Defective histone supply causes changes in RNA polymerase II elongation rate and cotranscriptional pre-mRNA splicing

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RNA polymerase II (RNAPII) transcription elongation is a highly regulated process that greatly influences mRNA levels as well as pre-mRNA splicing. Despite many studies in vitro, how chromatin modulates RNAPII elongation in vivo is still unclear. Here, we show that a decrease in the level of available canonical histones leads to more accessible chromatin with decreased levels of canonical histones and variants H2A.X and H2AZ and increased levels of H3.3. With this altered chromatin structure, the RNAPII elongation rate increases, and the kinetics of pre-mRNA splicing is delayed with respect to RNAPII elongation. Consistent with the kinetic model of cotranscriptional splicing, the rapid RNAPII elongation induced by histone depletion promotes the skipping of variable exons in the CD44 gene. Indeed, a slowly elongating mutant of RNAPII was able to rescue this defect, indicating that the defective splicing induced by histone depletion is a direct consequence of the increased elongation rate. In addition, genome-wide analysis evidenced that histone reduction promotes widespread alterations in pre-mRNA processing, including intron retention and changes in alternative splicing. Our data demonstrate that pre-mRNA splicing may be regulated by chromatin structure through the modulation of the RNAPII elongation rate.

Significance

The study of the role of histones in transcription in mammals has been hindered by the existence of large gene families encoding every histone subunit. Here we reduce the level of canonical histones in a human cell line by silencing stem-loop-binding protein (SLBP) gene. SLBP controls stability, processing, nuclear export, and splicing. Furthermore, histone depletion also causes several pre-mRNA splicing defects, including skipping of alternative exons and intron retention. Thus we demonstrate that the correct histone supply is required to control the RNAPII elongation rate and pre-mRNA splicing.


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and translation of canonical histone mRNAs (26). Our data demonstrate that a reduction in the level of available canonical histones increases the RNAPII elongation rate in vivo and causes multiple splicing defects.

Results

SLBP Depletion Disrupts Chromatin Structure and Alters Histone Abundance. To investigate how histone levels affect transcription elongation, we generated a stable cell line in HCT116 cells (named “Hct-shSLBP.1”) that expresses a doxycycline (Dox)-inducible shRNA targeting the SLBP transcript. Dox treatment of HCT-shSLBP.1 cells for different time periods (3, 7, or 14 d) provoked a decrease in SLBP protein levels (Fig. S1A). As already reported, strong and prolonged SLBP depletion caused an accumulation of cells in S phase (Fig. S1B and C and ref. 27). However, we did not observe cell-cycle alterations 3 d after Dox treatment, and SLBP levels were moderately but significantly reduced. Therefore, to avoid cell-cycle effects, all experiments were performed 3 d after Dox treatment. Importantly, SLBP depletion caused a significant drop in soluble and chromatin-bound canonical H2B and H3 histone levels (Fig. 1A) with no significant changes in the level of DNA (Fig. 1A and by flow cytometry in Fig. S1B), suggesting that the histone:DNA ratio was reduced. Surprisingly, the levels of the chromatin-bound histone variants H2A.Z and H2A.X were reduced slightly, although their mRNAs either are not controlled (H2A.Z) or are only partially controlled (H2A.X) by SLBP (26). In contrast, levels of the chromatin-bound variant histone H3.3, which is also not controlled by SLBP, increased in Dox-treated cells (Fig. 1A), indicating that the H3.3 can partially replace canonical H3.

SLBP depletion also increased chromatin accessibility to micrococcal nuclease I (MNase I), suggesting that the combined effect of the lower histone:DNA ratio and the modified histone composition promotes a more open chromatin configuration (Fig. 1B and C). However, nucleosome spacing was not altered by SLBP depletion.

