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Nucleosomes Can Form a Polar Barrier to Transcript Elongation by RNA Polymerase II

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Summary

Nucleosomes uniquely positioned on high-affinity DNA sequences present a polar barrier to transcription by human and yeast RNA polymerase II (Pol II). In one transcriptional orientation, these nucleosomes provide a strong, factor- and salt-insensitive barrier at the entry into the H3/H4 tetramer that can be recapitulated without H2A/H2B dimers. The same nucleosomes transcribed in the opposite orientation form a weaker, more diffuse barrier that is largely relieved by higher salt, TFIIS, or FACT. Barrier properties are therefore dictated by both the local nucleosome structure (influenced by the strength of the histone-DNA interactions) and the location of the high-affinity DNA region within the nucleosome. Pol II transcribes DNA sequences at the entry into the tetramer much less efficiently than the same sequences located distal to the nucleosome dyad. Thus, entry into the tetramer by Pol II facilitates further transcription, perhaps due to partial unfolding of the tetramer from DNA.

Introduction

Chromatin consists of repeating units called nucleosomes. Within each nucleosome core, a central H3/H4 tetramer is flanked on both sides by H2A/H2B dimers. Nucleosomes can be transiently displaced from eukaryotic genes when transcription levels are very high (Kristjuhan and Svejstrup, 2004; Schwabish and Struhl, 2004), but the majority of transcribed genes retain nucleosomal organization and thus each Pol II complex must encounter nucleosomes during elongation. Although Pol II can clearly traverse nucleosomes efficiently in vivo, nucleosomes form a high barrier to transcribing Pol II in vitro (Izban and Luse, 1991, 1992; Kireeva et al., 2002). Pol II itself can overcome this in vitro barrier only at 300 mM or higher ionic strength (Izban and Luse, 1991, 1992; Kireeva et al., 2002). During nucleosome traversal by Pol II, a single H2A/H2B dimer is released (Kireeva et al., 2002), which matches the apparent effect of Pol II passage in vivo (Kimura and Cook, 2001; Thiriet and Hayes, 2005). The elongation factor FACT can act as an H2A/H2B chaperone during nucleosome assembly, and this activity most likely mediates its stimulating effect on transcription of nucleosomal templates (Belotserkovskaya et al., 2003). TFIIS, which facilitates transcript cleavage and restoration of catalytic activity of Pol II in arrested transcription complexes, can also facilitate transcript elongation on nucleosomal templates (Kireeva et al., 2005).

A number of previous experiments have shown that transcription of mononucleosomal templates recapitulates many important aspects of chromatin transcription in vivo (Kireeva et al., 2002, 2005). In the present study, we employed mononucleosomal templates containing DNA sequences with high affinity for the histone octamer. These DNAs direct assembly of nucleosomes to single, well-defined locations. Our analysis indicates that there is considerable sequence and orientation-dependent variation in both overall height and location of the nucleosomal transcription barrier. Nucleosomes assembled on strong nucleosome-positioning sequences (NPS) can present very strong salt- and factor-insensitive blockades to Pol II, but these exceptional barriers occur in only one transcriptional orientation. A substantial part of these very strong nucleosomal barriers can be observed with only the H3/H4 tetramer. Thus, nucleosomes do not represent a uniform, symmetrical barrier to transcript elongation by Pol II. Efficient traversal of nucleosomes, at least in some cases, must involve mechanisms in addition to the transient loss of an H2A/H2B dimer.

Results

A Nucleosome Formed on a Strong Nucleosome-Positioning Sequence Can Present an Insurmountable Barrier to Pol II

In our previous studies, transcription complexes were directly assembled with yeast Pol II, template oligonucleotides, and short initial RNAs. DNA fragments bearing single nucleosomes assembled in multiple positions were then ligated downstream of the yeast transcription complexes (Kireeva et al., 2002). A major goal of the present study was to analyze Pol II transcript elongation through a variety of uniquely positioned mononucleosomes. We therefore utilized DNA sequences having high affinity for the histone octamer, which can precisely position nucleosomes (Lowary and Widom, 1998; Thastrom et al., 1999). The single expected positions of the mononucleosomes on the templates were verified by analysis in native gels (Figure S1 in the Supplemental Data available with this article online), restriction digestion analysis (Figure S2), and DNase I and hydroxyl radical footprinting (data not shown). Yeast Pol II elongation complexes containing 9 nt RNA (EC9) were ligated to these uniquely positioned nucleosomes and advanced to produce 45 nt EC45 complexes as described earlier (Kireeva et al., 2002; Figure 1A). We also wished to compare the elongation competence of the assembled yeast Pol II complexes with promoter-initiated human Pol II.

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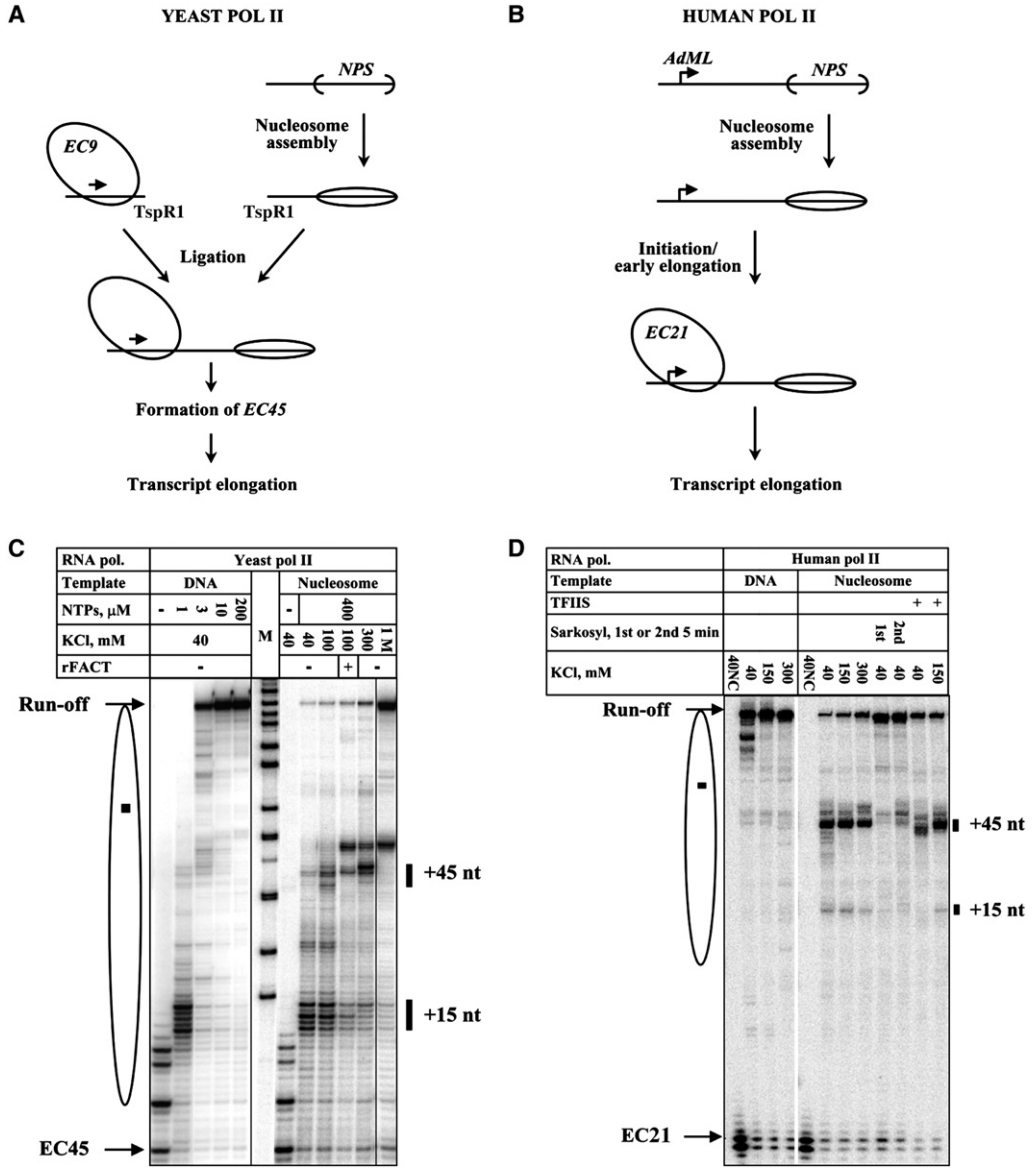

