Histone Sin mutations promote nucleosome traversal and histone displacement by RNA polymerase II

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Nucleosome traversal by RNA polymerase II (pol II) and recovery of chromatin structure after transcription are essential for proper gene expression. In this paper we show that nucleosomes assembled with Sin mutant histones present a much weaker barrier to traversal by pol II and are less likely to survive transcription. Increases in traversal from incorporation of Sin mutant histones and histones lacking H2A/H2B amino-terminal tails were in most cases additive, indicating that traversal can be facilitated by distinct mechanisms. We had identified a key intermediate in traversal, the zero (Ø)-loop, which mediates nucleosome survival during transcription. Sin mutations probably destabilize these intermediates and thus increase the likelihood of nucleosome disassociation.

Keywords: nucleosome; RNA polymerase II; transcript elongation

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INTRODUCTION

In the cell, polymerase II (pol II) efficiently traverses nucleosomes, but histone H3 (H3) and histone H4 (H4) are displaced only at very high levels of gene expression (Lee *et al*, 2004; Schwabish & Struhl, 2004; Thiriet & Hayes, 2005). Nucleosomes are formidable barriers to transcript elongation *in vitro* (Bondarenko *et al*, 2006; Újvári *et al*, 2008). Once pol II advances beyond a major pause, about 45 bp within the nucleosome, the remaining downstream DNA transiently unfolds from the octamer surface and the traversal barrier is largely overcome (Bondarenko *et al*, 2006; Újvári *et al*, 2008; Kulaeva *et al*, 2009). As traversal proceeds, retention of upstream DNA–octamer contacts ensures that the nucleosome generally survives transcription *in vitro* (Kulaeva *et al*, 2009).

The transient unwrapping that allows pol II to cross the +45 barrier depends on histone–DNA interactions from +86 to +102

(Kulaeva *et al*, 2009). Histone–DNA interactions in the region from +70 to +80 are particularly strong, suggesting that these are also important contributors to the nucleosomal transcription barrier (Hall *et al*, 2009). To evaluate the role of these latter interactions in the transcription barrier and in nucleosome survival, we have transcribed nucleosomes containing so-called Sin mutant histones. These variants of H3 or H4 partly relieve the requirement of a chromatin remodelling complex for the expression of certain yeast genes (Kruger *et al*, 1995). Sin mutations specifically affect the strong histone–DNA interactions in the region from +70 to +80 (Muthurajan *et al*, 2004). Sin mutant nucleosomes show increased mobility along DNA and reduced stability (Muthurajan *et al*, 2004).

In this study, we show that Sin mutants of H3 or H4 significantly reduce the nucleosomal transcription barrier for pol II. Sin mutant nucleosomes also have a greater tendency to dissociate completely from DNA during traversal by RNA pol II.

RESULTS

In our experiments, yeast or human pol II transcribed a template bearing a single downstream nucleosome assembled at a precise location (Bondarenko *et al*, 2006; Újvári *et al*, 2008). Transcripts were labelled during an initial pulse, followed by a rinse and chase with excess non-labelled NTPs. The two nucleosome assembly elements used in our study are designated 603 (Lowary & Widom, 1998), which is more permissive for traversal, and 603R, which is less permissive (Újvári *et al*, 2008).

Sin mutations affect gene expression to different extents (Kruger *et al*, 1995). Muthurajan *et al* (2004) incorporated yeast Sin mutations into analogous locations in *Xenopus* H3 or H4 and determined the structures and physical properties of nucleosomes containing the mutant histones. H3 T118I and H4 R45C showed the greatest effects in thermal mobilization studies of nucleosomes, whereas H3 R116H and H4 V43I had lesser effects. We used these recombinant *Xenopus* histones in our experiments.

