STRUCTURAL BIOLOGY

Structure of transcribed chromatin is a sensor of DNA damage

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Early detection and repair of damaged DNA is essential for cell functioning and survival. Although multiple cellular systems are involved in the repair of single-strand DNA breaks (SSBs), it remains unknown how SSBs present in the nontemplate strand (NT-SSBs) of DNA organized in chromatin are detected. The effect of NT-SSBs on transcription through chromatin by RNA polymerase II was studied. NT-SSBs localized in the promoter-proximal region of nucleosomal DNA and hidden in the nucleosome structure can induce a nearly quantitative arrest of RNA polymerase downstream of the break, whereas more promoter-distal SSBs moderately facilitate transcription. The location of the arrest sites on nucleosomal DNA suggests that formation of small intranucleosomal DNA loops causes the arrest. This mechanism likely involves relief of unconstrained DNA supercoiling accumulated during transcription through chromatin by NT-SSBs. These data suggest the existence of a novel chromatin-specific mechanism that allows the detection of NT-SSBs by the transcribing enzyme.

INTRODUCTION

Single-strand breaks (SSBs) are common DNA damages generated during various processes of cell metabolism (spontaneous DNA damage, aborted topoisomerase action, DNA repair by base or nucleotide excision pathways, etc.) (*1*–*5*). Unrepaired SSBs can interfere with transcription, replication, and DNA repair; induce accumulation of double-stranded DNA breaks; increase genomic instability and apoptosis; and lead to severe neurodegenerative diseases [reviewed in (*6*–*9*)].

The first step in SSB repair is detection of the breaks. In most cases, the 5′ and/or 3′ ends of SSBs are modified and therefore are not immediately available for ligation. Recognized by poly(adenosine diphosphate–ribose) polymerase 1 (PARP1), SSBs are readily removed by the base excision repair pathway (*6*, *9*–*12*). However, in eukaryotic cells, DNA is organized in chromatin, which considerably limits the access of DNA binding protein to DNA and likely blocks the recognition of at least some SSBs by PARP1. Some otherwise undetectable SSBs can be sensed by processive enzymes progressing along DNA. In particular, SSBs localized on the template DNA strand block the progression of RNA polymerase II (Pol II) in vitro and in vivo (*13*, *14*). Stalled Pol II serves as a signal to initiate the process of transcription-coupled nucleotide excision repair (TC-NER); thus, in transcriptionally active cells, Pol II–blocking SSBs can be repaired by this pathway (*15*). However, SSBs localized on the nontemplate DNA strand (NT-SSBs) do not considerably affect the transcription of histone-free DNA in vitro (*16*), although they are efficiently repaired in vivo (*17*, *18*).

During transcription through chromatin in vitro, Pol II, which is involved in TC-NER, encounters a high nucleosomal barrier (*19*, *20*): transcription is accompanied by the formation of intranucleosomal DNA loops and by the loss of H2A/H2B dimer (*20*, *21*). Although chromatin is unfolded upon gene activation in vivo, the DNA remains packed into nucleosomes in the coding region of transcribed genes (*22*). Nucleosomes can be transiently displaced from eukaryotic genes when transcription levels are high (*23*–*25*), but most transcribed genes retain nucleosomal organization; thus, the Pol II complex typically encounters nucleosomes during elongation.

These observations raise the possibility that nucleosomal structure could affect the process of detection and repair of DNA damages. This possibility was evaluated using mononucleosomal DNA templates containing NT-SSBs. NT-SSBs strongly affected the rate of transcription through the nucleosome, but not the transcription of histone-free DNA. This finding suggests the existence of a chromatin-specific, transcription-dependent mechanism that allows detection of NT-SSBs that are otherwise hidden in the chromatin structure. We propose that intranucleosomal DNA loops formed during transcription through the nucleosome likely contribute to the arrest of Pol II on NT-SSBs.

RESULTS

Experimental approach

To study the process of transcription through chromatin containing NT-SSBs, we used uniquely positioned mononucleosomes formed on the high-affinity DNA sequences (*26*). This experimental system recapitulates the major properties of transcription through chromatin in vivo, for example, survival of histones H3/H4 and displacement of histones H2A/H2B during transcription (*21*, *27*). Positioned nucleosomes present a polar barrier to transcription by Pol II (*28*). Here, 603 nucleosomes in permissive transcriptional orientation were used because they better recapitulate the properties of nucleosomes transcribed in vivo (*21*). The 603 nucleosomes were uniquely positioned and contained less than 5% of contaminating histone-free DNA.

Most of the experiments described below require high amounts of homogeneous complexes that can be obtained only with *Escherichia coli* RNA polymerase (RNAP) that uses the Pol II–specific mechanism of transcription through chromatin in vitro (*21*, *28*, *29*). Therefore, many experiments were conducted using this bacterial polymerase as a convenient experimental model with subsequent recapitulation of the key results using yeast Pol II.

To identify NT-SSBs that have overall positive or negative effects on transcription, 603 templates end-labeled at the nontemplate strand

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and containing single, randomly positioned breaks were fractionated after transcription for a limited time (Fig. 1A). Histone-free DNA labeled at the nontemplate strand was treated with hydrogen peroxide under single-hit conditions to introduce single breaks at random positions on the template. Nucleosomes were assembled using dialysis from 2 M NaCl and transcribed for a limited time after stalling of the elongation complex at position −39 (position of the active center of the enzyme relative to the promoter-proximal nucleosomal boundary, designated as EC−39) to synchronize the position of the RNAP complexes along DNA (Fig. 1A). Transcribed and nontranscribed fractions were separated by native polyacrylamide gel electrophoresis (PAGE). The nontranscribed, slow-mobility fraction contains a mix of elongation complexes arrested in different positions inside the nucleosome. The transcribed fraction is a mix of nucleosomes, hexasomes (nucleosomes missing one H2A/H2B dimer), and histone-free DNA (*29*, *30*). The breaks facilitating or inhibiting the progression of RNAP through a nucleosome are expected to be depleted or enriched, respectively, in the nontranscribed fraction. To reveal the effect of the breaks on transcription through the nucleosome, end-labeled DNA purified from the transcribed and nontranscribed fractions was separated by denaturing PAGE.

