Variant histone H3.3 marks promoters of transcriptionally active genes during mammalian cell division

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Variant histone H3.3 is incorporated into nucleosomes by a mechanism that does not require DNA replication and has also been implicated as a potential mediator of epigenetic memory of active transcriptional states. In this study, we have used chromatin immunoprecipitation analysis to show that H3.3 is found mainly at the promoters of transcriptionally active genes. We also show that H3.3 combines with H3 acetylation and K4 methylation to form a stable mark that persists during mitosis. Our results suggest that H3.3 is deposited principally through the action of chromatin-remodelling complexes associated with transcriptional initiation, with deposition mediated by RNA polymerase II elongation having only a minor role.

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INTRODUCTION

Cellular memory of gene expression states is a fundamental requirement for cell differentiation in multicellular organisms. Expression states are established by the binding of sequence-specific transcription factors and the recruitment of chromatin-remodelling complexes, but they must then be maintained even when binding of factors to the DNA is disrupted during DNA replication and mitosis. The relative stability of nucleosomes suggests that the core histones could act as repositories for epigenetic marks that would mediate cellular memory of transcriptional activation across large regions of chromatin during cell division.

Recently, attention has begun to focus on the role of variant histones in marking transcriptional states. In contrast to the replication-dependent core histones, which are expressed only during replication, the variant histones H3.3 and H2A.Z are expressed throughout the cell cycle. Mammalian histone H3.3 differs from replication-dependent H3.1 by four amino acids, and the presence of three conserved variant amino-acid residues has been shown to be necessary for replication-independent incorporation of H3.3 into nucleosomes in *Drosophila* (Ahmad & Henikoff, 2002). Current evidence indicates that replication-independent deposition of H3.3 occurs through a mechanism that involves interaction with the HIRA histone chaperone, whereas histone H3.1 is deposited at the replication fork in a process that is mediated by the histone chaperone CAF-1 (Tagami *et al*, 2004). H3.3 is enriched at transcriptionally active ribosomal gene arrays on metaphase chromosomes in *Drosophila* KC cells (Ahmad & Henikoff, 2002) and has also been shown to become associated with an artificially constructed transgene after transcriptional activation (Janicki *et al*, 2004).

Despite the observations of an association between transcription and H3.3 deposition, the mechanism that preferentially incorporates H3.3 into nucleosomes at transcribed genes is still unknown. One potential mechanism for replication-independent incorporation of H3.3 into nucleosomes would be through the disruption of the nucleosomal structure by the transcribing RNA polymerase (Henikoff *et al*, 2004; Workman & Abmayr, 2004). This would be expected to result in marking of the nucleosomes across the entire transcribing genes. Alternatively, H3.3 could be incorporated through localized remodelling of nucleosomes associated with binding of transcription factors to regulatory sequences. These different types of incorporation would be expected to generate quite different marks, which would have implications for the role that H3.3 has in mediating epigenetic inheritance.

In this study, we have used chromatin immunoprecipitation (ChIP) to carry out a detailed analysis of the location of variant histone H3.3 at active and inactive genes. We show that the principal site of H3.3 deposition at transcribing genes is at the

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Fig 1 | Location of variant histone H3.3 in the endogenous $\lambda 5$ -*VpreB1* locus. (A) The $\lambda 5$ -*VpreB1* locus. (B) Expression of Myc-H3.3 in stably transfected clones detected using an anti-c-Myc tag antibody (green, left panel). DNA was counterstained with TOTO (blue, right panel). (C) Chromatin immunoprecipitation analysis of H3.3. The positions of the primer pairs are shown on the locus map (below the graph) as vertical bars. DNase I-hypersensitive sites (HS) that have been mapped in the locus are shown as vertical arrows (red, constitutive HS; black, pre-B-cell-specific HS). Enrichment values represent the mean $\pm$ s.d. from two independently immunoprecipitated samples (see Methods for details of calculations).

gene promoters. We also show that H3.3 deposition combines with H3 acetylation and H3 K4 methylation to form a stable mark at a transcriptionally active gene that persists on metaphase chromosomes even when the gene is located in inhibitory pericentromeric heterochromatin.

