Considerable attention has been given to the understanding of how nucleosomes are altered or removed from the transcription start site of RNA polymerase II genes to enable transcription to proceed. This has led to the view that for transcriptional activation to occur, the transcription start site (TSS) must become depleted of nucleosomes. However, we have shown that this is not the case with different unstable histone H2A variant-containing nucleosomes occupying the TSS under different physiological settings. For example, during mouse spermatogenesis we found that the mouse homolog of human H2A.Bbd, H2A.Lap1, is targeted to the TSS of active genes expressed during specific stages of spermatogenesis. On the other hand, we observed in trophoblast stem cells, a H2A.Z-containing nucleosome occupying the TSS of genes active in the G1 phase of the cell cycle. Notably, this H2A.Z-containing nucleosome was different compared with other promoter specific H2A.Z nucleosomes by being hetertotypic rather than being homotypic. In other words, it did not contain the expected two copies of H2A.Z per nucleosome but only one (i.e., H2A.Z/H2A rather than H2A.Z/H2A.Z). Given these observations, we wondered whether the histone variant composition of a nucleosome at an active TSS could in fact vary in the same cell type. To investigate this possibility, we performed H2A.Z ChiP-H2A reChiP assays in the mouse testis and compared this data with our testis H2A.Lap1 ChiP-seq data. Indeed, we find that different promoters involved in the expression of genes involved in distinct biological processes can contain either H2A.Z/H2A or H2A.Lap1. This argues that specific mechanisms exist, which can determine whether H2A.Z or H2A.Lap1 is targeted to the TSS of an active gene.

Introduction

Eukaryotic genomic DNA is assembled into a highly compacted state with an equal mass of protein to form chromatin. The repeating unit of chromatin is the nucleosome, which is formed by wrapping ~150 base pairs of DNA around a core histone octamer (two molecules each of histone H2A, H2B, H3, and H4). Nucleosomes are connected by short linker DNA segments to form long arrays, which undergo short-range intra and long-range inter-nucleosomal interactions to contribute to the high degree of compaction observed in interphase chromosomes.1 This condensation of genomic DNA into chromatin has profound implications for the regulation of key DNA processes such as transcription. For example, this compaction and occupancy of canonical nucleosomes at the TSS of genes transcribed by RNA polymerase II prevents access to the transcription machinery.2-4 It therefore has been assumed that for transcription to proceed, a nucleosome free (NFR) or depleted region (NDR) must be created at the TSS.5-7 and the experimentally observed increase in accessibility to micrococcal nuclease digestion has been

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Histone variant selectivity at the transcription start site

H2A.Z or H2A.Lap1

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used as evidence to support this notion.38 However, such sensitivity to micrococcal nuclease digestion at the TSS does not necessarily imply a loss of nucleosomes but could also be the consequence of the formation of a highly unstable nucleosome.7 Indeed, our work19 and the studies of others20 indicate that this is the case and that histone variants play an important role in forming stable nucleosomes at the TSS.

H2A.Z/H2A Z Homotypic vs. H2A.Z/H2A Heterotypic Nucleosomes

Among the core histones, the H2A family shows the greatest divergence in primary sequence leading to the greatest number of variants known.21 The key amino acid differences between histone H2A variants and histone H2A are strategically placed within the nucleosome to affect nucleosome stability13-15 and also on the surface of the nucleosome (the acidic patch) to regulate the interactions between neighboring nucleosomes thereby determining the extent chromatin compaction.1,10,12,16,17 A highly conserved and extensively studied histone variant is H2A.Z.12 H2A.Z has a range of functions including being required for early metazoan development,20,21 chromosome organization and inheritance,13-15 as well as regulating promoter chromatin architecture.21,22 Notably in mammalian cells, the apparent NDR of active genes at the TSS is flanked on both sides by nucleosomes thereby determining the extent chromatin compaction.1,10,12,16,17