To study transcription through nucleosomes containing single, uniquely positioned NT-SSBs, DNA templates were prepared by annealing and subsequent ligation of synthetic oligonucleotides or by enzymatic nicking (Fig. 1B). After RNA pulse labeling of EC−39, transcription was resumed for a limited time in the presence of all NTPs at different concentrations of KCl. Transcripts were analyzed by denaturing PAGE.

Location of intranucleosomal NT-SSBs determines effect on transcription

The transcribed and nontranscribed nucleosomal templates were separated in a native gel (Fig. 2A). In addition to formation of nucleosomes and subnucleosomes, transcription results in the appearance of ~35% of histone-free DNA, most likely due to inefficient formation of the intermediate complex, allowing nucleosome survival in vitro (*21*, *30*). Analysis of the distribution of NT-SSBs between the fractions has revealed two distinct regions of nucleosomal DNA (Fig. 2, B and C). NT-SSBs were enriched in the −5 to +36 region and underrepresented in the +(40–105) region of the nontranscribed fraction, respectively. The opposite distribution of NT-SSBs was observed in the transcribed fraction (Fig. 2, B and C). A similar distribution of NT-SSBs was observed in transcribed hexasomes.

To evaluate whether the fractionation of NT-SSBs is nucleosome-specific, we conducted a similar fractionation after limited transcription of histone-free, NT-SSB–containing DNA (fig. S1). We conducted the fractionation after transcription for 60 or 160 s to allow completion

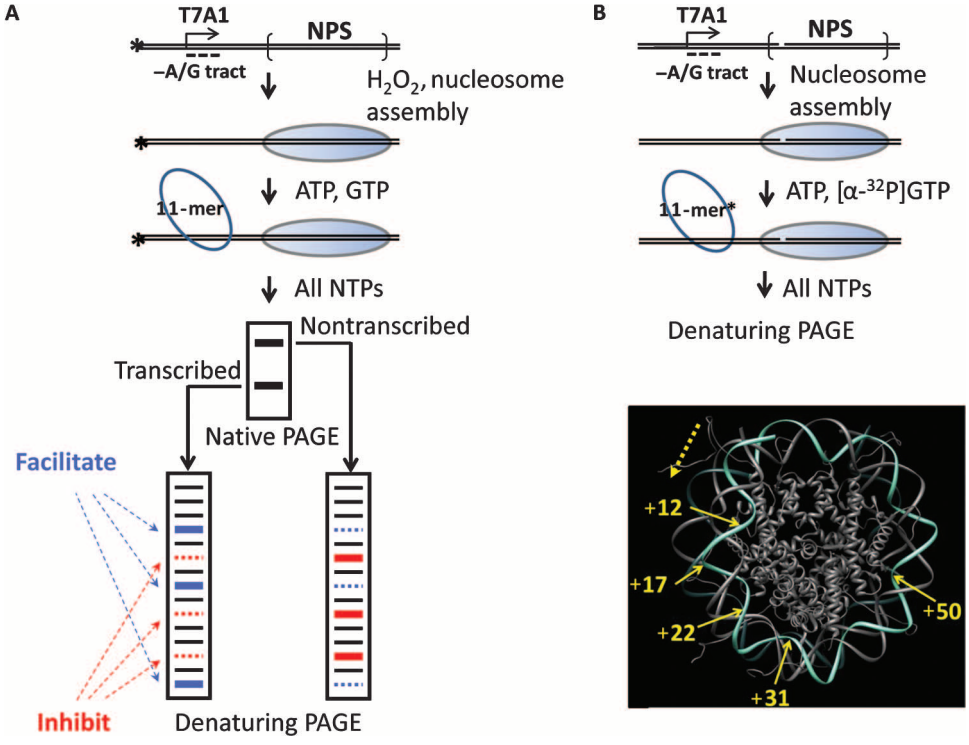

Fig. 1. Experimental strategies: Analysis of the effect of NT-SSBs on transcription through chromatin. (**A**) Identification of SSBs inhibiting or facilitating the progression of *E. coli* RNA polymerase through a nucleosome. Positioned nucleosomes were assembled on end-labeled (at nontemplate strand, asterisk) 603 DNA containing random single SSBs and transcribed for a limited time. Nucleosomes were separated by native PAGE. DNA was purified from transcribed and nontranscribed templates and analyzed by denaturing PAGE. NPS, nucleosome positioning sequence. NT-SSBs facilitating and inhibiting transcription through the nucleosome are shown in blue and red, respectively. (**B**) Transcription of nucleosomes containing unique SSBs. 603 nucleosomes containing SSBs in position +12, +17, +22, +31, or +50 (nontemplate DNA strand, insert at the bottom) were transcribed for different time intervals in the presence of various concentrations of KCl. Pulse-labeled RNA was separated by denaturing PAGE. The direction of transcription is indicated by the dashed yellow arrow.