RESULTS AND DISCUSSION

H3.3 is enriched at promoters of expressing genes

The mouse $\lambda 5$ -*VpreB1* locus was used as a model system to obtain a detailed picture of H3.3 deposition across a transcriptionally active gene locus (Fig 1A). The locus contains the $\lambda 5$ and *VpreB1* genes, which are expressed in pro- and pre-B cells (Melchers *et al*, 1993). The genes encode the components of the surrogate light chain, which forms part of the pre-B-cell receptor. All of the sequences required for position-independent and copy-dependent expression of the genes in transgenic mice are located within a 19 kb region (Sabbattini *et al*, 1999). This makes the locus an ideal system for studying the epigenetic marking of mammalian functional domains. The region analysed also includes the promoter of the *Topoisomerase-3 β* (*Topo3 β*) gene, which is expressed ubiquitously from a promoter located only 1.5 kb upstream from the *VpreB1* start site within a CpG island (Minaee *et al*, 2005).

It is not possible to distinguish histone H3.3 from H3.1 in chromatin using antibodies raised directly against the protein because the region containing the variant amino acids is occluded by the winding of the DNA strand around the nucleosome (Akhmanova *et al*, 1997). Therefore, we tagged the H3.3 protein

with an epitope from the human c-Myc protein and expressed the tagged protein in Abelson-transformed pre-B cells using a retroviral vector (see Methods). Expression was verified by staining of the cells with an antibody specific for the c-Myc epitope (Fig 1B).

Nuclei from non-transgenic pre-B cells that expressed the tagged H3.3 protein were digested with micrococcal nuclease to give predominantly mono- and dinucleosomes, and the chromatin was precipitated with anti-c-Myc epitope antibody. The precipitated DNA was analysed using real-time PCR primer pairs that cover the 19 kb region at a resolution of ~ 1.0 kb (Szutorisz *et al*, 2005). PCR values were normalized against values obtained with chromatin from non-transfected cells (see Methods). The results of the ChIP analysis are shown in Fig 1C. Peaks of histone H3.3 were observed in the promoter regions of the $\lambda 5$ and *VpreB1* genes and in the region between the two genes, but there was a sharp reduction in enrichment within the transcribed regions. The peaks that were observed in the intergenic region coincide with intergenic promoters for non-coding transcripts and the region around HS7/8 has also been shown to be a centre for recruitment of RNA polymerase II (RNA pol II; Szutorisz *et al*, 2005). Little or no H3.3 was detected in the region immediately 3' of $\lambda 5$ despite the presence of several DNase I-hypersensitive sites and the observation that this region is required for efficient expression of the locus in transgenic mice (Sabbattini *et al*, 1999). Interestingly, the promoter of the ubiquitously expressed *Topo3 β* gene showed no significant enrichment for H3.3 despite its close proximity to the *VpreB1* promoter.

Fig 2 | Comparison of the levels of Myc-H3.3 in the 5' promoter regions and the transcribed regions of different genes. Results were obtained by chromatin immunoprecipitation analysis of Myc-H3.3-expressing pre-B cells using anti-c-Myc tag antibody. PCR values were used to calculate enrichment relative to non-transfected cells (see Methods). Because the values are calculated as ratios relative to non-transfected cells, values ≤ 1 (blue shading) indicate no enrichment (1) or depletion (<1). For PCR primer positions, see supplementary Table 1 online. For analysis of transcription levels of the genes, see supplementary Fig 1 online. (A) Genes that are transcribed in pre-B cells. The significance of the differences between the promoters and the transcribed regions was tested for the entire transcribed gene data set using the non-parametric Wilcoxon's matched-pairs test ($P=0.01$). (B) Genes that are silent in pre-B cells. p, promoter; t, transcribed region; td, distal transcribed region; tp, proximal transcribed region.

The results obtained for the $\lambda 5$ -VpreB1 locus suggest a possible association of H3.3 deposition with active gene promoters. Therefore, we set out to obtain a more general picture of the association of H3.3 with RNA pol II-transcribed genes by extending the ChIP analysis in Myc-H3.3-transfected pre-B cells to a total of 18 genes. The results of this analysis are shown in Fig 2. Genes that are not expressed in pre-B cells (*MyoD*, pancreatic amylase and neurofilament light-chain) do not have significant amounts of H3.3 associated with either the promoters or the transcribed regions (Fig 2B). Most of the genes that are transcribed in pre-B cells (11 out of 15) show H3.3 enrichment at the gene promoters. For most of these genes (9 out of 11), little or no enrichment was observed in the transcribed regions

(Fig 2A), but there were two exceptions, the B-lineage-specific *mb1* gene and the ubiquitously expressed β -actin gene, which showed significant levels of enrichment at the promoters and within the transcription units (Fig 2C). Interestingly, of the genes that were analysed, *mb1* and β -actin showed relatively high levels of expression in pre-B cells (supplementary Fig 1 online). Four genes, *ADA* and *Topo3 β* , *HPRT* and *CD19*, showed little enrichment in either the promoters or the transcribed regions. The significance of the higher level of H3.3 enrichment in the gene promoters compared with the transcribed regions was tested by statistical analysis of the entire transcribed gene data set (see legend to Fig 2). The difference was shown to be highly significant ($P=0.01$).