Histone Depletion Accelerates RNAPII Elongation. We then studied the effect of histone depletion on the RNAPII elongation rate of two long human genes, utrophin (UTRN) and catenin beta-like 1 (CTNNBL1), commonly used to analyze transcription elongation (28). First, we investigated by ChIP how SLBP depletion affects histone levels at different regions of these two genes. In agreement with the Western blotting data, SLBP silencing caused a decrease in the occupancy of total histone H3, H2A.Z, and H2A.X and an increase in the variant H3.3 at UTRN and CTNNBL1 genes (Fig. 2A). Histone changes were not identical in all analyzed regions, suggesting that some regions are more susceptible to histone depletion than others (compare, for example, the levels of H3 in exon 1 with those in exon 4, exon 5, and exon 6 of CTNNBL1). To measure transcription elongation rate, initiating RNAPIIs were transiently inhibited with 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB). Three h later DRB was washed off to resume transcription elongation (28). The velocity of the transcription wave was measured using quantitative RT-PCR (RT-qPCR) with primers spanning different exon–intron junctions of UTRN and CTNNBL1. Exon 1 transcription in both genes was recovered 10 min after DRB removal from cells treated with Dox and from untreated cells. In contrast, expression of exon 3 of UTRN or exons 6 and 16 of CTNNBL1 was detected 10 min earlier in the presence of Dox than in the control cells (Fig. 2B and C), suggesting that the RNAPII elongation rate increases under conditions of canonical histone depletion.

Singh and Padgett (28) showed that pre-mRNA splicing of most introns occurs 5–10 min after transcription of the downstream exon. Because RNAPII elongates faster under histone depletion, we wondered whether splicing also was accelerated under these conditions. To measure the kinetics of pre-mRNA splicing, we determined the time between the new synthesis of an exon and the appearance of the splicing product of that exon and the immediately preceding exon by RT-qPCR (28). In agreement with previous results, introns 4 and 5 of the CTNNBL1 gene were spliced 5–10 min after synthesis of exons 5 and 6, respectively (Fig. 3). Interestingly, histone depletion delayed the splicing of these introns relative to the synthesis of the corresponding downstream exons (Fig. 3). In addition, CTNNBL1 intron 5 splicing was significantly impaired in the presence of Dox (Fig. 3B), suggesting that histone depletion promotes intron retention. Taken together, these results indicate that a correct chromatin organization is required for normal transcription elongation speed and for the temporary coupling between elongation and splicing.

Histone Depletion Promotes Exon Skipping at the CD44 Gene. To characterize the consequences of histone depletion on splicing further, we examined the effect of histone depletion on the CD44 gene as a well-known model of complex alternative splicing. CD44 contains 10 constant and 9 clustered variable exons (Fig. 4A) and generates many splice variants (29). Histone depletion provoked a 20–40% decrease in total H3, H2A.Z, and H2A.X and a concomitant increase in H3.3 occupancy along the CD44 gene (Fig. 4B and Fig. S2A). Once histone reduction was verified, the effect on CD44 alternative splicing was analyzed. Fig. 4C shows that histone depletion decreased the levels of CD44 mRNAs containing exons v4–v5 and v9–v10, indicating that a relaxed chromatin structure promotes skipping of the alternative exons. As a control, we verified that similar results were obtained in HCT-shRNA.2 cells, a stable cell line that expresses a different shRNA against SLBP (Fig. S2 B and C).

Next, we investigated how histone depletion affects the distribution of RNAPII along the gene body of CD44. Under control conditions RNAPII occupancy increased at the variable region (Fig. 4D), in agreement with previous observations (30). However, RNAPII accumulation decreased drastically at this region under conditions of histone depletion, suggesting that chromatin has a role in modulating the progression and pausing of RNAPII at the variable exons. Importantly, histone depletion also decreased the levels of the splicing factor U2AF65 on the
variable region of CD44 (Fig. 4E), consistent with the skipping of variable exons observed under conditions of histone depletion.