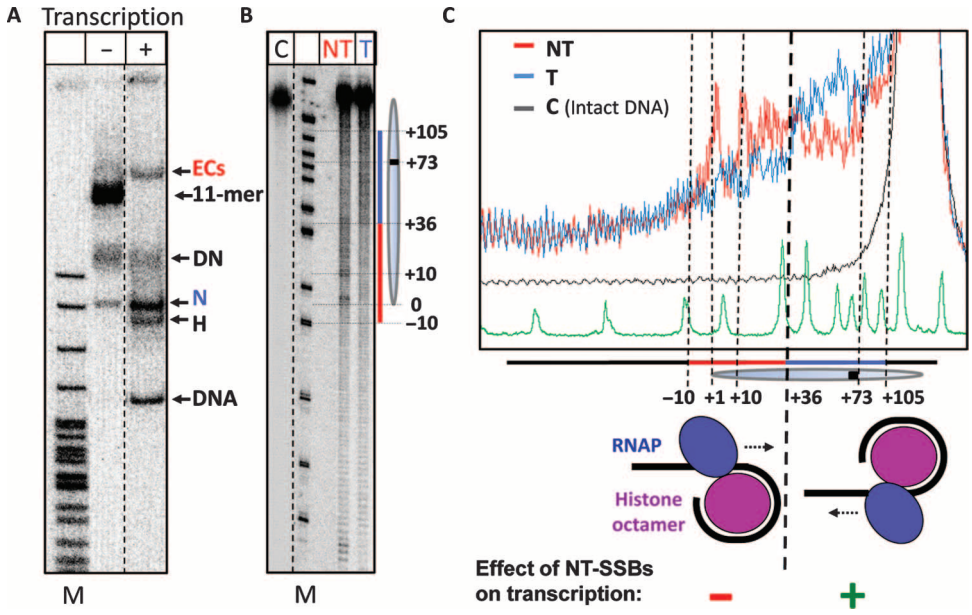

Fig. 2. Location of intranucleosomal NT-SSBs determines their effect on transcription. (**A**) Transcription of 603 nucleosomes containing end-labeled DNA and random single SSBs by *E. coli* RNAP for 10 min. Transcribed nucleosomes (N) and nontranscribed elongation complexes containing arrested RNAP (ECs) were separated by native PAGE before or after transcription. DN, nontranscribed dinucleosomes; H, hexasomes. Complexes containing 11-mer RNA are indicated. (**B**) Analysis of the distribution of NT-SSBs between transcribed (T) and nontranscribed (NT) templates by denaturing PAGE. C, control intact DNA. The nucleosome (blue oval) and the dyad (black rectangle) are indicated. Red and blue lines show DNA regions where breaks in the transcribed fraction were under- or overrepresented, respectively. (**C**) Quantitative analysis of the distribution of the SSBs shown in (B). M, pBR322 Msp I digest. The overall positive and negative effects of NT-SSBs on transcription (+ and –) roughly correlate with uncoiling of nucleosomal DNA in front and behind the RNAP, respectively (*21*, *30*). Only half of the nucleosomal DNA supercoil is shown.

of transcription on 20 or 80% of templates, respectively. This fractionation revealed only minimal differences between the transcribed and nontranscribed templates (fig. S1), suggesting that the presence of nucleosomes is essential for transcription-dependent fractionation of NT-SSBs. The observed, relatively minor differences in distributions of NT-SSBs between DNA recovered from completely transcribed templates and DNA with stalled polymerase (for example, in the −10 to +1 and +105 DNA regions) likely occur because of the sequence-specific effects of NT-SSBs on the progression of RNAP along histone-free DNA.

Accumulation of NT-SSBs in the nontranscribed fraction in the −5 to +36 region of nucleosomal templates indicates that these breaks likely interfere with the progression of the RNAP along nucleosomal DNA. Within this region, two distinct peaks (at positions +1 and +12) and a broad +(20–36) region were detected (Fig. 2C), perhaps related to the rotational orientation of NT-SSBs in nucleosomal DNA. On the contrary, accumulation of NT-SSBs localized within the +(40–105) region in the transcribed fraction (Fig. 2C) suggests that breaks in this region likely facilitate the progression of the enzyme through chromatin.

During transcription through the +(1–36) region and the region after position +50, nucleosomal DNA is uncoiled from the histone octamer primarily behind and in front of the enzyme, respectively (*21*). Thus, the locations of NT-SSBs that negatively and positively affect transcription through the nucleosome approximately correlate with locations of the regions where DNA is uncoiled behind and in front of the RNAP, respectively (Fig. 2C). This, in turn, suggests that the topology of complexes containing RNAP transcribing through the nucleosome dictates the effect of NT-SSBs on the efficiency of transcription through chromatin.

Intranucleosomal NT-SSBs can have opposite effects on transcription

To further study the effect of NT-SSBs on transcription through chromatin, we introduced single, uniquely positioned NT-SSBs in various positions on 603 nucleosomal DNA (Fig. 1B). Initially, single SSBs were introduced at positions +12 and +50 because here they were expected to strongly inhibit and facilitate transcription through the nucleosome, respectively (Fig. 2C). As expected, transcription of histone-free DNA templates containing single SSB at either position +12 or +50 did not result in a significant additional pausing as compared with transcription of intact DNA.

In contrast, transcription through the nucleosome having +12 SSB results in an almost quantitative arrest of the RNAP at 40 and 150 mM KCl (Fig. 3A). At 300 mM and 1 M KCl, ~50% and more than 80% of transcribing complexes proceed through the SSB, respectively. The more efficient progression of RNAP through the nucleosomal SSB is most likely explained by the less stable DNA-histone interactions at higher concentrations of KCl (*31*). Analysis of the time course of transcription through the nucleosome suggests that the overall efficiency of transcription is considerably affected by the SSB (Fig. 3, C and D, and fig. S2).