Figure 1. A Nucleosome Formed on a Strong Nucleosome-Positioning Sequence Can Present an Insurmountable Barrier to Pol II

(A) The experimental system for analysis of transcription through a nucleosome by yeast Pol II. Pol II elongation complex (EC9) was assembled on a 50 bp DNA fragment. The EC9 was immobilized on Ni^{2+}-NTA agarose beads, ligated to DNA or nucleosomal templates, and washed. Pol II was advanced to produce 45 nt transcripts (EC45 complex) by using a subset of NTPs and [α-^{32}P] GTP to label the RNA. The EC45 complexes were washed, and transcription was resumed by addition of all unlabeled NTPs.

(B) Experimental system for analysis of transcription through a nucleosome by human Pol II. DNA fragments preassembled into nucleosomes were attached to beads and then used for assembly of Pol II preinitiation complexes, which were advanced to EC21 and then chased with four NTPs. All templates for yeast and human Pol II were constructed in a similar way; in particular, strong nucleosome-positioning sequences (NPS) were always located on the downstream DNA end and overall length of template DNA was constant in most experiments.

(C) Nucleosome cores assembled on the 601 DNA sequence form a strong barrier to yeast Pol II. Histone-free 601 DNA or nucleosomes were transcribed in the presence of the indicated concentrations of NTPs and KCl, in the presence or absence of FACT. Arrows indicate positions of the labeled transcripts in the gel. Position of the nucleosome on the template is indicated by the oval (the nucleosomal dyad is shown as a black square). The positions of strong nucleosomal pausing are indicated by black rectangles. M, end-labeled pBR322-Msp digest.

(D) The 601 nucleosome core presents a strong barrier to human Pol II. Pol II transcription complexes were assembled on bead-attached pure DNA or nucleosomal templates bearing the 601 sequence. In all lanes except 1 and 5 (marked nc), the initial U21 ternary complex was chased at the KCl concentration and with the addition of sarkosyl or TFIIS as indicated in the figure.

However, preliminary experiments indicated that the ligation procedure was not tolerated by human Pol II. Thus, for the human polymerase, templates containing both a promoter and an NPS were employed. After nucleosomes were assembled on these longer DNA fragments, human Pol II preinitiation complexes were assembled with purified RNA polymerase and purified or recombinant general transcription factors (Pal et al., 2005; Figure 1B). Human Pol II complexes were initially advanced with a subset of the NTPs to produce stalled EC21 elongation complexes (Figure 1B). The yeast EC45 and human EC21 complexes were extended in

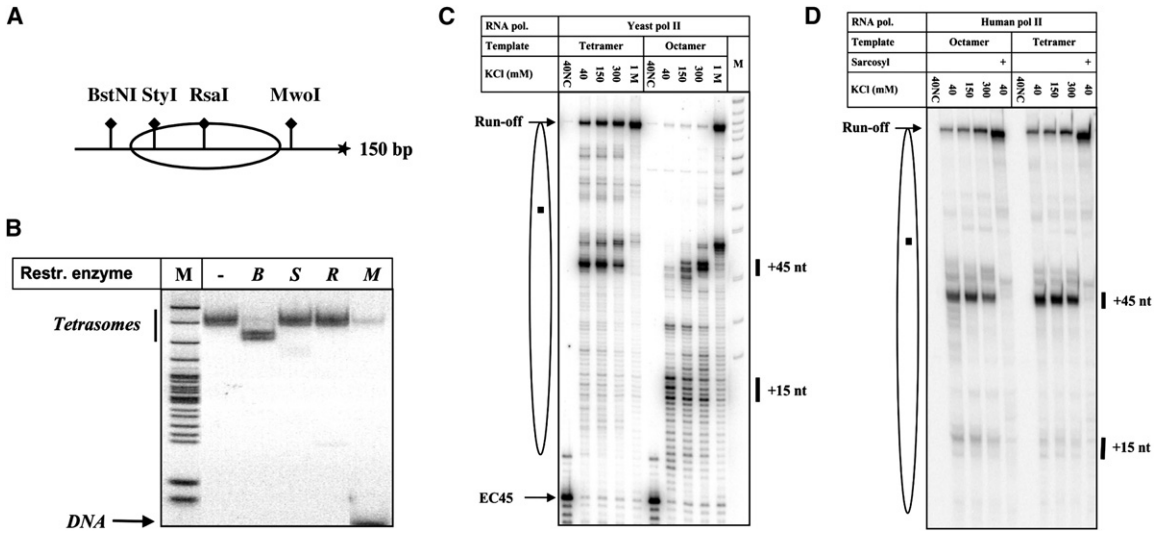

Figure 2. The Insurmountable Barrier to Pol II Persists after Removal of H2A/H2B Dimers from the 601 Nucleosome

(A) Schematic diagram of tetramer positioning on the 601 NPS. Cleavage sites for restriction enzymes and the expected position of the tetramer on the template (oval) are indicated.

(B) Mapping of the position of the tetramer on the 601 template using a restriction enzyme sensitivity assay. Analysis of DNA-protein complexes by nondenaturing PAGE. M, end-labeled pBR322-Msp digest.

(C) Omission of H2A/H2B dimers from the 601 nucleosome leaves the major barrier to yeast Pol II intact. Pulse-labeled RNA in EC45 was extended in the presence of unlabeled NTPs at the indicated concentrations of KCl on 601 nucleosomes or DNA-bound tetramer.

(D) Omission of H2A/H2B dimers from the 601 nucleosome leaves the major barrier to human Pol II intact. Pol II transcription complexes assembled on 601 templates bearing either nucleosomes or DNA-bound tetramers were extended as in Figure 1D.

the presence of all four NTPs, and the resulting transcripts were analyzed.

For our initial experiments, templates bearing the 601 sequence element (Lowary and Widom, 1998; Thastrom et al., 1999) were used to direct nucleosome assembly. Histone-free 601 templates were transcribed to completion by both yeast and human Pol II provided that NTPs were present at a sufficiently high concentration (100 μM or higher, Figures 1C and 1D). However, in contrast to the results of earlier studies (Izban and Luse, 1991, 1992; Kireeva et al., 2002, 2005), both RNA polymerases failed to efficiently traverse the 601 nucleosome, even at 300 mM salt (Figures 1C and 1D). Yeast Pol II recognized major pauses at ~10–20 nt and at ~45 nt into the 601 nucleosome (Figure 1C); less than 10% traversal occurred even at 300 mM KCl (summary in Figure 4). Human Pol II observed very similar pause sites, although the ~+45 pause predominated at all salt concentrations (Figure 1D). Human Pol II traversed the 601 nucleosome more efficiently than the yeast enzyme, but only about 40% readthrough was achieved at 300 mM KCl (Figure 4). The height and salt resistance of the 601 nucleosomal barrier to Pol II exceeds that observed previously with any other nucleosome.