Figure 1 shows the results of transcription of 603 nucleosomal templates by yeast (Fig 1A) or human (Fig 1B) pol II. The histone composition is abbreviated as (H2A–H2B type)/(H3–H4 type). For H2A/H2B, 'n' designates native and 'g' designates globular (lacking amino-terminal tails). For H3/H4, histones were either native (n) or the indicated Sin mutant was substituted. Traversal

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Fig 1 | Nucleosomes containing Sin mutant histones or lacking amino-terminal tails provide a reduced barrier to traversal by polymerase II. (A) Nucleosomes were assembled on the 603 template using wild-type (n/x) or tailless (g/x) H2A/H2B, and wild-type (x/n) or Sin mutant H3 and H4. RNA in yeast polymerase II complexes was initially pulse-labelled and then extended at the indicated KCl concentrations with excess unlabelled NTPs. The locations of the nucleosome (oval), the nucleosome dyad (square), the main pol II pause sites and the position of the run-off transcript are shown. (B) As in (A), except that human pol II was used. KCl concentrations, sarkosyl addition (1%) and no-chase controls (nc) are indicated. (C) As in (B), except that the template was 603R. g, globular; H3, histone H3; H4, histone H4; n, native; pol II, polymerase II.

of wild-type 603 nucleosomes by either yeast (Fig 1A) or human (Fig 1B) pol II is highly inefficient at 40 mM salt and increased only slightly at 150 mM KCl, as expected (Bondarenko *et al*, 2006; Újvári *et al*, 2008; quantitative results are shown in supplementary Fig S1 online). Prominent stops were observed at +15 and in the +45 region.

The 603 transcription barrier was reduced significantly when either H3 T118I or H4 R45C was incorporated (Fig 1A,B; supplementary Fig S1 online). Pausing was primarily lowered at or near +45. By contrast, traversal of 603 nucleosomes with H3 R116H histones was almost unchanged from that of wild type (primary data not shown; supplementary Fig S1 online). Similarly, for yeast pol II, no increase in traversal was observed after substitution of H4 V43I for wild-type H4 (supplementary Fig S1 online). Thus, the extent of relief of the 603 nucleosomal barrier to pol II transcription by various Sin mutations seems to be correlated with their effect on thermal mobilization of nucleosomes (Muthurajan *et al*, 2004).

Nucleosomes assembled on the 603R template provide a consistently stronger barrier to transcript elongation in comparison with 603 nucleosomes (Bondarenko *et al*, 2006). Yeast pol II was not used for our 603R studies, as the +45 region on this template represents a strong barrier to transcript elongation for the yeast enzyme as pure DNA. Substitution of either H3 T118I or H4 R45C reduced the pausing by human pol II at +45 and strongly reduced (R45C) or eliminated (T118I) the +55 pause (Fig 1C). Traversal of the 603R nucleosomes with H3 T118I by human pol II increased about threefold relative to wild type at both 40 and 150 mM KCl (supplementary Fig S1 online). Interestingly, the barrier relief relative to wild type provided by H4 R45C was distinctly lower than the barrier reduction with H3 T118I; further, barrier relief for human pol II on 603R was comparable for H3 R116H and H4 R45C, in contrast to the results with 603 nucleosomes. In preliminary tests (data not shown), the barrier reduction for human pol II on the 603R template provided by incorporation of the H4 V43I mutant was also comparable to the reduction obtained with the H4 R45C mutant. The effects of Sin mutations on human pol II traversal efficiency are clearly not identical on all templates. As Sin mutations affect mostly the +45/+55 barrier, the differences between the effects of different mutations on traversal could be more evident on the non-permissive templates.

Removal of some or all of the N-terminal histone tails can also result in significant decreases in the barrier to complete nucleosomal traversal by both yeast and human pol II (Újvári *et al*, 2008). To determine whether the Sin mutations and tail removal reduce the nucleosome traversal barrier by a common mechanism, we tested nucleosomes assembled with tailless H2A and H2B and wild-type H3/H4 tetramers, or tetramers containing Sin mutants H3 T118I or H4 R45C. A significant reduction in the traversal barrier was expected from the removal of only the H2A/H2B tails (Újvári *et al*, 2008). Incorporation of tailless H2A/H2B (designated g in Fig 1) further reduced the nucleosomal barrier for human pol II with the Sin mutant histones (Fig 1B; supplementary Fig S1 online). Similar effects were observed for yeast pol II on the 603 template with tailless H2A/H2B and each of the four Sin mutations (Fig 1A; supplementary Fig S1 online). Importantly, relief of the 603 traversal barrier by tail removal and by incorporation of H3 T118I and H4 R45C Sin mutations seems to occur by independent mechanisms. As shown in supplementary