Our data indicate that chromatin structure has an influence on alternative splicing. Two models (not mutually exclusive) have been proposed to explain how chromatin might affect alternative splicing (9, 22, 23): nucleosomes and histone posttranslational modifications may be involved in recruiting splicing factors, or, alternatively, nucleosomes might affect splicing by modulating the RNAPII elongation rate. We have shown that canonical histone depletion increases the RNAPII elongation rate, but we cannot exclude the possibility that chromatin has a role independent of elongation. To assess the relevance of transcription elongation rate on the skipping of CD44 variable exons, we tested whether a “slow” mutant of RNAPII could rescue the splicing defects observed under conditions of histone depletion. We used the catalytic mutant of the large subunit of RNAPII, hC4, that causes a reduced transcription rate (14). hC4 and WT α-amanitin–resistant alleles were expressed in HCT-shSLBP.1 cells while endogenous RNAPII was inactivated by addition of α-amanitin (Fig. 4F). Fig. 4G shows that the slow hC4 RNAPII suppressed the skipping of variable exons promoted by histone depletion. Hence, we conclude that the effect of chromatin on CD44 alternative splicing depends on the RNAPII elongation rate.

Histone Depletion Causes Widespread Splicing Defects. To determine the global effects of histone depletion in gene expression and splicing, we hybridized RNAs isolated from three biological replicates of HCT-shSLBP.1 cells, cultured either in the presence or absence of Dox, to splicing-sensitive Human Transcriptome Arrays (HTA) 2.0 from Affymetrix. This array contains probe sets covering 560,472 exons from protein-coding genes and 109,930 exons from non–protein-coding genes. Exons are covered by one to four probe sets. To perform standard microarray normalization procedures, we verified that SLBP depletion did not globally affect the total level of RNA (Fig. S3A). Analysis of the results at the gene level demonstrated that histone depletion caused only mild changes in transcripts levels (Fig. S3B). As expected, expression of 58 of the 74 canonical histone-encoding genes present in the array decreased in the presence of Dox compared with control conditions (Fig. S3B), confirming that SLBP silencing decreased histone mRNA levels. Histone depletion affected the expression of 290 genes (P < 0.05 and linear fold change ≥1.5), of which 152 were down-regulated and 138 were up-regulated (Dataset S1). Only eight transcripts changed more than twofold; three were histone transcripts, and the other five were noncoding RNAs. Therefore, our data indicate that the canonical histone-depletion conditions used in our experiments caused only mild changes in gene expression.

Next we analyzed whether histone depletion promotes splicing defects. To do so, we calculated the splicing index of each probe set of the array. The splicing index represents the change in probe set inclusion (Dox versus control) normalized to the change of gene-level expression. A positive splicing index value indicates higher inclusion of this probe set with respect to the whole transcript; a negative splicing index indicates skipping of this probe set. Using a threshold of |splicing index| ≥1 and P < 0.05, we identified significant splicing defects in 943 exon probe sets corresponding to 734 genes. One hundred eighty probe sets showed higher skipping in the presence of Dox than in control conditions; 763 probe sets showed higher inclusion (Dataset S1). Consistent with the results described above, several CD44 variable exons presented negative splicing index values (Fig. S4). Positive splicing indexes were associated mainly with genes with a high level of expression (P = 1.39 × 10−22; hypergeometric test), whereas negative splicing indexes were present mostly in genes with medium or low levels of transcription (P = 2.04 × 10−24) (Fig. 5 panels). Pre-mRNA values are normalized to the values of the prior-DRB treatment sample, which was set to 1. Results are shown as means ± SEM from three independent experiments.

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A and B), suggesting that the effect of canonical histone depletion on splicing depends on the transcription intensity.