Some major stops (such as the pauses at ~+15) on the 601 nucleosomal template matched corresponding transient pauses seen on free DNA at low NTP levels (Figure 1C), in agreement with previous studies of nucleosome transcription (Izban and Luse, 1991, 1992; Kireeva et al., 2002, 2005). However, the major pause at +45 was very selectively and strongly amplified in the presence of the nucleosome (Figures 1C and 1D). Because nucleosomal pauses were not observed on free DNA at the high NTP levels used for nucleosome transcription, they are nucleosome specific. As expected, transcription of the nucleosomal 601 template in the presence of reagents that strongly destabilize DNA-histone interactions (1 M KCl or the detergent sarkosyl) resulted in efficient synthesis of run-off transcripts (Figures 1C and 1D).

The elongation factor TFIIS can relieve a large part of the nucleosomal barrier to transcript elongation by yeast Pol II (Kireeva et al., 2005). However, TFIIS could only partially relieve the 601 nucleosome barrier for human Pol II. When the run-off bands were quantified, we found that readthrough transcription increased from 12% to 38% at 40 mM salt and from 20% to 33% at 150 mM salt in the presence of TFIIS (Figure 1D). The strongest 601 pause site is located approximately where nucleosomal DNA begins to interact with the central H3/H4 tetramer, suggesting that this part of the barrier is conferred primarily by the tetramer. Consistent with this idea and the proposed role for FACT as an H2A/H2B chaperone (Belotserkovskaya et al., 2003; Formosa et al., 2002), FACT could not increase traversal of the 601 nucleosome by yeast Pol II at 100 mM KCl (Figure 1C). The FACT preparation was active, because it allowed yeast Pol II to penetrate further into the 601 nucleosome at 100 mM salt, relieving the barrier formed by the H2A/H2B dimers (Figure 1C). Similar results were obtained with human Pol II and FACT (Figure S3).

Because the barrier formed by the 601 nucleosome is localized in the tetramer and FACT cannot relieve it, we next tested whether the tetramer alone assembled on the 601 sequence can recreate the barrier imposed by the entire 601 nucleosome. As expected, a single tetramer is bound to the central part of the 601 positioning sequence (Figures 2A and 2B). Neither polymerase paused significantly in the +15 region on the tetramer-containing templates, even at 40 mM KCl, but strong pausing in the

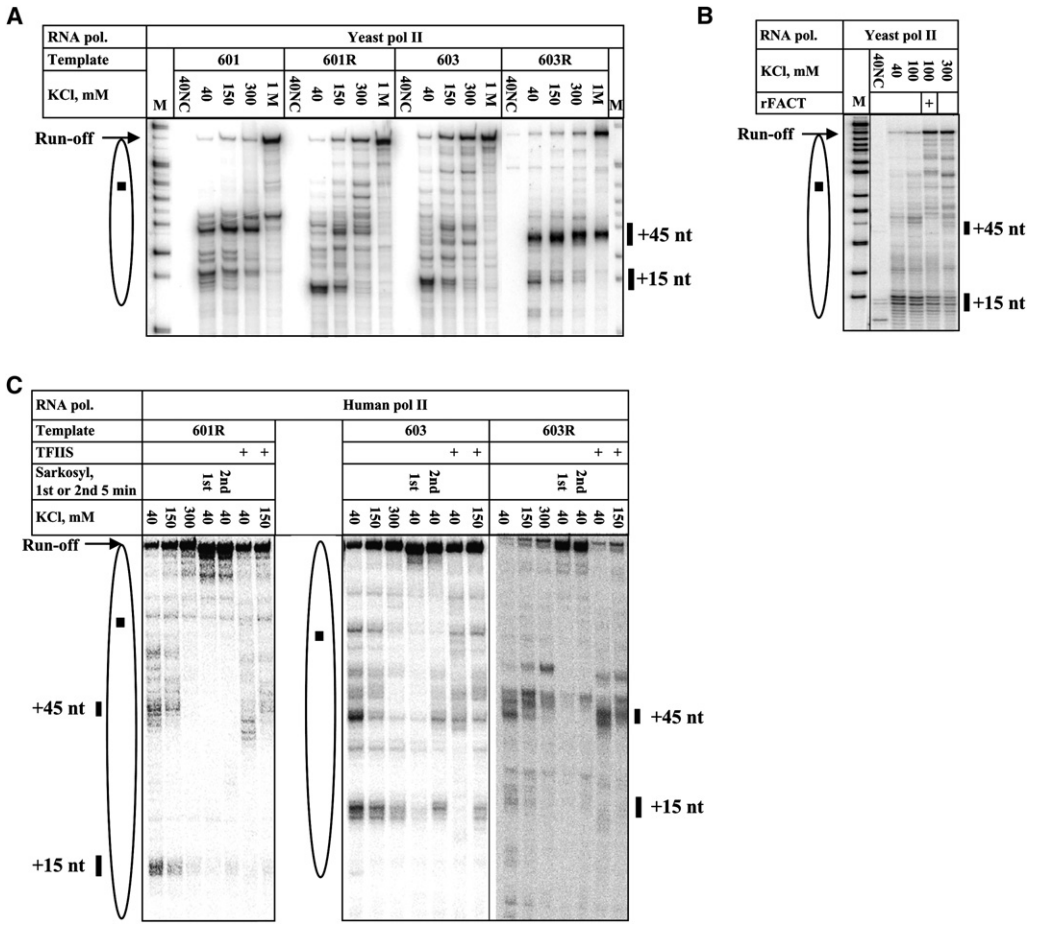

Figure 3. The Height and Organization of the Nucleosomal Barrier to Transcription by Pol II Are Orientation Dependent

(A) The 601 and 603 nucleosomes present orientation-dependent barriers to transcription by yeast Pol II. RNA-labeled EC45 complexes were extended in the presence of unlabeled NTPs at the indicated concentrations of KCl.

(B) FACT can relieve the 601R nucleosomal barrier to yeast Pol II. Transcription of the 601R nucleosomes was conducted as described above; the 45-mer was extended in the presence or in the absence of FACT.

(C) The height and location of the nucleosomal barrier to transcription by human Pol II is variable and depends on the underlying DNA sequence and its orientation. Pol II transcription complexes assembled on nucleosomal templates bearing the 601R, 603, or 603R sequences were extended as in Figure 1D.

+45 region occurred at all salt concentrations tested and at nearly the same intensity on the tetramer- and octamer-containing 601 templates (Figures 2C and 2D).

The Height of the Nucleosomal Barrier to Pol II Depends on Both the DNA-Histone Affinity and the Orientation of the Nucleosome

Why is the nucleosomal barrier on the 601 template so high? The 601 DNA sequence was selected based primarily on the affinity of the central ~70 bp for the H3/H4 tetramer (Thastrom et al., 2004). Thus, strong binding of the H3/H4 tetramer to 601 DNA could account for the high barrier to Pol II, particularly at the point of entry into the tetramer. If this were true and all DNA-histone interactions are equally strong along the tetramer-bound DNA region, one would expect that (1) the 601 sequence would provide an equally strong barrier regardless of its orientation on the template and (2) nucleosomes assembled on other DNA sequences with comparable affinities for histones would provide comparably strong barriers to transcript elongation in analogous locations.

