as described above. Unligated oligos were removed using a 4 ml S-300 (Amersham Pharmacia Biotech, Piscataway, NJ) gel filtration column equilibrated with 10 mM Na-HEPES, pH 8.0, 10 mM NaCl, 0.1 mM EDTA, and 0.5 mg/ml BSA. Transcription by SP6 RNAP was performed as described (Studitsky et al., 1995).

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