To investigate this issue we performed H2A.Z ChIP followed by H2A reChIP assays.9 Basically, ChIP assays were first performed using affinity purified H2A.Z antibodies to immunoprecipitate all H2A.Z-containing nucleosomes followed by a second ChIP employing H2A antibodies to pull down heterotypic H2A.Z/H2A nucleosomes. Significantly, we observed that only -5% of H2A.Z nucleosomes at G1 were heterotypic but by M phase, this dramatically increased to -35%. At active promoters, there was a clear increase in heterotypic H2A.Z/H2A nucleosomes at S and M compared with G1.8 Why a heterotypic H2A.Z/H2A nucleosome only exists at the TSS in G1 is unclear but probably reflects a different chromatin configuration at the TSS for genes expressed in G1 vs. genes expressed in M and S.8

Finally, we addressed the question of why heterotypic H2A.Z/H2A nucleosomes formed during S phase was not fully restored to the homotypic state by M phase (Fig. 1B and C); respectively.19 Consistent with this heterotypic nucleosome being unstable, we also found it at other dynamic genomic loci including CTCF binding sites and Dnase1 hypersensitive sites. In addition, we observed replacement of this heterotypic H2A.Z/H2A nucleosome following the passage through S phase at all of these sites.8 On the other hand, it is worth noting that a homotypic H2A.Z/H2A.Z nucleosome is more stable than a canonical nucleosome, which can be attributed to an enhanced interaction between the L1 loop regions of each H2A.Z molecule compared with H2A.20 Why a heterotypic H2A.Z/H2A nucleosome only exists at the TSS in G1 is unclear but probably reflects a different chromatin configuration at the TSS for genes expressed in G1 vs. genes expressed in M and S.
heterochromatin.22,23 We therefore wondered whether the loss of H2A.Z at promoters was coincident with a gain of H2A.Z at centromeres and indeed this was found to be the case. Taken together, this dynamic net transfer of H2A.Z from promoters to the centromere at M phase brings into question whether H2A.Z has a role in providing transcriptional memory during the cell cycle. Interestingly, it has also been shown that histone post-translational modifications and other histone variants are likewise restored slowly after DNA replication, and in some cases requiring more than one round of cell division to be fully re-established.30-32

H2A.Lap1 and Spermatogenesis

While H2A.Z is ubiquitously expressed, human H2A Bbd (H2A.B) 33 is expressed primarily in the adult testis and brain (NCBI GEO data sets). It is also aberrantly expressed in a number of tumors and cancer cell lines34 (NCBI GEO data sets). In contrast to H2A.Z which promotes nucleosome stability15 and chromatin compaction, 1 H2A.Bbd-containing histone octamers and mononucleosomes are unstable and the latter only protects ~120 base pairs of DNA from micrococcal digestion compared with 145 base pairs for canonical nucleosome core particles.13,35 This is consistent with FRET, AFM, and cryo-EM studies showing the unwrapping of nucleosomal DNA from the nucleosome surface at the DNA entry and exit points.13,35,36 Assembly of nucleosomal arrays with H2A.Bbd completely inhibits array folding and compaction leading to a de-repression of chromatin mediated transcription.17 This ability of H2A.Bbd to prevent chromatin compaction is due to the loss of three acidic amino acid residues, which partially neutralizes the acidic patch located on the surface of the nucleosome.17 To gain new insights into the possible role(s) of H2A.Bbd and its orthologs, we turned to the mouse where we identified four major “H2A.Bbd-like” proteins. We designated these variants as H2A.Lap1–4 (lack of an acidic patch)10 (H2A.B.1, H2A.L.1, H2A.L.2, and H2A.M, respectively)33 to more accurately reflect their partially neutralized acidic patch, with H2A.Lap1 being most similar to H2A.Bbd. Analogous to H2A.Bbd, analogous to H2A.Bbd, H2A.Lap1 is expressed primarily in the testis and the brain.10 Intriguingly though, while H2A.Lap1 also prevented chromatin compaction and also only protected ~120 base pairs of DNA from micrococcal digestion in vitro, it was less efficient in decompacting chromatin compared with H2A.Bbd indicating that these histone variants are not completely functionally equivalent (which may explain why the mouse has four major “H2A.Bbd-like” proteins whereas human has only one protein).
Mammalian spermatogenesis is a complex developmental and differentiation process that occurs in seminiferous tubules. A rapidly dividing pool of stem cell spermatogonia is located at the outer edge of these tubules. Some spermatogonia stop dividing and differentiate into primary spermatocytes, which enter the first meiotic prophase. The first meiotic prophase consists of five sequential stages: leptotene (condensation of interphase chromosomes), zygotene (synapsis begins), pachytene (recombination and crossing over occurs), diplotene (desynapsis initiates), and diakinesis (transition to metaphase I). Cells then complete division I of meiosis to produce secondary spermatocytes, which then undergo meiotic division II to produce haploid round spermatids. Differentiation (spermiogenesis) continues toward the center of the tubule where mature spermatozoa ultimately escape into the lumen.