To test these predictions, we constructed 601R, a template with the reverse orientation of the 601 positioning sequence. We also made four other templates (603, 603R, 605, and 605R) utilizing the 603 and 605 DNA sequences in both transcriptional orientations. The 601, 603, and 605 positioning elements are essentially unrelated in DNA sequence, but they have equally high affinities for core histones (Lowary and Widom, 1998; Thastrom et al., 1999). Yeast and human Pol II elongation complexes were assembled on these nucleosomal templates as described in Figures 1A and 1B. Transcripts were extended at different concentrations of KCl in the presence or absence of TFIIS (human Pol II) or FACT. The results are shown in Figure 3 and Figure S3 and summarized in Figure 4 and Figure S5.

We observed a clear orientation dependence of the height and location of the transcriptional barrier formed on these nucleosomes. Polymerases paused primarily at ~+45 on the 603R and 605R nucleosomal templates, and this barrier was only slightly reduced by 300 mM KCl or TFIIS, as was the case with the 601 template. In

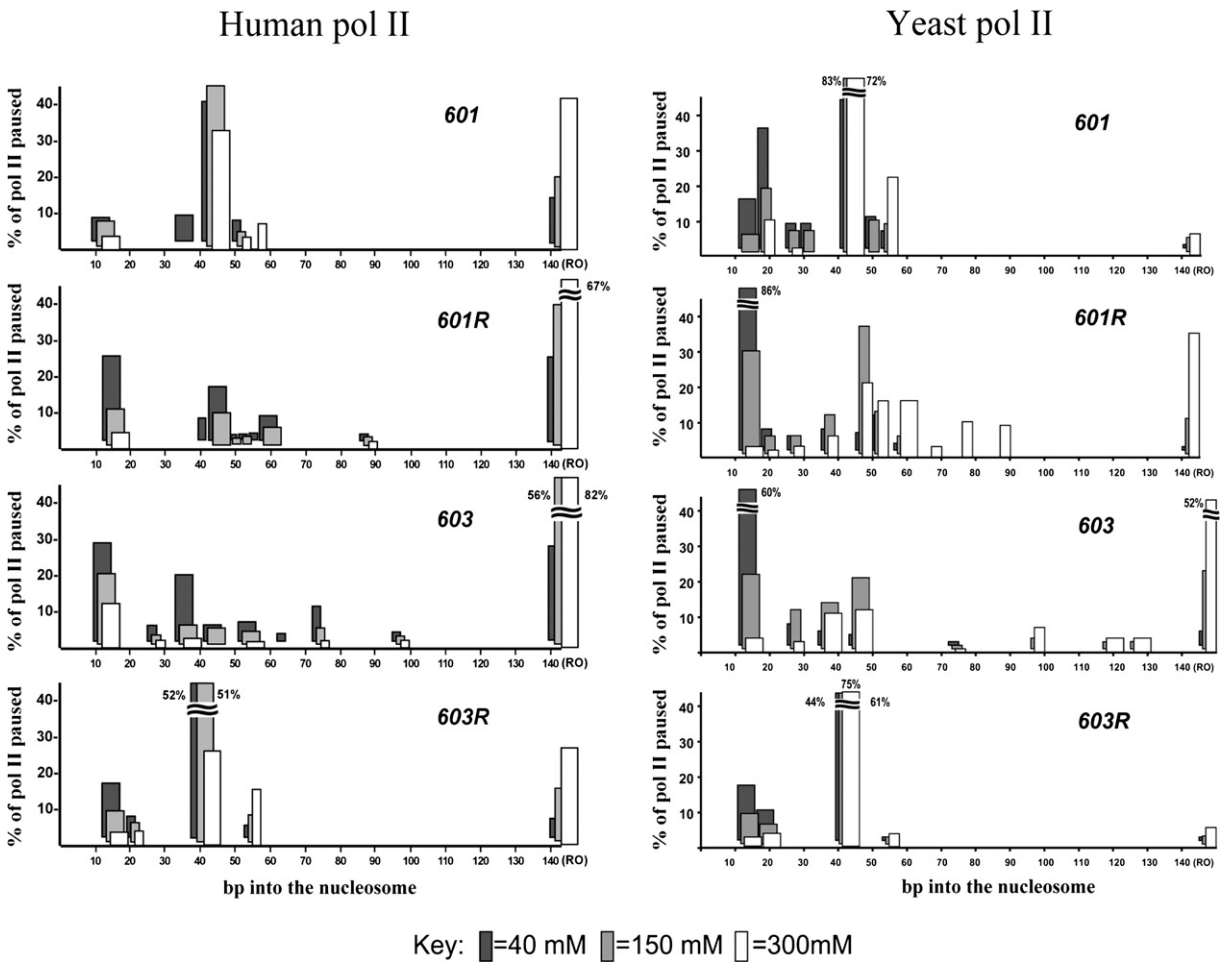

Figure 4. Summary of the Positions and Intensities of Nucleosome-Specific Pauses Formed during Transcription of 601 and 603 Templates by Yeast or Human Pol II

The extent of pausing at different sites was quantified. The location of each pause within the given nucleosome is shown on the x axis (RO, run off). Narrow-width bars show pausing at a single location, whereas wide bars indicate pausing in closely spaced groups. Bar height shows the amount of pausing at the indicated location, with the amount of run off at 1 M KCl (yeast) or 1% sarkosyl (human) set to 100%. Dark bars show results at 40 mM KCl, gray bars show results at 150 mM KCl, and white bars show results at 300 mM KCl.

contrast, the 601R, 603, and 605 barriers were less localized at 40 or 150 mM KCl and significantly relieved by 300 mM salt (Figures 3 and 4 and Figures S4 and S5). At 300 mM KCl, the amount of run-off transcript generated by yeast Pol II increased from <10% on 601 and 603R, and <20% on 605R, to 35% on 601R and >40% on 603 and 605. With human Pol II as well, traversal at 300 mM was considerably lower for the less-permissive group of templates (42% on 601 and 30% on 603R) as compared to traversal levels for the more permissive template group (67%, 82%, and 57% on 601R, 603, and 605, respectively). On the 601R, 603, and 605 templates, a significant fraction of yeast and human Pol II complexes transcribed past the +45 pausing area even at 150 mM KCl. The barrier on the nucleosomal 603 template is lower than the barrier formed by nucleosomes bound to the 5S DNA sequence (Kireeva et al., 2002), although 5S DNA has at least 100-fold lower affinity for the octamer than the 603 sequence (Lowary and Widom, 1998; Thastrom et al., 1999).

The 601, 603R, and 605R transcriptional barriers have similar structures and heights. In contrast, pause sites on 601R, 603, and 605 differed in both intensity and location (Figures 3 and 4 and Figures S4 and S5). Although pause locations between roughly +10 to +20 and +35 to +55 were seen for all nucleosomes, the details of the pausing patterns were quite distinct for each template (Figure 4 and Figure S5). For a given salt concentration, the human enzyme always produced a greater proportion of run-off transcripts in comparison with yeast Pol II. Comparison of the nucleosomal pause locations with patterns of transient pausing on the corresponding histone-free DNA at lower concentrations of NTPs demonstrates that only a subset of the nucleosomal pauses represents increases in free DNA pausing (Figure 1C and data not shown). Thus, our results indicate that the extent of the nucleosomal barrier to transcript elongation by Pol II is determined by a combination of the underlying DNA sequence and the position of this sequence within the nucleosome. Some major pauses on nucleosomal templates occurred at or beyond the nucleosomal dyad (601R, 603, and 605 templates; see Figure 4 and Figure S5), suggesting that nucleosomes remain bound to the DNA template after Pol II has crossed the dyad.