Table S1 online, the percentage increase in traversal on 603 templates conferred by the incorporation of both a Sin mutant histone and tailless H2A/H2B was either roughly equal to or greater than the sum of the increases provided by either nucleosome alteration alone. The effect of tail removal on traversal of 603R Sin mutant nucleosomes by human pol II at 150 mM KCl was less straightforward. In particular, tail removal provided no additional stimulation at 150 mM KCl in the context of nucleosomes containing the T118I H3 variant. However, the T118I mutation alone relieves almost all the transcriptional barrier on 603R at 150 mM KCl; hence, there was little opportunity for further barrier reduction in that case.

The TFIIIS transcript elongation factor stimulates traversal of nucleosomal 603 templates in the presence or absence of the histone N-terminal tails (Újvári *et al*, 2008). We found that yeast TFIIIS stimulated traversal of wild-type and Sin mutant 603 nucleosomal templates by 2–2.5-fold, in the presence or absence of the H2A/H2B N-terminal tails (supplementary Figs S2, S3 online). Relief of the 603 nucleosomal barrier by tail removal, as well as by Sin mutations and TFIIIS, seems to occur by independent mechanisms.

Sin mutations affect the histone–DNA interactions in the region from +70 to +80 of nucleosomal DNA that make a significant contribution to overall nucleosome stability (Muthurajan *et al*, 2004; Hall *et al*, 2009). We therefore tested whether the Sin mutant nucleosomes were more likely to dissociate from the template on traversal by pol II. DNA-labelled nucleosomes were transcribed by yeast pol II and analysed in a native gel to monitor the histone-free DNA generated. The amount of DNA released from Sin nucleosomes was larger than the amount released from intact nucleosomes (Fig 2; supplementary Fig S4 online). In the case of the H3 T118I mutant at 150 mM KCl, the octamer was displaced from ~40% of templates. These data indicate that interactions between H3/H4 histones with the region from +70 to +80 of nucleosomal DNA are important for nucleosome integrity and survival during transcript elongation by pol II.

Sin mutations reduce the major +45 pause by pol II; they also increase complete nucleosomal traversal and decrease nucleosomal survival on traversal. Given that Sin mutations reduce crucial DNA–octamer interactions across the segment of nucleosomal DNA from +70 to +80, uncoiling of downstream DNA from the octamer surface should be more probable when RNA polymerase arrives at the major pause site on Sin mutant nucleosomes. DNase I footprinting can be used to test this idea, but to obtain interpretable footprints it is essential to analyse nearly homogeneous transcript elongation complexes. Even under optimal conditions, a substantial fraction of templates fail to be transcribed by pol II; in addition, it is not possible to advance pol II by any distance into a nucleosome without stalling many complexes at various upstream positions. Thus, it was necessary to perform the footprinting studies with *Escherichia coli* RNA polymerase (RNAP; Kulaeva *et al*, 2009). Results with RNAP are relevant to pol II transcription because pol II and RNAP share the same mechanism of nucleosome traversal (Walter *et al*, 2003). To further confirm the similarities of the mechanisms of transcription through the 603 nucleosome by RNAP and pol II, wild-type and H3 T118I Sin mutant nucleosomes were transcribed by RNAP at 150 mM KCl (supplementary Fig S5 online). As expected, Sin mutant nucleosomes showed a reduction in +45 and +55

Fig 2 | Nucleosomes containing Sin mutant histones are more likely to dissociate during pol II transcription. (A) DNA-labelled nucleosomes containing wild-type or Sin mutant histones were transcribed by yeast pol II at the indicated KCl concentrations; intact complexes were then resolved from free DNA by native polyacrylamide gel electrophoresis. Only the region of the gel in which free DNA appears is shown; an example of the entire native gel from such an experiment is shown in supplementary Fig S4 online. No-chase control (nc): Transcription was performed in the absence of UTP and pol II was stalled upstream of the nucleosome. (B) The amounts of histone-free DNA produced after transcription at 150 mM KCl were quantified and normalized to the overall amount of active ECs. EC, elongation complex; H3, histone H3; H4, histone H4; pol II, polymerase II.

pauses (supplementary Fig S5a online) and an increase in nucleosome traversal (supplementary Fig S5b online) relative to wild-type nucleosomes.