Fig 3 | Localization of variant histone H3.3 to a variegating $\lambda 5$ transgene on metaphase chromosomes. (A) The $\lambda 5$ transgene. (B) RNA fluorescence *in situ* hybridization (FISH) analysis of a variegated pre-B-cell clone showing expression of the $\lambda 5$ transgene (green) and endogenous β -actin (red). Scale bar, 10 μ m. (C) Immuno-DNA FISH analysis showing colocalization of Myc-H3.3 (green) and the $\lambda 5$ transgene (red) on metaphase chromosomes in a clone with greater than 70% of cells expressing the transgene (clone 1) and one with less than 2% of cells expressing the transgene (clone 2). DNA was counterstained with TOTO (blue). Immuno-FISH was also carried out on metaphase spreads from clone 1 using antibodies specific for phosphorylated histone H3 (green, bottom panel). Arrows indicate the pericentromeric region. Scale bar, 2 μ m. (D) Comparison of Myc-H3.3 colocalization with the transgene during mitosis with numbers of expressing cells in two different pre-B-cell clones. Confocal images of metaphase spreads were randomized and blind-scored by an operator not involved in the analysis. The number of cells or metaphase spreads analysed for each clone is indicated below each histogram bar. χ^2 analysis of the values showed that the differences between the clones were highly significant ($P < 0.0001$).

To exclude the possibility that the differential enrichment was due to nucleosome depletion in the transcribed regions, we analysed histone H3 K4 dimethylation, which has been shown in the promoters and in the transcription units of active genes (Schneider *et al*, 2004). In three out of five genes analysed, significant levels of H3 lysine 4 dimethylation were observed in the transcribed regions (supplementary Fig 2 online), indicating that the patterns of H3.3 deposition that we observe cannot be explained by nucleosome depletion. High levels of H3 K4 dimethylation have also been shown in the promoters and in the transcribed regions of the $\lambda 5$ and *VpreB1* genes in pre-B cells (Szutorisz *et al*, 2005).

The link between transcription and H3.3 deposition was further tested by ChIP analysis of Ba/F3 early pro-B cells, which do not express $\lambda 5$ and *VpreB1*. Ba/F3 cells that were transfected with c-Myc-tagged H3.3 showed no significant incorporation of H3.3 at the $\lambda 5$ and *VpreB1* genes (supplementary Fig 3 online). Enrichment for H3.3 was observed at the promoters of the $\beta 2$ -microglobulin and glucose phosphate isomerase genes, which are expressed in Ba/F3 cells.

Our data show that the main site of H3.3 incorporation at transcribed genes is located upstream from the transcription initiation site. This suggests that H3.3 is incorporated into nucleosomes principally through the action of chromatin-remodelling complexes associated with promoter function. Deposition of H3.3 linked to transcriptional elongation may also occur, but our results suggest that the contribution of this mechanism is relatively minor compared with that of promoter remodelling.

H3.3 marks the active state of a pol II-transcribed gene

To determine whether H3.3 association with RNA pol II-transcribed genes is stable enough to act as an epigenetic mark for transcription in dividing cells, we analysed H3.3 marking of a $\lambda 5$ transgene that had integrated into pericentromeric heterochromatin. The transgene, which is integrated as a 40-copy tandem array, is subject to position-effect variegation with expression observed in $\sim 30\%$ of pre-B cells (Lundgren *et al*, 2000). The active and inactive states are maintained through many cell divisions, but analysis of clones generated from single pre-B cells also showed that the position-effect variegation is unstable,