Surprisingly, the RNAP is arrested at a single position (+24) located 12 base pairs (bp) downstream of the +12 SSB. Because the arrest is nucleosome-specific and is observed after the active center of the enzyme progresses through the SSB, it is likely related to nucleosome-specific effects on the DNA structure. How can the presence of the SSB affect the structure of upstream DNA during transcription through a nucleosome? One obvious possibility is the formation of an intranucleosomal

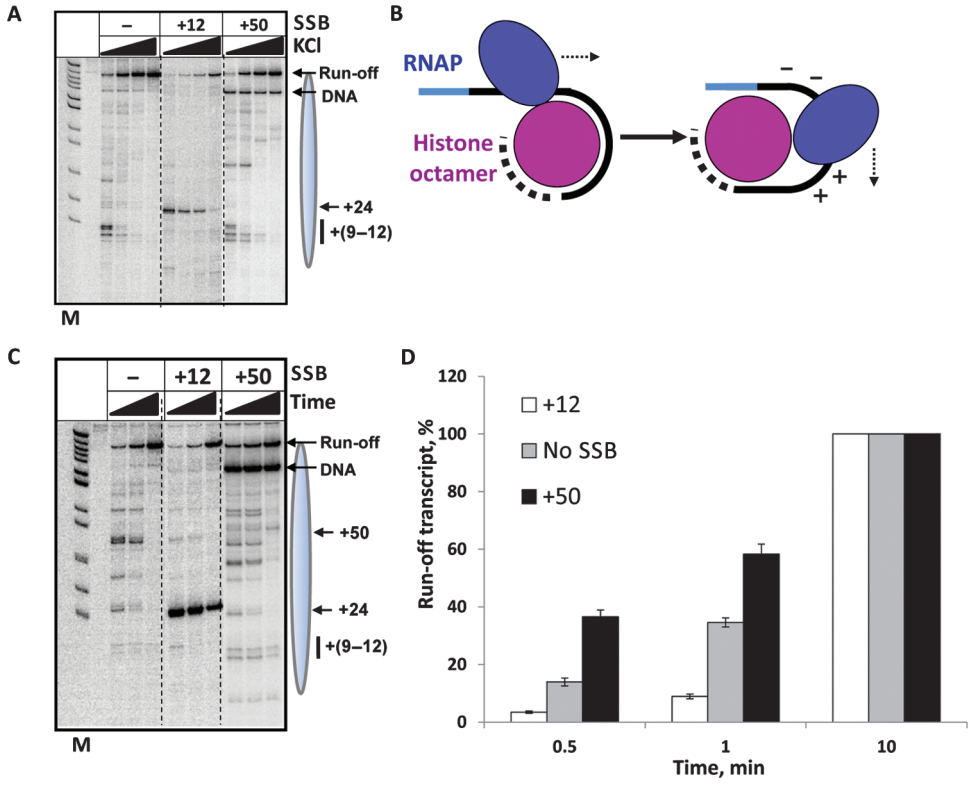

Fig. 3. Individual SSBs differentially affect transcription through a nucleosome. (**A**) Transcription through 603 nucleosomes containing single SSBs at position +12 or +50 by *E. coli* RNAP for 10 min at 40, 150, 300, or 1000 mM KCl. Analysis of pulse-labeled RNA by denaturing PAGE. (**B**) A model describing the effect of SSBs on transcription through a nucleosome. As RNAP encounters the histone octamer and proceeds past the SSB, a transient DNA loop could be formed. Formation of the loop would result in accumulation of unconstrained negative (––) and positive (++) DNA supercoiling behind and in front of the enzyme, respectively. We propose that SSBs facilitate or inhibit transcription through a nucleosome when present in front or behind RNAP, and relieve positive or negative unconstrained DNA supercoiling, respectively. (**C**) Transcription through 603 nucleosomes containing single SSBs by *E. coli* RNAP for 0.5, 1, and 10 min at 300 mM KCl. Analysis of pulse-labeled RNA by denaturing PAGE. (**D**) Quantitative analysis of run-off transcripts shown in (C). The quantified signals were normalized to the corresponding 10-min signals. The signals without normalization are shown in fig. S2.

DNA loop containing the transcribing enzyme that was previously observed in a different location of the enzyme (position +49) (*21*). Formation of such a loop (Fig. 3B) would result in establishment of DNA-histone interactions behind the RNAP. These interactions could affect transcription in at least two nonexclusive ways: (i) They serve as an additional physical barrier to rotation of the enzyme. (ii) Because a small, topologically constrained DNA loop is formed, the rotation of RNAP likely results in accumulation of unconstrained DNA supercoiling having opposite signs in front of and behind the enzyme (Fig. 3B) (*32*). In this case, the SSBs introduced in nucleosomal DNA could strongly and negatively affect the accumulation of both negative and positive unconstrained DNA supercoiling and act as topoisomerases.

More generally, an encounter between a processive enzyme (such as RNA or DNA polymerase) and a nucleosome is expected to result in the formation of "topological locks" of different types (fig. S3). In the topological locks, the DNA region between the enzyme and the octamer is topologically constrained. Because RNAP is a powerful molecular motor (*33*), its rotation likely induces high levels of positive and/or negative unconstrained DNA supercoiling (++ and ––, respectively), depending on the exact topology of the complexes (fig. S3).

In addition to causing a strong decrease in the overall yield of run-off transcripts and arrest at position +24, the presence of +12 SSB results in a considerable relief of nucleosomal pausing at the +(9–12) region (Fig. 3A). This region is positioned upstream of the SSB, and the SSB has an opposite effect on nucleosomal pausing. These data suggest that the effect of an SSB on transcription through the nucleosome depends on the SSB location relative to the active center of the enzyme (Fig. 3B).

Although the +50 SSB does not strongly affect the yield of run-off transcripts when transcription is conducted for 10 min at various concentrations of KCl (Fig. 3A), analysis of the time courses (Fig. 3, C and D, and fig. S2) revealed almost three- and twofold increases in the yield of run-off RNA after transcription at 300 mM KCl for 0.5 and 1 min, respectively. In contrast to +12 SSB, the +50 SSB did not induce a strong arrest of RNAP in a unique downstream position (Fig. 3, A and C). Instead, the presence of the +50 SSB results in a complex change in nucleosome-specific pausing pattern, with stronger pausing at positions +30, +45, +55, and +65 and with partial relief of pausing at positions +48 and +80 (Fig. 3C). These complex changes in the pattern of nucleosomal pausing are likely explained by the complex conformational transitions in the nucleosome

structure that occur during transcription through the +50 region of nucleosomal DNA (*34*).