An RNAP elongation complex (EC) stalled at +41 in the 603 nucleosome was selected for the footprinting studies because it was shown that DNA–histone interactions are maintained in front of RNAP in this nucleosomal transcription complex when wild-type histones are used for nucleosome assembly (Kulaeva *et al*, 2009). EC + 41 complexes stalled on pure 603 DNA or nucleosomal templates were digested with DNase I under single-hit conditions. When the bacterial polymerase was stalled at +41 on the nucleosome assembled with wild-type histones, nucleosomal DNA was accessible to DNase I digestion upstream from the RNAP, but the DNA downstream remained inaccessible (Fig 3A,B), as expected from earlier studies (Kulaeva *et al*, 2009). By contrast, the nucleosomal DNA both upstream and downstream from the EC + 41 RNAP was accessible to DNase I in the Sin nucleosome (compare the free DNA and Sin EC + 41 traces in the lower panel of Fig 3B). These results are fully consistent with the observations that (i) Sin mutant nucleosomes provide a significantly reduced transcriptional barrier at +45 for pol II (Fig 1) and (ii) traversal of Sin mutant nucleosomes by pol II is more likely to result in complete dissociation of the template DNA from the histone octamer (Fig 2).

DISCUSSION

Sin mutations reduce the barrier to nucleosome traversal by pol II and increase the probability of nucleosome loss on transcription. Why would Sin mutant nucleosomes have these properties? It seems probable that Sin mutations further destabilize an

Fig 3 | Advancing RNA polymerase 41 bp into a Sin mutant nucleosome results in nuclease accessibility of downstream DNA. (A) DNase I footprinting of non-transcribed 603 nucleosomes (left gel) or EC + 41–603 nucleosome complexes (right gel). Histones were wild type (W) or H3 T118I (S); D lanes are free DNA controls. The dotted line indicates protection by the EC + 41 complex. (B) Scans of the footprints in (A): naked DNA (black), wild-type (upper, red) or Sin (lower, red) nucleosomes, and corresponding EC + 41 complexes (green). EC, elongation complex; H3, histone H3; M, size markers; N, nucleosome; RNAP, RNA polymerase; wt, wild type.

intermediate that already has a limited number of DNA–protein interactions. During the initial stages of nucleosome traversal, a crucial intermediate called a zero (Ø)-loop complex can form when pol II swings away from the octamer surface at +39 (Fig 4; Kulaeva *et al*, 2009). As pol II approaches the nucleosome (Fig 4, complex A), it initially displaces the promoter-proximal DNA (complex B), which subsequently begins to reassociate with the octamer surface, forming the +39 Ø-loop (complex C). At the same time, uncoiling of the downstream template (complex C–D) is facilitated by a steric clash between the advancing polymerase

Fig 4 | Proposed mechanism of transcription through nucleosomes. Pol II enters the nucleosome (A), partly displaces upstream DNA (B) and forms a zero (Ø)-loop at +39 (C), inducing reversible uncoiling of downstream DNA (D). On wild-type nucleosomes, continued transcription is accompanied by nucleosome recovery. However, strong DNA–histone interactions (blue squares) are weakened in Sin nucleosomes (purple squares), causing a larger downstream DNA region to be displaced (E), favouring nucleosome displacement. Inset panel, from Luger *et al* (1997): DNA, grey; H3, blue; H4, green; H3 residues Arg 116 and Thr 118, yellow; H4 residues Val 43 and Arg 45, orange; histone–DNA contacts disrupted by Sin mutants (Muthurajan *et al*, 2004), red. The white diamond indicates the nucleosome dyad. H3, histone H3; H4, histone H4; pol II, polymerase II.