To determine precisely which mouse testis cell types express H2A.Lap1, we performed indirect immunofluorescence analyses using affinity purified H2A.Lap1 antibodies on testis sections, displaying all 12 stages of the cell cycle of the seminiferous tubule, as well as on surface spreads of individual spermatocytes and spermatids. We found that H2A.Lap1 is not ubiquitously expressed throughout spermatogenesis but is expressed in a temporally specific manner, between pachytene and the round spermatid stage of spermatogenesis.

To investigate the genomic location of H2A.Lap1 and its link with transcription, we performed H2A.Lap1 ChIP-seq experiments and examined global gene expression using whole mouse expression microarrays in the 30 d testis (where the vast majority of tubules contained round spermatids). The striking observation was that H2A.Lap1 was located at the TSS of active genes analogous to the position of heterotypic H2A.Z/H2A nucleosomes in T5 cells (Fig. 2A). This allowed us to conclude that H2A.Lap1 is a new component of genes expressed during spermatogenesis and moreover, confirmed...
the view that the TSS of an active gene is not nucleosome free. Taken together, our results show that H2A.Z or a H2A.Lap1-containing nucleosome can be precisely positioned at the TSS dependent upon their physiological context.

Our immunofluorescence analysis of spermatids revealed an unexpected observation: while H2A.Lap1 was largely excluded from heterochromatin regions, as we expected, it was targeted to the inactive X or Y chromosome specifically during the latter stages of round spermatid differentiation.10 In mammals, the X or Y chromosome are subjected to meiotic sex chromosome inactivation during pachytene and significantly, this silencing of the sex chromosomes persists postmeiotically throughout spermatogenesis.21,37,38 However, Namekawa and colleagues showed that a small number of genes on the X chromosome, required for spermatogenesis, are reactivated in round spermatids (~13%).37

Based on this knowledge, our hypothesis therefore became that H2A.Lap1 was specifically targeted to these X-linked and round spermatid specific genes to enable their activation. To test this hypothesis, we used our H2A.Lap1 ChIP-seq data and combined it with published X chromosome gene expression data in round spermatids.37 Confirming our hypothesis, we found that H2A.Lap1 was not present on inactive genes but selectively enriched at the TSS of X chromosome genes that were only active in round spermatids (Fig. 2B). Moreover, maximal transcription was coincident with the highest H2A.Lap1 signal at their transcription start site at the latter stages of round spermatid differentiation suggesting that H2A.Lap1 is directly involved in the transcriptional activation process.39 Combined with our structural data, this allowed us to propose a model whereby H2A.Lap1 coordinately activates transcription of genes on the X chromosome by creating an open chromatin configuration at the TSS (Fig. 2C). A major problem for the future is to uncover the specific H2A.Lap1 sequence and/or ATP-dependent remodelling complex that targets H2A.Lap1 to the TSS (see below).

To answer the first question we performed H2A.Z ChIP H2A reChIP assays using 30 d testes and combined this data with our gene expression analysis. All mouse genes were separated into quartiles according to their expression level. A clear positive correlation between the abundance of a heterotypic H2A.Z-H2A nucleosome at the TSS and the level of expression is clearly observed analogous to active promoters in TS cells (Fig. 3A). Interestingly, the heterotypic H2A.Z-H2A nucleosome is not uniquely positioned at the TSS but is present at several locations around the TSS including at nucleosome position –2 and +1 indicating a different chromatin structure for genes active in the mouse testis compared with TS cells. Indeed, we previously performed H2A.Z
CHIP experiments in the 30 d testis and reported the unexpected observation that while H2A.Z was broadly enriched at the -2 nucleosome position, it was depleted at the +1 position. This suggests that in addition to the TSS, the +1 nucleosome is also unstable being predominantly in the heterotypic form. We conclude that a heterotypic H2A.Z-H2A nucleosome is present at the TSS, arguing that this special heterotypic nucleosome may be a universal feature of an active mammalian gene.