In summary, the data suggest that the effect of SSBs on transcription through the nucleosome depends on their location relative to the active center of the enzyme: SSBs localized downstream or upstream of transcribing RNAP facilitate or inhibit transcription through the nucleosome, respectively. These effects of SSBs on transcription are likely explained by the formation of a small intranucleosomal DNA loop containing the transcribing enzyme and by the accumulation of unconstrained DNA supercoiling within the loop during transcription of intact nucleosomal DNA (Fig. 3B). NT-SSBs relieve unconstrained DNA supercoiling and thus affect the progression of RNAP through the nucleosome.

+12 NT-SSB relieves nucleosomal pausing and induces a strong arrest of Pol II in a nucleosome

The negative effect of NT-SSBs located in the −5 to +36 region of nucleosomal DNA on transcription is of immediate interest because the effect of +12 SSB is strong and distinct and because arrested Pol II could serve as a marker of DNA containing NT-SSBs (see Discussion). Therefore, our further studies focused on analysis of the negative effect of NT-SSBs on transcription through chromatin.

To evaluate the effect of the +12 SSB on transcription through chromatin by Pol II and further validate *E. coli* RNAP as the experimental model, yeast Pol II elongation complexes were assembled on nucleic acid scaffold [set of oligonucleotides allowing formation of the proper "transcription bubble" (*20*)]. This experimental system (Fig. 4A) recapitulates multiple properties of transcription through chromatin by Pol II in vivo (*20*, *21*). Assembled elongation complexes were ligated to preassembled 603 mononucleosomes (containing DNA either with or without +12 SSB) and transcribed at 40, 150, 300, or 1000 mM KCl (Fig. 4B). As expected, the presence of the SSB resulted in a lower yield of run-off transcripts and a considerable change in nucleosome-specific pausing pattern in the +(9–25) region of nucleosomal DNA. Specifically, the +(9–12) nucleosomal pausing characteristic of intact nucleosomal DNA was nearly quantitatively relieved and replaced with a strong pausing at the +(22–24) region after introduction of the +12 SSB (Fig. 4B). Thus, the primary data obtained using RNAP were recapitulated with yeast Pol II.

NT-SSBs induce arrest of RNAP in discrete positions spaced with 10-bp intervals along nucleosomal DNA

Next, critical predictions of the looping model for SSB-dependent arrest of RNAP in the nucleosome (Fig. 5A) were further evaluated. The model predicts that intranucleosomal DNA loops could be formed only in certain rotational orientations of the RNAP on DNA; these orientations are largely dictated by the topology of DNA looping (*21*). Different sets of closely positioned NT-SSBs are localized within the same DNA loop and are expected to induce arrest of RNAP at the same locations, but these locations are expected to be different for different sets (Fig. 5A).

To evaluate the predictions of the looping model, single NT-SSBs having different rotational orientations were introduced in various closely related locations in 603 DNA (+12, +17, +22, or +31; Fig. 1A). Transcription of the nucleosomes containing NT-SSBs in these positions at 150 and 300 mM KCl revealed clear 10-bp periodicity of arrest/pausing of RNAP induced by the SSBs (Fig. 5B). Thus, SSBs at positions +12 and +17 induce +24 arrest/pausing, whereas +22 and +31 SSBs induce arrest at positions +34 and +(43, 44), respectively. Thus, NT-SSBs having different rotational and translational positioning on nucleosomal DNA induce 10-bp periodic arrest at positions +24, +34, and +(43, 44). Therefore, pausing/arrest locations are dictated not by the position of the SSB but rather by the ability of the intranucleosomal loop to form at the discrete positions of RNAP.

The second critical prediction of the looping model was also confirmed by the data. Indeed, closely positioned +12 and +17 NT-SSBs induced arrest of RNAP at the same +24 location on nucleosomal DNA, suggesting that these SSBs are localized within the same DNA loop. Thus, the data obtained with the templates containing single NT-SSBs at position +12, +17, +22, or +31 provide a strong support for 10-bp periodic formation of intranucleosomal DNA loops containing RNAP paused/arrested at positions +24, +34, and +(43, 44). Note that some much weaker pausing occurs at the positions of SSB-induced pausing/arrest [+24, +34, and +(43, 44)] even during transcription through nucleosomes having intact DNA (Fig. 5B). The data suggest that during transcription of the intact nucleosomes, similar intranucleosomal loops are formed, but in the absence of SSBs, they are resolved at a higher rate.

In all cases, NT-SSBs are localized 7 to 13 bp upstream of the paused/arrested RNAP, suggesting that putative DNA loops are relatively small, with DNA "arms" between the active center of the enzyme and DNA-histone interactions in the nucleosome having a length of 15 to 20 bp, with an overall loop size of 40 to 60 bp (Fig. 6). In agreement with the previous data (Fig. 2C), all NT-SSBs within the −5 to +36 region negatively affect the yield of run-off transcripts (Fig. 5B). However, the efficiencies of pausing/arrest are different for various SSBs in the following order: +12 ≥ +22 > +31 > +17. The least efficient pausing/arrest was observed at position +17, most likely

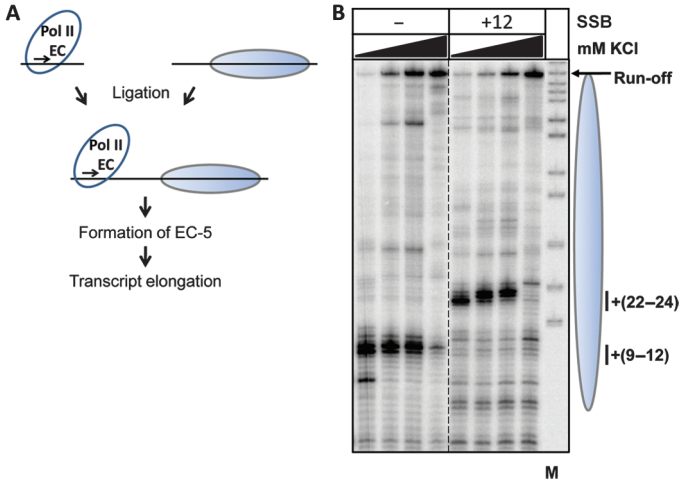

Fig. 4. +12 NT-SSB relieves nucleosomal pausing and induces a strong arrest of Pol II in a nucleosome. (**A**) The experimental approach for analysis of transcription through 603 nucleosome by yeast Pol II. Pol II elongation complex (EC) was assembled, immobilized on Ni^{2+}-NTA agarose beads, and ligated to DNA or nucleosomal templates. Pol II was advanced to produce EC-5 complex using a subset of NTPs and [α-^{32}P]GTP (guanosine triphosphate) to label the RNA. Then transcription was resumed by the addition of all unlabeled NTPs. (**B**) Transcription through 603 nucleosomes containing single SSB at position +12 by Pol II for 10 min at 40, 150, 300, or 1000 mM KCl. Analysis of pulse-labeled RNA by denaturing PAGE.