Fig 4 | Localization of acetylated and methylated histone H3 to a variegating $\lambda 5$ transgene on metaphase chromosomes. (A) Immunofluorescence *in situ* hybridization (Immuno-FISH) analysis showing colocalization of diacylated histone H3 (green) and the $\lambda 5$ transgene (red) during mitosis in a clone with greater than 90% expressing cells (clone 3) and one with less than 5% expressing cells (clone 6). DNA was counterstained with TOTO (blue). Arrows indicate the position of the transgene. Scale bar, 10 μ m. The insets show the chromosome containing the transgene at higher magnification. (B) Correlation of histone H3 acetylation at the transgene with numbers of expressing cells in four different pre-B-cell clones. The number of cells or metaphases analysed for each clone is indicated below each histogram bar. Correlation analysis is shown in supplementary Fig 5B online ($R = 0.99$). (C) Immuno-DNA FISH analysis of the transgene on metaphase chromosomes using antibodies specific for the di- or trimethylated form of H3 K4. χ^2 analysis showed that the difference between the clones was highly significant ($P < 0.01$).

with reversible transitions occurring periodically between the two states in individual cells. A cell line was generated by transformation of pre-B cells from the transgenic mouse line with the Abelson murine leukaemia virus. RNA fluorescence *in situ* hybridization (FISH) analysis showed that the transgene gave variegated expression in these cells (Fig 3B). Cloning of these transformed cells by limiting dilution was used to generate several clones from single cells. When individual clones were expanded to 10^5 – 10^6 cells, the proportion of cells expressing the transgene in different clones ranged from <5% to close to 100% (supplementary Fig 4A–C online). These proportions were retained through several rounds of cell division in short-term culture (15–20 divisions). Long-term culture of the clones resulted in a gradual conversion to the steady-state frequency of $\sim 30\%$ of cells expressing the transgene.

The ability to isolate clones with different proportions of expressing cells allowed us to correlate expression of the transgene with epigenetic marking during mitosis. Cells were transfected with the Myc–H3.3 construct and the H3.3 was visualized on metaphase chromosomes using an immuno-FISH technique that allows antibody staining of metaphase chromosomes to be carried out in conjunction with detection of the transgene by *in situ* hybridization. Cells from different clones were each divided into two aliquots. One aliquot was subjected to RNA FISH to determine the proportion of expressing interphase cells at the time of the experiment. The remaining cells were treated with colcemid to increase the number of mitotic cells, and metaphase spreads were prepared and stained with the appropriate antibodies (see Methods). The chromosomes were then fixed, denatured and hybridized with a $\lambda 5$ probe.

When mitotic spreads from Myc–H3.3-expressing cells were analysed by immuno-FISH using anti-c-Myc epitope antibody and a $\lambda 5$ probe, a proportion of spreads had a strong c-Myc–H3.3 signal that showed clearly against the dark heterochromatin background and coincided with the hybridization signal for the $\lambda 5$ transgene (Fig 3C). Comparison of the number of mitotic cells that had H3.3 signals at the $\lambda 5$ transgene with the proportion of cells that expressed the transgene in interphase cells showed a close correspondence between the two values (Fig 3D). As a control to demonstrate that localization of the H3.3 staining to the transgene was specific, chromosomes were stained with an antibody against phosphorylated histone H3 serine 10, which has been shown to be widely distributed on condensed metaphase chromosomes (Jenuwein & Allis, 2001). The anti-phospho-H3 S10 antibody gave a broad staining of the long arm and the pericentromeric region, with staining largely excluded from the transgene hybridization signals (Fig 3C). These results show that the H3.3 mark is stable enough to be retained on pol II-transcribed genes during the mitotic shutdown of transcription even when the gene is surrounded by inhibitory heterochromatin.

H3 modifications mark active genes during mitosis

Acetylation and methylation of the amino-terminal tails of histones H3 and H4 have been shown to be associated with transcriptional activation (Turner, 2000; Jenuwein & Allis, 2001; Kouzarides, 2002). Analysis of total histone H3.3 in *Drosophila* KC cells also showed that it is enriched in acetylated residues and depleted for K9 methylation (McKittrick *et al*, 2004). To determine whether histone acetylation and methylation form stable marks at

actively transcribed genes, the immuno-FISH procedure was used to analyse the modification status of histone H3 at the variegating $\lambda 5$ gene in mitotic cells. The results of this analysis are shown in Fig 4. Antibodies raised against diacetyl histone H3 (acetylated at K9 and K14) showed a close correlation with the number of cells expressing the transgene. Analysis of the specificity of the antibody by enzyme-linked immunosorbent assay against peptides that contained individual modified residues showed that the anti-diacetyl H3 recognizes acetyl-K9 but not acetyl-K14 (supplementary Fig 2A online). Immuno-FISH analysis of high- and low-expressing clones for di- and trimethyl H3 K4 also showed that the proportion of metaphase spreads with positive signals for each antibody was similar to the proportion of cells expressing the transgene in interphase (Fig 4C; supplementary Fig 6 online).