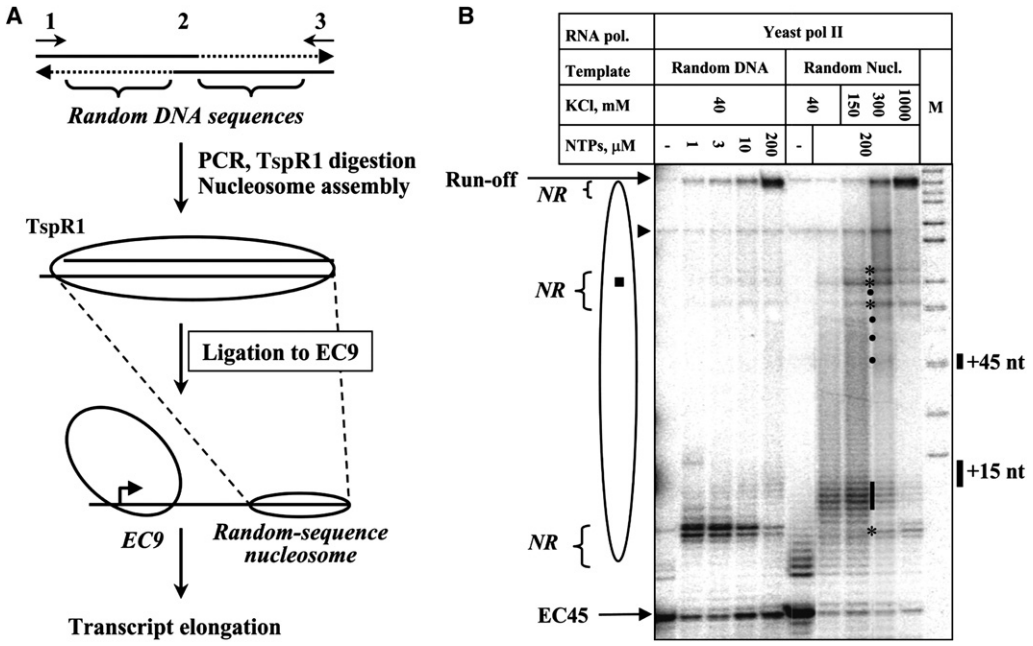

Figure 5. Nucleosome Organization Does Not Necessarily Specify Distinct Pausing by Yeast Pol II

(A) Experimental approach.

(B) Transcription of the random-sequence DNA and nucleosome. DNA or nucleosomes were transcribed in the presence of the indicated concentrations of NTPs and KCl. The clusters of nucleosome-specific pauses are indicated by the black rectangle and by black dots. Nonrandom DNA sequences (NR) are indicated; positions of sequence-specific pauses are indicated by asterisks. Note that nucleosomal DNA was labeled (unligated DNA is indicated by arrowhead). M, end-labeled pBR322-Msp digest.

In contrast to the case with the 601 nucleosome (Figure 1C), FACT stimulated RNA synthesis on the 601R template for yeast Pol II (Figure 3B), raising run-off levels to those seen with 300 mM KCl. With human Pol II, FACT essentially eliminated pausing in the promoter-proximal segment of the 601R nucleosome, whereas pausing in the analogous region of the 601 nucleosome was only slightly reduced by FACT (Figure S3). TFIIS stimulated elongation by human Pol II on the 601R, 603, and 605 nucleosomal templates (Figure 3C and Figure S4), increasing run-off levels in 40 mM salt to those seen with 150 mM salt in the absence of TFIIS. As we observed with the 601 template, run-off transcription on 601R and 605 nucleosomes at 150 mM KCl in the presence of TFIIS was not significantly greater than run-off levels at 40 mM salt in the presence of TFIIS. However, TFIIS did stimulate elongation at 150 mM salt on the 603 template, increasing readthrough to roughly the same level (over 80%) seen at 300 mM KCl in the absence of TFIIS. Thus, in agreement with earlier results (Kireeva et al., 2005) with yeast Pol II, stimulation of human Pol II by TFIIS on nucleosomal templates was easily evident at low-salt concentrations, but at physiological ionic strength, TFIIS increased run-off transcription beyond the 50% level on only one of our nucleosomal templates.

Nucleosome Organization Does Not Necessarily Determine the Location of the Barrier to Transcription by Pol II

The variability of the nucleosome-specific pausing patterns on various templates (Figure 4) raises the question of if there are any pausing features determined entirely by nucleosome structure. To eliminate sequence bias, templates for yeast Pol II containing mostly random DNA sequence were constructed as shown in Figure 5A. Transcription of these histone-free templates at lower NTP concentrations resulted in transient, apparently sequence-specific pauses at positions +4 and +5 and at several locations near the dyad (Figure 5B). These pauses occurred within the defined-sequence linkers used to assemble the otherwise random-sequence DNA templates (Figure 5A).

Pausing by yeast Pol II on the random-sequence nucleosome at 40 mM KCl occurred at a discrete set of nucleosome-specific positions near +15 and a broader range of sites centered within the promoter-proximal 30–40 bp of the nucleosome (Figure 5B). These features generally resembled those seen on the specific-sequence nucleosomes under these conditions (Figure 4 and Figure S5). The tight distribution of these pauses suggests that the majority of random-sequence nucleosomes were positioned on the template with a variation of no more than 2–3 bp. The mean distribution of the pausing pattern progressively shifted into the nucleosome as the salt concentration was increased to 150 and 300 mM KCl. The only discrete pauses seen at 150–300 mM KCl (downstream of the +15 pause) were very faint bands within the proximal half of the nucleosome (marked by dots in Figure 5B), which lacked the 10 bp periodicity observed during transcription through a nucleosome by yeast Pol III (Studitsky et al., 1997). Very little run-off RNA was obtained until the salt concentration was raised to 300 mM; at this ionic strength, most of the pausing was localized within the boundaries of the central H3/H4 tetramer. Some pausing was observed within the random-sequence nucleosome at or