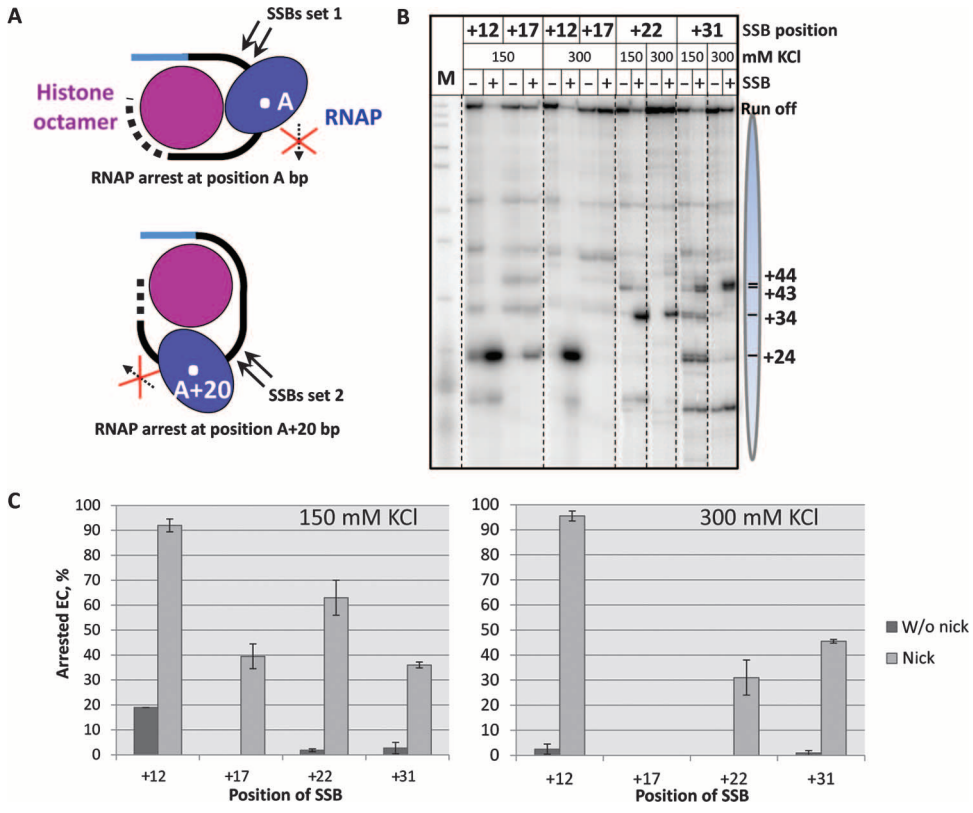

Fig. 5. NT-SSBs induce a strong arrest of RNAP in discrete 10-bp periodic positions of nucleosomal DNA. (**A**) Predictions of the looping model (Fig. 3B) for the effect of NT-SSBs on the arrest of RNAP during transcription through chromatin. Different sets of closely located SSBs (larger arrows) are expected to induce arrest of RNAP at different, distinct locations on nucleosomal DNA (see Results for detail). (**B**) Transcription through 603 nucleosomes containing single SSBs at position +12, +17, +22, or +31 by *E. coli* RNAP for 2 min at 150 or 300 mM KCl. Analysis of pulse-labeled RNA by denaturing PAGE. (**C**) Quantitative analysis of SSB-induced arrest of RNAP in the nucleosome.

because this SSB is positioned only 7 bp upstream of the position of the pausing (+24) and therefore remains with the transcriptional "bubble" and could be stabilized by RNAP interactions with the bubble. In this case, it could be more difficult to relieve DNA supercoiling accumulated in the loop during transcription. SSBs in multiple positions (+12, +22, and +31) induce nearly quantitative, nucleosome-dependent arrest of RNAP at physiological ionic strength (150 mM KCl); the higher efficiencies of arrest are characteristic of the SSBs that are more hidden in the nucleosome structure.

Mechanism of NT-SSB–induced arrest of RNAP in chromatin

In summary, the data show that NT-SSBs in multiple intranucleosomal locations induce a strong arrest of RNAP. Because the arrest is nucleosome-dependent and in all cases occurs downstream of the SSBs, the data suggest that it is accompanied by formation of intranucleosomal DNA loops. The loops likely have a small size (40 to 60 bp) and are formed with 10-bp periodicity (at +24, +34, and +44 positions of the active center of RNAP), indicating that they have similar geometries and rotational orientations of RNAP and similar properties. The loops are likely formed both in the presence and in the absence of NT-SSBs, but the SSBs stabilize the loops, inducing the arrest of RNAP in the loop.