Our results demonstrate that the promoters of many actively transcribed mammalian genes are marked by deposition of variant histone H3.3. This finding is consistent with a mechanism of H3.3 deposition through the action of chromatin-remodelling complexes associated with binding of transcription factors and the basal transcription machinery to gene promoters. The low levels of H3.3 associated with transcribed regions of most genes argue that nucleosome disruption by the RNA polymerase as it tracks along the DNA has only a minor role in H3.3 incorporation into nucleosomes. The presence of a strong H3.3 mark at an active gene on metaphase chromosomes suggests that H3.3 deposition is important for maintaining cellular memory of active transcription states during mitosis. It is notable that TFIID, which is the only factor that has been shown to bind to gene promoters of pol II-transcribed genes during mitosis (Christova & Oelgeschlager, 2002), interacts specifically with acetylated K9 and K14 residues of histone H3 (Agalioti *et al*, 2002). Our observations support the idea that H3.3 deposition combines with H3 acetylation and TFIID binding to mark gene promoters for reactivation after exit from mitosis.

METHODS

Cell cultures. Abelson-transformed pre-B-cell line 1028 was maintained in RPMI/15% serum (Invitrogen, Paisley, UK). Primary pre-B cells were isolated from transgenic mice carrying 40 copies of the pericentromeric $\lambda 5$ transgene (Lundgren *et al*, 2000). The cells were transformed using the Abelson leukaemia virus and cloned by limiting dilution as described previously (Lundgren *et al*, 2000).

RNA FISH. RNA FISH was performed essentially as described by Lundgren *et al* (2000). Slides were examined using a confocal laser scanning microscope (TCS SPII system, Leica, Milton Keynes, UK). Serial images were collected with a focus increment of $0.2\ \mu\text{m}$ using a $\times 100$ oil immersion lens.

Antibodies. Mouse monoclonal antibody 9E-10, which is specific for the c-Myc epitope tag, was used for ChIP and immunostaining of Myc–H3.3 by ChIP. The following rabbit polyclonal antisera against modified histones were used for immuno-FISH staining of metaphase chromosomes: anti-diacetyl histone H3 (1:200), anti-dimethyl histone H3 lys4 (1:300; Upstate, Milton Keynes, UK) and anti-trimethyl histone H3 lys4 (1:200; Abcam, Cambridge, UK).

Expression of Myc-tagged H3.3 in cells and ChIP analysis. A human histone H3.3 complementary DNA was tagged at the 5' end by the addition of an oligonucleotide encoding the 12-amino-acid c-Myc epitope recognized by the 9E10 monoclonal antibody

and introduced into transgenic and non-transgenic pre-B-cell lines using the pBabe puro retroviral vector (for details, see supplementary information online). Expression of the tagged H3.3 protein was confirmed by immunofluorescent staining with the mouse monoclonal anti-c-Myc tag antibody (1:10) and the donkey fluorescein isothiocyanate-conjugated anti-mouse antibody (1:100; Jackson ImmunoResearch, PA, USA) as primary and secondary layers. The ChIP procedure is described in supplementary information online. H3.3 enrichment was calculated according to the following equation: $\text{fold enrichment} = 2^{(\text{Ct no ab1} - \text{Ct Myc1})} / 2^{(\text{Ct no ab2} - \text{Ct Myc2})}$, where 'Ct no ab' is the cycle above threshold (Ct) value obtained by precipitation without antibody and 'Ct Myc' is the Ct value from precipitations with the c-Myc antibody. 'No ab1' and 'Myc1' are the values obtained for transfected cells and 'no ab2' and 'Myc2' are the values for non-transfected cells. Statistical analysis was performed using the software package Genstat 5 (version 4.1).

Immuno-FISH of metaphase chromosomes. Cells were collected onto microscope slides with a Cytospin 3 centrifuge. Modified histones or histone H3.3 were detected by indirect immunofluorescence and $\lambda 5$ transgene signals were detected by FISH (Lundgren et al, 2000). Details of the immuno-FISH procedure can be found in supplementary information online. Slides were examined using a Leica confocal microscope.

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

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