As a second tool for studying alternative splicing, we used AltAnalyze software (31). In this case, using the default parameters of the MiDAS algorithm, 875 probe sets with splicing defects were identified in 702 genes (Dataset S1). Of the 875 misregulated splicing events, 644 showed a positive splicing index, and 231 had a negative splicing index. AltAnalyze incorporates a library of splicing annotations from University of California Santa Cruz (UCSC) KnownAlt database (Fig. S5A) (32). About 50% of the detected splicing defects were annotated in the KnownAlt database (Dataset S1). Alternative cassette exons were highly represented among the exons with a negative (57%) or positive (28%) splicing index. Importantly, ~40% of the exons with increased inclusions (splicing index >1) were annotated as intron retentions and bleeding exons (which also cause intron retention) (Fig. S5C and Fig. S5A). However, only 5% intron-retention and bleeding-exon annotations were found in skipped probe sets (splicing index <−1; Fig. S5C). Fig. S5B shows multiple examples of intron retentions and bleeding exons in HNRNPK, UBA2P2L, and FUS genes. Manual examination of nonannotated exons showed that under conditions of histone depletion 42% of the up-regulated probe sets expanded regions cataloged as introns in the RefSeq database. In contrast, only 6% of the down-regulated probe sets expanded intronic regions, indicating a strong increase of intron-retention defects under histone depletion. Intron retentions and bleeding exons were found mostly in genes with high levels of expression (87%; P = 1.3 × 10−53; hypergeometric test) (Fig. S6). Increased levels of intron-containing pre-mRNAs in histone-depleted cells were confirmed by RT-qPCR at the HNRNPK, UBA2P2L, and FUS genes (Fig. S5D). Therefore, our data suggest that depletion of canonical histones causes multiple types of splicing defects including increased or decreased inclusion of cassette exons and intron retention.

Discussion

How chromatin affects the RNAPII elongation rate in vivo is still not fully understood. Partial loss of core histones at gene bodies during intense transcription has been reported (33, 34), suggesting that a certain degree of histone removal is a prerequisite for or a consequence of active transcription. However, recent genome-wide studies have shown either little (4) or no (3) negative correlation between nucleosome occupancy and elongation rates in mammals. To investigate the role of histones in transcription elongation in vivo, we reduced the levels of available canonical histones by SLBP silencing. SLBP-depleted cells showed a reduction in the total levels of H3, H2B, and H2A.X. Interestingly, expression of the variant histone H2A.Z, which is not controlled by SLBP, also was decreased, suggesting that reduction of H2B may cause loss of H2A.Z–H2B heterodimers from some nucleosomes. However, the levels of the histone variant H3.3 increased strongly in SLBP-silenced cells. H3.3 normally is enriched in active genes and in regulatory regions (35). In fact, H3.3-containing regions display a looser and more open chromatin structure (35, 36). Consistent with the partial reduction in the level of histones and the increased level of the
open-chromatin–specific H3.3, we observed that chromatin was more accessible to MNase I in SLBP-silenced cells (Fig. 1B). Importantly, these chromatin conditions, characterized by a general depletion of canonical histones together with an alteration in the level of noncanonical histones, caused a faster RNAPII elongation rate in two different genes, indicating that nucleosomes hinder RNAPII elongation in vivo as they do in vitro (5, 6). We also show that chromatin has an unanticipated role in the temporary coupling between transcription and splicing because histone depletion delayed pre-mRNA splicing from RNAPII elongation.

We have observed that canonical histone depletion produces skipping of the variable exons of CD44 gene. RNAPII accumulates at the variable region of the CD44 gene, a pause that facilitates variable exon inclusion (30, 37). We show here that variable exon inclusion and RNAPII accumulation decrease with histone depletion, suggesting that a correct chromatin structure is required for the inclusion of these exons. Why is this region so sensitive to histone depletion? It has been proposed that binding of the chromatin factors hBRM and HPV1 to this region decreases the elongation rate of RNAPII (30, 37). Because hBRM and HPV1 harbor histone-interaction domains able to interact with posttranslationally modified histones (38, 39), it is possible that the absence of a correct nucleosomal structure under conditions of histone depletion impairs the recruitment of these factors to the chromatin. Our data also show that exon skipping in CD44 is rescued by a slow mutant of RNAPII, indicating that the role of chromatin on CD44 alternative splicing mostly depends on the RNAPII elongation rate and not on other putative roles of chromatin in recruiting splicing factors. This finding is consistent with the classical kinetic model that predicts that fast elongation rates can favor the skipping of alternative exons with weak splice sites (14). Certainly, we also detected a defect in the recruitment of the splicing factor U2AF65 at the CD44 variable region. However, because U2AF65 interacts with the C-terminal domain of RNAPII (11), this phenotype may result from the reduced occupancy of RNAPII observed at this region.