Together with our previous data (*21*, *27*), the current results suggest the following model for transcription through the nucleosome (Fig. 6). As RNAP encounters a nucleosome, the rotation of the enzyme around DNA is sterically blocked by the histone octamer. This topological block could result in accumulation of positive unconstrained DNA supercoiling in front of the enzyme (Fig. 6, complex 1). Because NT-SSBs that relieve positive supercoiling likely facilitate transcription (Fig. 3), positive supercoiling probably inhibits the progression of the enzyme. Positive supercoiling accumulated in front of RNAP could inhibit transcription by inducing unfolding of the histone octamer (*35*, *36*). This reaction competes with the partial DNA displacement required for further transcription and thus likely inhibits the progression of the enzyme. Alternatively, positive supercoiling could inhibit DNA melting (*37*) and therefore the unwinding of DNA strands in the transcriptional bubble, thus inducing the arrest of transcription. The presence of an NT-SSB immediately in front of the enzyme would allow rotation of DNA strands around the remaining phosphodiester bond, relieve the positive supercoiling, and allow further progression of RNAP.

As RNAP proceeds further, the DNA binding surface of the histone octamer is exposed behind the enzyme (Fig. 6, complex 2) and allows the formation of an intranucleosomal DNA loop containing the transcribing enzyme within the loop, initially at position +24 (Fig. 6, complex 3). Because of the small size of the loop, two independent topological domains are likely formed (in front of and behind the enzyme, respectively). As bulky RNAP rotates in the loop, positive and negative DNA supercoiling accumulate in front of and behind the enzyme, respectively (Fig. 6, complex 3). As negative supercoiling accumulates in

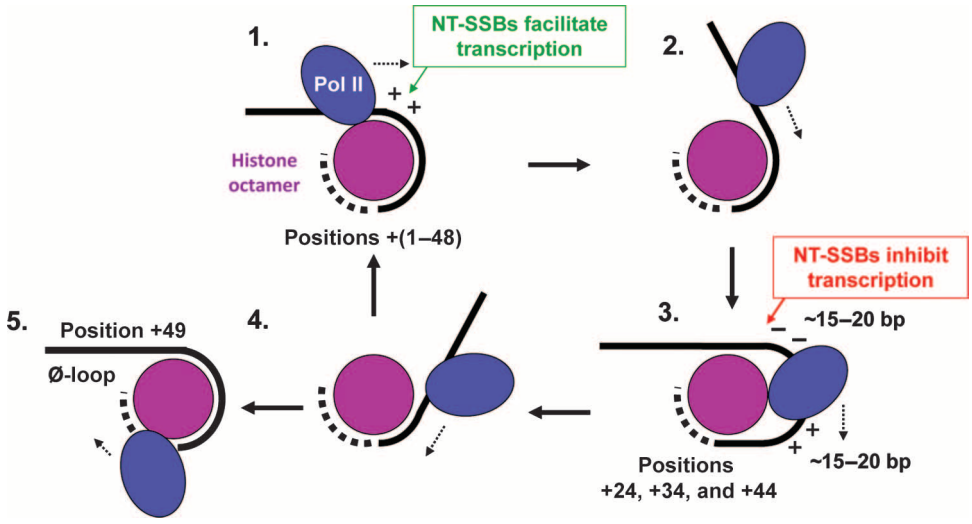

Fig. 6. Proposed mechanism of NT-SSB–induced arrest of Pol II in chromatin. Only half of the nucleosomal DNA supercoil is shown. As Pol II encounters the histone octamer, its progression is hindered, and positive unconstrained DNA supercoiling (++) accumulates in front of the enzyme. We propose that single-strand breaks in DNA facilitate transcription through a nucleosome when present in front of the enzyme (perhaps by relieving unconstrained positive DNA supercoiling) (complex 1). Then, nucleosomal DNA is partially uncoiled from the octamer (complex 2), and transient DNA loops are formed [likely at the position of the active center of RNA polymerase +24, +34, or +44 (complex 3)]. Here, SSBs could inhibit transcription and DNA displacement (steps 3 to 4) when present behind RNAP, perhaps by relieving negative supercoiling behind the enzyme. Once Pol II proceeds to position +49, a very small intranucleosomal DNA loop is formed (complex 5), DNA is uncoiled from the octamer in front of the enzyme, and transcription continues efficiently (*21*).

the loop, the loop opens behind the enzyme (Fig. 6, complex 4). SSBs likely inhibit this partial DNA displacement by relieving DNA supercoiling and thus inhibit transcription when present behind RNAP.

Once the enzyme escapes from the +24 loop (Fig. 6, complex 4), the cycle described above is likely repeated at least two more times (accompanied by formation of similar loops at positions +34 and +44). As the enzyme escapes from the +44 loop, the +49 loop is formed and resolved in front of the transcribing complex (Fig. 6, complex 5) (*21*). Then, the enzyme proceeds more efficiently and completes the transcription of nucleosomal DNA; this is typically accompanied by nucleosome recovery at the original position on DNA and by the loss of one H2A/H2B dimer (*21*).

DISCUSSION

Role of intranucleosomal DNA supercoiling during transcription through chromatin

Transcription of right-handed DNA double helix requires rotation of the RNAP around the DNA. If the DNA ends and the enzyme are attached to a scaffold, transcription results in accumulation of unconstrained positive and negative supercoiling in DNA ahead of and behind RNAP, respectively (*32*, *33*). This mechanism most likely explains the accumulation of unconstrained DNA supercoiling during transcription of chromatin in vivo and typically involves large domains of chromatin [reviewed in (*38*)]. Similar conditions for accumulation of transcription-dependent DNA supercoiling are present after formation of a small intranucleosomal DNA loop—DNA ends are immobilized on the surface of the histone octamer, and RNAP cannot rotate because of the small size of the loop. Our present data suggest that formation of such loops occurs several times during transcription through a nucleosome (Fig. 6) (*21*). Because RNAP is a powerful molecular motor (*33*), formation of such loops should result in accumulation of unconstrained positive and negative DNA supercoiling in front of and behind RNAP, respectively. Unconstrained supercoiling likely plays multiple important roles during transcription through chromatin (see Supplementary Discussion).