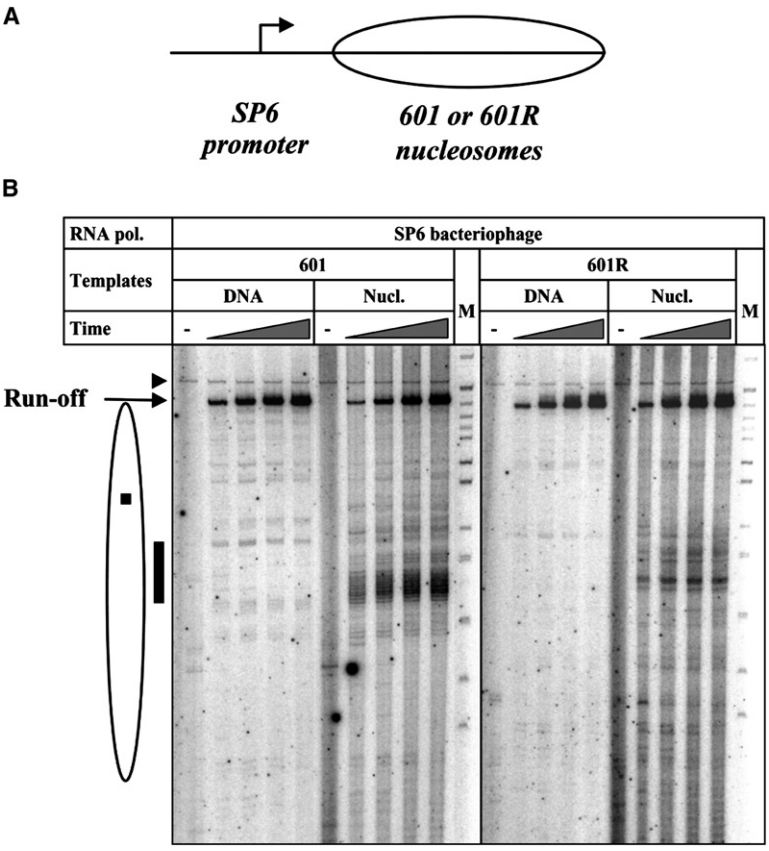

Figure 6. RNA Polymerase Specificity of the Nucleosomal Barrier to Transcription

(A) Nucleosomal templates for transcription by SP6 RNA polymerase.

(B) The nucleosomal barrier to SP6 RNA polymerase formed on the 601 and 601R templates is low. Preformed RNA-labeled EC14 complexes were extended in the presence of all cold NTPs at 40 mM KCl on 601 or 601R DNA or nucleosomes. Arrow at the left indicates the run-off transcripts; arrowhead indicates the end-labeled DNA used as a loading control.

beyond the nucleosome dyad, particularly at 300 mM salt.

The data in Figure 5 indicate that the histone octamer presents a barrier for Pol II that extends continuously into the nucleosome, including a considerable distance past the dyad. Except for the discrete pause at ~+15, no specific position within the nucleosome predominated in directing pausing. The random-sequence nucleosome transcriptional barrier was of intermediate strength, between the 601/603R barriers and the 601R/603 barriers, as judged by the extent of run-off RNA production by yeast Pol II at 300 mM KCl.

Polymerase Specificity of the Nucleosomal Barrier to Transcription

Pol II and *E. coli* RNA polymerase (RNAP) encounter a strong nucleosomal barrier to transcription. Traversal of the nucleosome by these polymerases results in nucleosome remodeling (loss of one H2A/2B dimer), but the nucleosome remains in place on the template (Kireeva et al., 2002; Walter et al., 2003). In contrast, eukaryotic Pol III and bacteriophage SP6 RNAP see a much lower nucleosomal barrier to transcription (Bednar et al., 1999; Studitsky et al., 1995, 1997). As these latter polymerases proceed, nucleosomes remain intact but are displaced from their original locations. To evaluate polymerase specificity of the nucleosomal barrier formed on high-affinity positioning sequences, templates were constructed with the 601 and 601R positioning elements attached to promoters for bacteriophage SP6 RNAP (Figure S6). These templates were transcribed, as free DNA or mononucleosomes, for various time intervals at 40 mM KCl (Figure 6). The nucleosomal barriers to SP6 transcription were much lower than those encountered by Pol II: up to 70% of SP6 RNAP molecules synthesized run-off transcript under these low-salt conditions (Figure 6). Therefore, even a nucleosome that cannot be traversed by Pol II at 40 mM salt (601, Figure 1) does not form a high barrier to SP6 RNAP under the same conditions. However, some features of the barrier to the SP6 RNAP are in common with the Pol II barrier on both the 601 and 601R templates. The locations of the major ~+45 pause sites in both 601 and 601R for SP6 RNAP were essentially identical to those seen with Pol II. In addition, the level of pausing by SP6 polymerase was weaker and more diffuse on the 601R template as compared with 601, which is similar to the results of transcription of 601 and 601R by Pol II. Thus, the barrier imposed by the H3/H4 tetramer is recognized by both Pol II and SP6 RNAP, although the barrier is much lower for the phage polymerase. Note that, unlike Pol II, the SP6 polymerase did not pause beyond the dyad on the 601R template. This is consistent with the observation that transcription past the dyad by SP6 polymerase results in relief of the nucleosomal barrier by nucleosome relocation (Studitsky et al., 1995).

Discussion

We have established an experimental system for analysis of the mechanism of Pol II transcript elongation through uniquely positioned nucleosomes. To guarantee that nucleosomes occupy a single position, templates

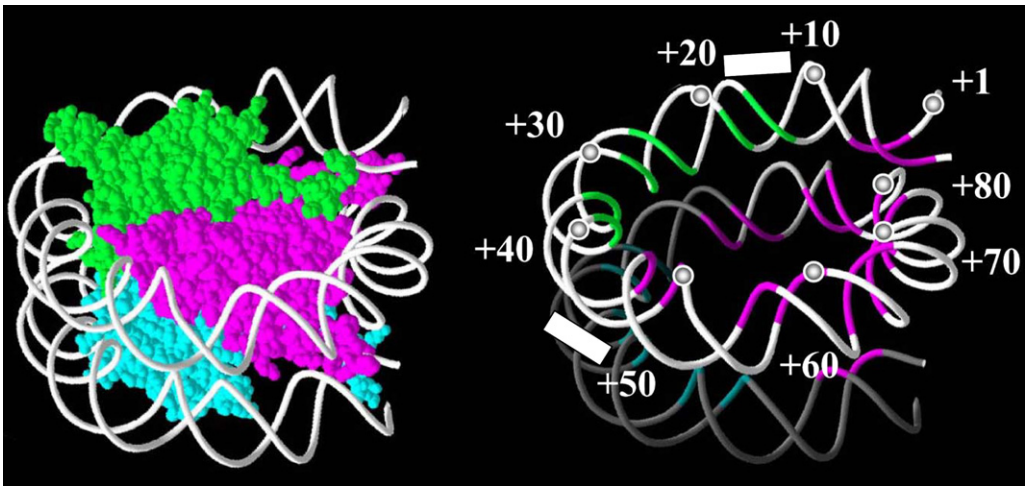

Figure 7. Positions of Nucleosome-Specific Pauses within the Nucleosomal Structure

The structure of the nucleosome core (Luger et al., 1997) is shown on the left and the path of nucleosomal DNA on the right. The backbone of nucleosomal DNA is shown in white and gray, the H3/H4 tetramer in purple, and the H2A/H2B dimers in green and blue. The regions of histone interactions with DNA are colored correspondingly (on the right). The locations of ten base intervals on the template DNA strand (numbered from the upper end of the nucleosome) are indicated along the DNA backbone. The major positions of strong Pol II pausing that occur in the 601, 603R, and 605R nucleosomes are shown by white rectangles.

containing high-affinity nucleosome assembly sequences were used. Striking features of the transcriptional blockades imposed by these nucleosomal templates include the strength and polarity of the barriers. These critical features of the barriers are Pol II specific. The 601, 603R, and 605R templates provided very high, factor- and salt-insensitive barriers. Because such high-strength barriers have never been observed using DNA sequences with lower affinities to core histones, the high-affinity DNA sequences must dictate a primary characteristic of the barriers. The high-strength barriers are always localized at the point of entry into the H3/H4 tetramer and were never detected within the dyad-distal part of the histone tetramer.