A severe histone reduction provokes strong replication defects, accumulation of cells in the S and G2 cell-cycle phases, and genetic instability (27, 40). However, the subtle histone depletion used in our work has allowed us to analyze the transcriptional phenotypes caused by histone depletion without affecting cell cycle. Under these conditions only mild changes in gene expression were observed (most of them less than twofold), splicing defects being the most prominent phenotypes observed. Genes encoding splicing factors were not significantly up- or downregulated in the SLBP-silenced cells (Fig. S3C). However, a high proportion of splicing genes were slightly down-regulated in the presence of Dox (less than −1.4 lineal fold change). Although this effect can be attributed to the recently reported important role for RNAPII elongation rate in alternative splicing regulation of pre-mRNA splicing factor genes (13), it is too mild to be considered significant. Nevertheless, we cannot rule out the possibility that this subtle down-regulation acts as a positive feedback that increases splicing defects. Our genome-wide analysis showed that histone depletion caused different types of effects, including the firing of alternative promoters, changes in inclusion of cassette exons, alternative termination sites, and intron retention. Both increased and decreased cassette-exon inclusions were detected, in agreement with the proposal that the classical kinetic model can explain only part of the observed elongation-dependent splicing defects (15). Although we have shown that histone depletion promotes a faster transcription elongation rate, we cannot discard the possibility that some...
splicing defects are caused directly by chromatin changes in splicing and not by the change in elongation rate.

The increase in intron retention with the depletion of canonical histones also is consistent with an extended view of the kinetic model in which elongation rate affects constitutive splicing (41). Thus, retention of intronic regions was observed mostly in highly expressed genes. It has been shown that highly transcribed genes have higher elongation rates (3, 4) and elevated histone replacement (42, 43). Therefore, the chromatin structure of highly expressed genes may be more severely affected by histone depletion, and, as a consequence, the RNAPII elongation rate might be further accelerated, affecting the recognition of constitutive splicing sites and hence promoting intron retention.

A reduction in canonical histones has been observed during replicative aging in yeast (44) and in senescent cells in yeast (45) and human fibroblasts (46). Furthermore, in senescent human fibroblasts depletion of canonical histones also is accompanied by an increase in histone H3.3 (47). It is currently unclear to what extent the transcriptional changes that occur during aging or/and senescence in mammals are a consequence of histone reduction. However, and interestingly, intron retentions are the most abundant age-related splicing changes found in the human brain (48). Therefore, our results prompt us to hypothesize that the splicing alterations found during human aging might be caused by the associated histone depletion.

Materials and Methods

Detailed methods for plasmids, cell culture, generation of inducible cell lines, pre-mRNA analysis, MNase I treatment, and immunoblotting are provided in SI Materials and Methods. For exon array analysis, total RNA was isolated in triplicate from HCT116/SLBP1 cells cultured in the presence or absence of Dox by using the RNeasy Mini Kit (Qiagen). Details about GeneChip HTA Array hybridization and data analysis are provided in SI Materials and Methods. Exon array data are available from the Gene Expression Omnibus database (accession number GSE68307). Chip experiments were carried out as previously described (7). Five to ten micrograms of the indicated antibody (Table S1) were used per Chip. Quantification of immunoprecipitated DNA was performed by qPCR, using three qPCR determinations per biological replica. Provided data are the average of three independent biological replicas ± SEM. The primers used are described in Table S2.

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