Detection of NT-SSBs through intranucleosomal DNA loop–dependent arrest of transcribing Pol II

Pol II arrest is a signal to transcription-coupled repair cascade (see Introduction); therefore, the ability of NT-SSBs to induce transcriptional arrest suggests their role in DNA repair. Typically, SSBs are detected by PARP1 (*6*, *10*, *11*), but chromatin-organized DNA is likely less accessible to PARP1, and at least some lesions could remain undetected. Indeed, mutation rates are elevated in more heterochromatin-like domains and repressed in more open chromatin in cancer cells (*39*). SSBs localized in the template strand of genes can be directly detected by Pol II arrest during transcription of damaged DNA and become repaired by the TC-NER pathway (*40*, *41*). Furthermore, repair of NT-SSBs requires the activity of CSB, the major TC-NER factor in mammalian cells (*42*), suggesting that the TC-NER pathway is also involved in repair of NT-SSBs. However, in vitro studies failed to detect the arrest of Pol II (the first step in TC-NER) on NT-SSBs during transcription of histone-free DNA in vitro [(*16*) and see Results]. Our data show that chromatin structure enables detection of NT-SSBs by inducing the arrest of transcribing Pol II through formation of small intranucleosomal DNA loops. The NT-SSBs localized on the side of nucleosomal DNA facing the histone octamer [and therefore most efficiently hidden from the enzymes repairing DNA (positions +12, +22, and +31; Fig. 1B)] induce a more efficient arrest of RNAP than SSB +17 that is exposed on the surface of nucleosomal DNA and

therefore could be accessed by the excision repair machinery. Although the specific mechanism dictating this preference is unknown (see Results), the data suggest that it is designed to allow detection of SSBs hidden in the nucleosome structure.

NT-SSBs induce a stronger arrest of RNAP when they are localized in the ~50-bp-long, promoter-proximal region of nucleosomal DNA (Fig. 5). How could NT-SSBs localized in the remaining ~100 bp of nucleosomal DNA be detected? Because nucleosomes rarely occupy strictly defined positions in vivo [see (*43*) for discussion] and because nucleosome positioning during transcription is dynamic (*44*), the position of every NT-SSB in a nucleosome is likely to be variable. For example, if nucleosome positions are randomized, during every round of transcription, each NT-SSB has ~20 to 30% probability to be localized in the ~50-bp-long, promoter-proximal region of nucleosomal DNA and to be detected by transcribing Pol II through the loop-dependent mechanism.

In summary, our data suggest the existence of a novel, chromatin-specific mechanism of detection of NT-SSBs by transcribing Pol II that relies on the formation of multiple small intranucleosomal DNA loops. The formation of the loops induces the arrest of transcribing Pol II, particularly efficient in the case of SSBs that are hidden on the side of nucleosomal DNA facing the histone octamer. Thus, DNA supercoiling accumulated after loop formation serves as a sensor of NT-SSBs and mediates their effect on the progression of RNAP along nucleosomal DNA. Pol II arrest initiates the DNA repair cascade in vivo and thus likely allows repair of otherwise hidden NT-SSBs. The dynamic structure of transcribed chromatin could facilitate DNA repair and render the vast majority of SSBs accessible to the repair machinery.

MATERIALS AND METHODS

Proteins and DNA templates

Saccharomyces cerevisiae Pol II and hexahistidine-tagged *E. coli* RNAP were purified as described (*45*, *46*). Plasmid containing the 603 nucleosome positioning sequence (*47*) was provided by J. Widom. The 603 sequence was modified at four positions to construct the s603-25A template and allow stalling of *E. coli* RNAP at position +24. Templates containing single NT-SSBs in different positions were prepared by annealing of synthetic purified oligonucleotides. After annealing and ligation, templates were purified by native PAGE (*48*). Alternatively, NT-SSB–containing templates were obtained by mutagenesis of the s603-25A template to introduce single sites for nicking endonuclease *Nt.Bsm*A1. NT-SSB–containing DNA templates were purified with a QIAquick PCR Purification Kit (Qiagen). The sequences of the templates will be provided upon request.

Hydroxyl radical treatment

Hydroxyl radical treatment of DNA template with labeled noncoding or coding strand was performed as described in the published protocol (*49*). Conditions were selected to introduce single, randomly positioned NT-SSBs in less than 25% of templates. The level of digestion was controlled by denaturing PAGE.

Nucleosome assembly

Nucleosomes were assembled by octamer transfer from chicken erythrocyte donor H1 chromatin after dialysis from 1 M NaCl (*20*).

Transcription

Transcription of nucleosomal and DNA templates by Pol II was performed as described earlier (*20*). Transcription by *E. coli* RNAP (200 nM) was started with formation of open complex on template DNA (40 nM) in transcription buffer TB40 [20 mM tris-HCl (pH 7.9), 5 mM $MgCl_2$, 40 mM KCl, and 1 mM β-mercaptoethanol] by incubation for 7 min at 37°C. Elongation complexes containing 11-mer RNA (EC–39, the number indicates the position of the active center of the enzyme relative to the promoter-proximal nucleosomal boundary) were formed by the addition of 5′-ApUpC and adenosine triphosphate to 20 μM each and [α-^{32}P]GTP (3000 Ci/mmol) in TB40 for 10 min at room temperature. Then, unlabeled GTP was added to 20 μM concentration, and the reaction mixture was incubated for 5 min at room temperature. To prevent multiple rounds of transcription initiation, rifampicin (20 μg/ml) was added. Transcription was resumed by the addition of four NTPs to a final concentration of 200 μM each to pulse-labeled EC–39 in transcription buffer with different concentrations of KCl for the limited time intervals at room temperature (see the figure legends for detail). The reaction was terminated by the addition of EDTA or phenol.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/1/6/e1500021/DC1

Discussion

Fig. S1. Effect of NT-SSBs on transcription of histone-free DNA.

Fig. S2. Quantitative analysis of run-off transcripts shown in Fig. 3C without normalization to the corresponding 10-min signals.

Fig. S3. Encounter between RNAP and nucleosome likely results in formation of topological locks.

Reference (*50*)

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