Transcription of the high-strength barrier templates in the reverse orientation did not reveal high-strength barriers. Traversal of the 601R, 603, and 605 nucleosomes by Pol II resulted in a diffuse collection of pause sites that could be largely relieved by higher salt or the elongation factors TFIIS or FACT. The characteristics of the nucleosomal barrier are therefore dictated both by the local nucleosome structure (which is likely to be a function of the strength of the histone-DNA interactions) and the location of the DNA sequence elements within the nucleosome. The fact that Pol II transcribes DNA regions at the entry into the tetramer much less efficiently than the same regions when encountered distal to the nucleosomal dyad axis suggests that after entering the tetramer, Pol II facilitates its own progression through the nucleosome. A likely explanation for this effect would be a partial unfolding of the histone tetramer from DNA as a result of the entry of Pol II into the tetramer.

The Nature of the Nucleosomal Barrier to Transcribing Pol II

None of the nucleosomes in our study could be efficiently traversed by either human or yeast Pol II at (or below) physiological ionic strength in the absence of elongation factors. For one orientation of each of the templates, at least one-third (and in many cases, a majority) of the polymerases could cross the nucleosome at 300 mM KCl (Figure 4 and Figure S5), in agreement with earlier work (Izban and Luse, 1991, 1992; Kireeva et al., 2002). However, the 601, 603R, and 605R nucleosomes could not be crossed efficiently under any condition tested. The location of the most prominent pause sites on these three templates, ~45 bp into the nucleosome (Figure 7), suggested that the H3/H4 tetramer alone can impose the major transcriptional barrier on these sequences. This hypothesis was confirmed by demonstrating that the pausing at +45 directed by a 601 DNA-bound histone tetramer is similar to the pausing produced at this location by the complete 601 nucleosome (Figures 2C and 2D). Consistent with this observation, the H2A/H2B chaperone FACT was not able to significantly decrease the height of the major 601 barrier, although FACT can stimulate transcription through other nucleosomes (Belotserkovskaya et al., 2003), including 601R (Figure 3B and Figure S3). In preliminary tests (data not shown), we found that FACT also stimulates traversal of the 603, but not the 603R, nucleosome by human Pol II. These results indicate that traffic of the H2A/H2B dimers does not always control nucleosome traversal by Pol II. The other factor known to facilitate transcription through nucleosomes, TFIIS (Guermah et al., 2006; Kireeva et al., 2005), could only partially stimulate readthrough on the nucleosomal 601 and 603R templates. Thus, nucleosomes can form a barrier to transcript elongation by Pol II that cannot be significantly relieved by 300 mM salt or any known elongation factor.

All of the templates employed in this study contained nucleosome-positioning elements that have high, and comparable, affinities for the core histones, particularly for the H3/H4 tetramer (Lowary and Widom, 1998; Thastrom et al., 1999, 2004). Because positioning sequences used in earlier studies did not support assembly of high-strength nucleosomal barriers that resisted salt and elongation factors, exceptional barrier strength

must require high affinity of DNA for the histone core. However, simply inverting the 601, 603, or 605 sequence elements, which would not change the affinity of the nucleosomes for the underlying DNA, significantly changed the height and predominant location of the nucleosomal barrier to transcription (Figure 4 and Figure S5). This barrier asymmetry suggests that the high affinity of the 601, 603, and 605 assembly sequences for the H3/H4 tetramer does not extend over the entire central segment of the elements but instead can be localized to only one side of the H3/H4 tetramer-bound DNA region. This model can explain the sharp blockade to entry into the tetramer provided by nucleosomes assembled with the high-affinity sequences on the promoter-proximal side of the nucleosomal dyad (i.e., 601, 603R, and 605R). However, if localized high affinity of DNA for the underlying H3/H4 tetramer always provides a strong barrier to transcription, why is such a barrier not observed on the promoter-distal side of the dyad with the 601R, 603, and 605 nucleosomes? Our data suggest that after entering the tetramer Pol II induces a cooperative partial unfolding of the tetramer from DNA, facilitating continued transcription through the nucleosome. If polymerase entry into the tetramer reduces downstream barriers, then high-affinity elements located downstream of the dyad would not be expected to provide an exceptionally strong blockade to Pol II after it passes the +45 region.

Interestingly, the only significant pause by SP6 RNAP on the 601 and 601R nucleosomes (Figure 6) occurred at the entry into the H3/H4 tetramer, suggesting that this is a particularly difficult step for any transcriptional machinery. At this stage of our analysis, we cannot identify the critical contact between Pol II and the histones that blocks further progress at tetramer entry. In this context, it is important to note that bacterial RNAP, which is very similar to Pol II at its catalytic core, can extend transcripts to within 7 bp of the exonuclease III boundary of EcoRI bound to DNA (Pavco and Steege, 1990) and up the base preceding a psoralen crosslink in the template (Shi et al., 1988). Thus, because the leading edge of Pol II halted at the +45 barrier should be located ~20 nt downstream of +45 (Samkurashvili and Luse, 1996), the major barrier in the 601, 603R, and 605R nucleosomes could actually be located within the range from 1 to 20 bp downstream of the +45 region.

Does Pol II pausing within nucleosomes simply reflect difficulties in transcribing the underlying DNA sequence that are made more severe by the association of the template with the nucleosome surface? The amplification of free DNA pausing within the corresponding nucleosomes has been frequently reported (Izban and Luse, 1991, 1992; Kireeva et al., 2002, 2005; Studitsky et al., 1995). Such nucleosome-specific amplification of free DNA pausing could be explained in part by the results obtained with nucleosomes formed on random-sequence DNA (Figure 5). These data show that nucleosome organization does not necessarily determine the location of the barrier to transcription by Pol II. Thus, given the tight association of DNA with histones on the nucleosome surface, sequence-specific DNA pausing is likely to be generally increased by the presence of nucleosomes. However, within some nucleosomes, particular free DNA pauses can be very selectively amplified. A striking example is the strong +45 pause on the 601 nucleosome, which was barely detectable during transcription of histone-free 601 DNA (Figure 1D) even at low concentrations of NTPs (Figure 1C). We conclude that the strength of Pol II pausing on a nucleosomal template cannot be predicted with certainty either from the exact location of that sequence within the nucleosome or from the tendency of Pol II to pause on that sequence when it is transcribed as free DNA.

Polymerase Specificity of the Nucleosomal Barrier to Transcription

A major goal in these studies was the comparison of nucleosomal transcription by assembled yeast Pol II elongation complexes and promoter-initiated mammalian Pol II complexes. Although yeast Pol II was uniformly less effective in elongating transcripts at a given salt concentration, the two polymerases showed similar responses to the nucleosomal templates, particularly to the very strong 601 and 603R barriers (Figure 4). Thus, both systems should be suitable for further analysis of the mechanism of transcription through chromatin.

Critical features of the nucleosomal barrier are specific to Pol II. In contrast to Pol II, SP6 RNAP crossed the 601 nucleosome efficiently at 40 mM KCl (Figure 6). Nucleosome traversal by Pol II and *E. coli* RNAP (Kireeva et al., 2002; Walter et al., 2003) involves coincident displacement of one H2A/H2B dimer. In contrast, yeast Pol III and bacteriophage SP6 RNAP relocate the nucleosome upstream of the transcribing enzyme (Bednar et al., 1999; Studitsky et al., 1994, 1997). Because nucleosome translocation by these latter enzymes occurs before they approach the nucleosomal dyad, the transcription barrier never extends past this point (Figure 6, Kireeva et al. [2002], and Studitsky et al. [1995, 1997]). Importantly, in the present study, significant Pol II pauses were detected on some nucleosomes near or downstream of the nucleosome dyad axis, including some locations in the distal half of the nucleosome (Figures 3 and 4). Nucleosome-specific pauses on random-sequence nucleosomes were also observed past the dyad (Figure 5). Thus, even though the contacts between the H3/H4 tetramer and the underlying DNA may be transiently unfolded during Pol II traversal, the results very strongly suggest that Pol II does not completely displace the octamer into solution during transcription through nucleosomes.