and downstream DNA (Kulaeva *et al*, 2009). We predict that, on wild-type nucleosomes, strong histone–DNA interactions flanking the nucleosome dyad (Fig 4, blue squares) prevent full uncoiling of the downstream DNA (complex D), resulting in a high probability of histone survival. However, on nucleosomes with Sin mutant histones, critical dyad–proximal interactions are weaker (complex E, purple squares). This should further destabilize the +39 complex, favouring the full unwinding of the downstream DNA. Note that DNA–histone association is already weakened in an RNAP complex stalled at +41 in a Sin mutant nucleosome (Fig 3). An increased probability of unwinding facilitates traversal, but also increases the likelihood that the nucleosome will be displaced during transcription.

On the 603 nucleosome, barrier reduction from the removal of the H2A/H2B tails is additive with the reduction obtained with the Sin mutant histones. Loss of H2A/H2B tails reduces the second-strongest histone–DNA interactions, located 25–35 bp from each nucleosome boundary (Brower-Toland *et al*, 2005; Hall *et al*, 2009). Consistent with this, the major barrier at +15 on the 603 nucleosome is reduced specifically in the absence of H2A/H2B tails (Fig 1; Újvári *et al*, 2008). However, H2A/H2B tail removal also causes a significant increase in nucleosome traversal on the 603R template by human pol II (Fig 1C; supplementary Fig S1 online), where there is no +15 pause. In this case, H2A/H2B tail removal is presumably disrupting the interaction 25–35 bp from the downstream edge of the nucleosome (the region from +112 to +122), which should also facilitate partial displacement of the DNA end from the octamer surface during the complex C to D transition in Fig 4 (Kulaeva *et al*, 2009). In summary, different segments of the histone–DNA interface are expected to be disrupted by Sin mutations and tail removal, which would explain the generally additive stimulation of traversal caused by these two alterations in nucleosome structure.

Histones are not normally lost from genes during moderate levels of transcription. However, our *in vitro* studies suggest that Sin mutations could lead to increased histone displacement

in transcription units. In yeast, transcription-induced histone displacement can be monitored by the activation of cryptic promoters localized in transcribed genes (Kaplan *et al*, 2003; Cheung *et al*, 2008). It is interesting to note that a Sin-like mutation in histone H3 (H3 T118F, similar to Sin H3 T118I used in this study) results in activation of cryptic promoters in transcribed regions *in vivo* (Cheung *et al*, 2008). Thus, at least some Sin mutations are likely to induce transcript elongation-dependent histone displacement *in vivo*.

METHODS

Preparation of proteins, nucleosome reconstitution by salt dialysis, assembly of transcription complexes and transcription of nucleosomal templates were performed as described (Bondarenko *et al*, 2006; Újvári *et al*, 2008). Briefly, yeast pol II complexes were assembled from RNA, template and non-template DNAs; they were then immobilized on Ni-nitrilotriacetic acid beads, washed, eluted and ligated to nucleosomes. After pulse-labelling of 45-mer RNA, complexes were chased into the nucleosomes with excess non-labelled NTPs. For the Fig 2 assay, complexes were resolved by native polyacrylamide gel electrophoresis (PAGE) after transcription. For human pol II, nucleosomes were reconstituted on template DNAs and bound to beads, followed by assembly of pre-initiation complexes with pol II and transcript initiation factors. After pulse-labelling of 21-mer RNA and rinsing, transcripts were chased into the nucleosomes with excess non-labelled NTPs. Salt concentrations and additions to reactions are described in the figure legends. Labelled RNAs were resolved by denaturing PAGE and quantified using a PhosphorImager.

Transcription by *E. coli* RNAP and DNase footprinting were carried out as described previously (Kulaeva *et al*, 2009). In brief, nucleosomes were reconstituted on end-labelled 147 bp DNA, followed by ligation of the T7A1 promoter upstream. Transcription by RNAP was stalled 41 bp in the nucleosome, followed by treatment with DNase I for 30 s at 37 °C. DNase-digested complexes were resolved by native PAGE. DNA was extracted

from the complexes, separated by denaturing PAGE and quantified using a PhosphorImager.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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