To address the second possibility that an active promoter can selectively choose between H2A.Lap1 or H2A.Z, we took the top 250 genes in the 30 d testis enriched either with a H2A.Lap1-containing nucleosome or a heterotypic H2A.Z-H2A-containing nucleosome at their TSS, and examined the relative level of expression of these 250 genes at four stages of spermatogenesis (spermatogonia stage A, spermatogonia stage B, pachytene, and the round spermatid stage) normalized to the level of expression at spermatogonia stage A using published gene expression data.7–9 Fig. 3B, Significantly, these top 250 heterotypic H2A.Z-H2A or H2A.Lap1-nucleosome containing genes were different (Table 1).

We find that heterotypic nucleosomes are present on genes active at different stages of spermatogenesis with the normalized mean expression for the top 250 heterotypic H2A.Z-H2A-containing genes peaking at the pachytene stage (Fig. 3B). The normalized mean expression of H2A.Lap1-containing genes also increases at pachytene but this increase is more significant compared with heterotypic containing genes. In addition, this level of expression is maintained in the round spermatid stage, which is expected given that H2A.Lap1 is expressed at pachytene and the round spermatid stage (Fig. 3B). It is worth highlighting that the presence of H2A.Lap1 at the TSS of genes active in the round spermatid stage is not due to a simple mass action effect because the expression of H2A.Z protein actually peaks in round spermatids.10

Given that the genes that contained either H2A.Lap1 or H2A.Z-H2A were different, we wondered whether their respective genes were involved in different biological processes. To investigate this possibility, we performed gene ontology analysis on these two groups of genes. While H2A.Z-H2A heterotypic nucleosomes were associated with genes that display a range of different functions, such as metabolic processes, no single process displayed a significant enrichment of H2A.Z-H2A indicating that this histone variant nucleosome has a more ubiquitous distribution not involved in any specific biological process (Table 1). On the other hand, processes involving transcription, RNA metabolism, and spermatogenesis were significantly overrepresented among H2A.Lap1 enriched genes (Table 1).

Taken together, these new observations show that during the same stages of spermatogenesis the TSS of active genes can selectively contain either a H2A.Z-H2A heterotypic or a H2A.Lap1-containing nucleosome. This argues that specific mechanisms exist that can determine whether H2A.Z or H2A.Lap1 is targeted to the TSS. These mechanisms most likely involve histone variant specific chaperones and/or ATP-dependent remodelling complexes and promoter specific transcription factors. Experiments are in progress to identify the nature of such putative complexes specific for H2A.Lap1. Concerning H2A.Z, our hypothesis is that ATP-dependent complexes that load H2A.Z into chromatin (e.g., P400 or SRCAP43) will exchange only a single copy of H2A with H2A.Z when a nucleosome is positioned at the TSS (perhaps regulated by H3.3 incorporation11 and/or histone acetylation12). Alternatively, or in addition, ATP-dependent complexes that are capable of removing H2A.Z (e.g., INO8013) may function to exchange H2A.Z with H2A.Lap1 at the TSS to create an unstable heterotypic nucleosome.

The reason why the nucleosome composition at the TSS is regulated in this manner is unclear but the specific targeting of H2A.Lap1 may ensure genes are activated in the correct temporal and spatial manner and/or might facilitate higher levels of transcription compared with a H2A.Z-H2A heterotypic nucleosome. Indeed, the highest level of overall transcription does occur between pachytene and the round spermatid stage, which is when H2A.Lap1 is expressed.44 Moreover, it is particularly intriguing that H2A.Lap1 is associated with genes that are, themselves, required for gene expression. In conclusion, we believe that this selectivity of histone variants at the TSS adds another layer to the process of transcriptional regulation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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