The Height of the Nucleosomal Barrier to Transcription by Pol II Can Be Regulated

We observed nucleosome traversal levels with yeast Pol II that ranged, at physiological salt, from a few percent on the 601 and 603R templates (Figure 4) to over 50% for the 603 template in presence of FACT (Figure 3B and data not shown). This emphasizes both the potential dynamic range and sequence dependence of such regulation. It has been suggested that an intrinsically high nucleosomal barrier might be important for regulation of the rate of transcript elongation (Brown et al., 1996; Kireeva et al., 2002). ATP-dependent chromatin-remodeling activities are obvious candidates for transcriptional regulators at the nucleosome level, particularly because such effects have already been demonstrated (Brown et al., 1996; Corey et al., 2003; Sullivan et al., 2001).

Chaperones that facilitate the transient removal of a subset of the core histones should function to assist the intrinsic ability of Pol II to remodel nucleosomes and thereby stimulate transcript elongation on nucleosomal templates. The H2A/H2B chaperone FACT can perform this role (Belotserkovskaya et al., 2003; Pavri et al., 2006), but we have shown here that FACT does not facilitate traversal of nucleosomes in which the primary barrier is provided by entry into the H3/H4 tetramer. We may therefore expect that activities that function analogously to FACT, but act through the tetramer, remain to be discovered. An important role for the histone H3/H4 chaperone Asf1 in displacement/deposition of H3/H4 histones during transcript elongation in yeast has recently been reported (Schwabish and Struhl, 2006).

Transcription through nucleosomes could also be regulated by factors that act directly on Pol II to maintain elongation competence. TFIIS performs this function in vitro (Kireeva et al., 2005; Pavri et al., 2006) and likely has a role in vivo as well (Kulish and Struhl, 2001). We found that TFIIS increased nucleosome traversal levels, particularly at low-salt concentrations, but it could not stimulate all polymerases to cross any of the nucleosomes we tested. In addition, most human Pol II complexes were released into elongation by removal of the nucleosome with sarkosyl in the absence of TFIIS. Thus, at least the majority of human Pol II complexes paused within a nucleosome have not entered the same state as complexes halted at well-characterized arrest sites on pure DNA templates (see for example Izban and Luse [1993]). Additional insight into the molecular basis for Pol II pausing within a nucleosome will be critical for uncovering activities that can confer elongation competence to Pol II on chromatin templates.

Experimental Procedures

DNA Templates

Plasmids containing nucleosome positioning sequences 601, 603, and 605 were kindly provided by Dr. Widom (Lowary and Widom, 1998). To prepare the templates for yeast Pol II, the nucleosome positioning sequences were amplified by PCR, digested with TspRI, gel purified, and used for nucleosome reconstitution and subsequent ligation to yeast EC9 transcription complexes as described earlier (Kireeva et al., 2002). For the human Pol II templates, the TspRI fragments were ligated to a promoter-bearing fragment containing the segment from −56 to +41 of the pML20-42 plasmid (Pal et al., 2005). PCR with a biotinylated upstream primer was then used to amplify this ligation product. The complete templates were gel purified and used for nucleosome reconstitution. To obtain the random-sequence DNA templates for transcription by yeast Pol II, two single-stranded DNA fragments were synthesized. One DNA fragment was 5′ end-labeled with T4 kinase. After annealing, the fragments were extended with Klenow(exo-) DNA polymerase and PCR amplified. The products of the reaction were digested with TspRI restriction enzyme, and the resulting 150 bp DNA fragment was purified and used for nucleosome reconstitution.

To prepare the 601 and 601R templates for transcription by SP6 RNAP, oligonucleotides containing an SP6 promoter were ligated to TspRI-cleaved fragments bearing the 601 and 601R assembly sequences. The final templates were PCR amplified, gel purified, and then used for nucleosome reconstitution. Details of the construction procedures as well as the sequences of the templates will be provided upon request.

Protein Purification and Nucleosome Assembly

Yeast Pol II with hexahistidine-tagged RPB3 subunit was purified as described (Kireeva et al., 2002). Human TFIIH and Pol II (purified from HeLa cells) and recombinant human transcription factors TBP, TFIIB, TFIIE, TFIIF, and TFIIS were prepared as described (Pal et al., 2005; Újvári and Luse, 2006). Human FACT was provided by Dr. D. Reinberg.

Nucleosomes for yeast Pol II transcription were reconstituted on the DNA templates by octamer exchange from chicken erythrocyte donor chromatin (Kireeva et al., 2002). Tetrasomes for yeast or human Pol II and nucleosomes for human Pol II or SP6 RNAP were reconstituted by decreasing salt dialysis using core histones purified from chicken erythrocytes (Studitsky, 1999). Assembly of nucleosomes from purified histones was conducted in the presence of a 2-fold excess of sheared salmon competitor DNA.

Transcription

Transcription of nucleosomal and DNA templates by yeast His-tagged Pol II was performed as described (Kireeva et al., 2002). In short, Pol II elongation complex (EC9) was assembled on a 50 bp DNA fragment. The EC9 was immobilized on Ni^{2+}-NTA agarose beads, ligated to DNA or nucleosomal templates, and washed. Pol II was advanced to the +45 position by using [α-^{32}P] NTPs to label the RNA. The ECs were washed, and transcription was resumed by addition of all unlabeled NTPs. Transcription in the presence of human recombinant FACT was conducted as described (Belotserkovskaya et al., 2003). Transcription by SP6 RNAP was conducted as described (Studitsky et al., 1995). Human Pol II preinitiation complexes (PICs) were assembled as described (Újvári and Luse, 2006), using 132 ng (free DNA or nucleosomes) or 264 ng (tetrasomes) of bead-attached DNA template per 100 μl. Template was doubled for tetrasome reactions because the promoter was occluded by the presence of a second tetramer on about half of these templates. To obtain U21 complexes, PICs were incubated with 0.25 mM CpA, 100 μM UTP, 0.7 μM α-^{32}P CTP, and 50 μM dATP at 30°C for 5 min, followed by the addition of nonlabeled CTP to 100 μM for 5 more min at 30°C. The U21 complexes were washed twice with 30 mM Tris-HCl (pH 7.9), 40 mM KCl, 10 mM β-glycerophosphate, 0.5 mM EDTA, 8 mM $MgCl_2$, 1 mM DTT, and 10% (v/v) glycerol and then resuspended in this same buffer. Rinsed complexes were supplemented with KCl, sarkosyl (1%), or TFIIS (24 μg/ml) as indicated in the figures, and then all complexes were incubated with 1 mM NTPs for 5 min at 30°C. For some reactions, sarkosyl was added after the first 5 min and transcripts were elongated for an additional 5 min at 30°C. All transcription reactions were stopped by the addition of EDTA to 19 mM, followed by extraction with phenol/chloroform and ethanol precipitation.

Transcripts made with yeast and human Pol II were resolved on denaturing polyacrylamide gels. Transcripts were quantified (Figure 4 and Figure S5) by using a Storm imager and ImageQuant software (GE Medical Systems).

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.molecule.org/cgi/content/full/24/3/469/DC1/.

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