Histone modification patterns associated with the human X chromosome

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X inactivation is associated with chromosome-wide establishment of inactive chromatin. Although this is classically regarded as facultative heterochromatin that is uniform in nature, the exact distribution of associated epigenetic marks is not well defined. Here we have analysed histone modifications in human somatic cells within two selected regions of the X chromosome. Intergenic, coding and promoter regions are segregated into differentially marked chromatin. H3K27me3 is most prominent in intergenic and silenced coding regions, but is associated with some active coding regions as well. Histone H3/H4 acetylation and H3K4me3 are locally enriched at promoter regions but do not necessarily mark continuing transcription. Remarkably, H3K9me3 is predominant in coding regions of active genes, a phenomenon that is not restricted to the X chromosome. These results argue against the exclusiveness of individual marks to heterochromatin or euchromatin, but rather suggest that composite patterns of interdependent or mutually exclusive modifications together signal the gene expression status.

Keywords: facultative heterochromatin; histone methylation; X inactivation; escape

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INTRODUCTION

Facultative heterochromatin of the inactive X chromosome (Xi) is hallmarked by association of the Xi-specific non-coding transcript (XIST; Brown *et al*, 1991), histone deacetylation (Jeppesen & Turner, 1993), demethylation of histone H3 lysine 4 (H3K4; Boggs *et al*, 2002; Goto *et al*, 2002), dimethylation of histone H3 lysine 9 (H3K9me2; Heard *et al*, 2001), trimethylation of histone 3 lysine 27 (H3K27me3; Plath *et al*, 2003; Silva *et al*, 2003), mono-methylation

of histone H4 lysine 20 (H4K20me1; Kohlmaier *et al*, 2004), methylation of promoter-containing CpG islands (Norris *et al*, 1991) and enrichment of the histone variant macroH2A (Costanzi & Pehrson, 1998). Facultative heterochromatin can be distinguished from constitutive heterochromatin by differential methylation of H3K9, H3K27 and H4K20. Facultative heterochromatin contains H3K27me3, H3K9me2 and H4K20me1, whereas constitutive heterochromatin contains H3K27me1, H3K9me3 and H4K20me3 (Peters *et al*, 2003; Kohlmaier *et al*, 2004; Schotta *et al*, 2004).

About 15% of human X-linked genes escape X inactivation to some extent, whereas another 10% show variable patterns of inactivation between individuals (Carrel & Willard, 2005). Although histone H3/H4 acetylation and H3K4 methylation have been correlated with escape from X inactivation, the exact distribution of these marks as well as H3K9/27 methylation is not well defined. Here we analysed two non-contiguous X-chromosomal regions using chromatin immunoprecipitation (ChIP). Our analyses show segregation of different types of chromatin at intergenic, coding and promoter regions. Intergenic and silenced coding regions have H3K27me3 as the most prominent mark. In contrast, promoter regions have high H3/H4 acetylation and H3K4me3, although their presence does not necessarily mark continuing transcription. Remarkably, we find that coding regions of active genes are enriched for H3K9me3. Analyses of several autosomal genes suggest that these patterns are general rather than X specific.

RESULTS

Gene expression analysis of selected genes

Because monoallelic expression of X-linked genes in XX cells complicates interpretation of ChIP-based histone modification and gene expression analysis, we first turned to hamster somatic hybrid cell lines containing the human Xi or active X chromosome (Xa), and subsequently to human XX and XY lymphoblastoid cell lines. We selected two chromosomal regions located on different arms of the X chromosome but outside the pseudoautosomal region, which is known to contain mostly genes that escape X-chromosome inactivation (XCI). In each case, we selected a single escape gene adjacent to X-inactivated genes, on the basis of expression analysis data described previously (Carrel & Willard, 2005).

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Fig 1 | Complementary DNA expression analysis using reverse transcription-quantitative PCR. (A) Xp22.22. BMX, bone marrow non-receptor tyrosine kinase; FIGF, c-Fos-induced growth factor/VEGF-D; PIGA, phosphatidylinositol *N*-acetylglucosaminyltransferase; PIR, pirin. (B) Xq13.1. CITED1, CBP/p300-interacting transactivator 1; HDAC8, class I histone deacetylase; PHKA1, phosphorylase B kinase α ; RPS4X, ribosomal protein S4. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase.

Expression analysis using reverse transcription-quantitative PCR showed that within Xp22.22, PIGA (phosphatidylinositol *N*-acetylglucosaminyltransferase) was subject to XCI, whereas PIR (pirin) escaped XCI (Fig 1A). FIGF (c-Fos-induced growth factor/VEGF-D) and BMX (bone marrow non-receptor tyrosine kinase) were unexpressed in hybrids and lymphoblastoids and several other XX cell lines (Fig 1A; data not shown), which suggests that these genes are subject to cell-type-dependent repression. Within Xq13.1, RPS4X (ribosomal protein S4) escaped XCI, whereas HDAC8 (class I histone deacetylase) was subject to XCI (Fig 1B). CITED1 (CBP/p300-interacting transactivator 1) and PHKA1 (phosphorylase B kinase α) showed differential expression patterns: in the hybrids, CITED1 was subject to XCI, whereas PHKA1 escaped XCI; however, both genes were unexpressed in lymphoblastoid cells. CITED1 and PHKA1 were active in several other XX cell lines (data not shown), and their unexpressed state seemed to be specific for lymphoblastoid cells. This suggests that cell-type-dependent repression mechanisms may be dominant over the established XCI profiles. Although we did not discriminate between monoallelic or biallelic expression in XX lymphoblastoid cells, expression was probably monoallelic for PIGA and HDAC and bi-allelic for PIR and RPS4X, on the basis of their expression measured in hybrid cells. An expected twofold reduction between XX and XY cells was not evident for the XCI

genes PIGA and HDAC8. We tentatively designated the selected genes as 'subject to X inactivation' (HDAC8 and PIGA), 'escaping from X inactivation' (RPS4X and PIR), 'unexpressed' (FIGF and BMX) or 'facultative' (CITED1 and PHKA1).

Euchromatic histone modification patterns

To correlate gene activity with histone modification patterns, we carried out ChIP experiments using antibodies against di-acetylated histone H3 (lysines 9 and 14, H3ac), tetra-acetylated histone H4 (lysines 5, 8, 12 and 16, H4ac) and trimethylated histone H3 lysine 4 (H3K4me3). These modifications colocalized and were concentrated at promoter regions of the genes, where they probably constitute an active chromatin conformation (Fig 2). This active code generally extended over a region about 1 kb in length. Intergenic and coding regions were devoid of such active histone marks: signals decreased to background levels at a distance of over 1 kb upstream or downstream of the transcriptional start site. For PIGA, PIR, RPS4X and HDAC8, the presence of active histone marks correlated with their expression status; they were present only at active alleles. The correlation between active histone marks and gene activity was not evident for the facultative genes CITED1 and PHKA1; inactive alleles still contained active histone marks in hybrid Xi cells or lymphoblastoid XY and XX cells (Fig 2B). We conclude that H3/H4

acetylation and H3K4me3 colocalized at active promoter regions, except for the facultative genes *CITED1* and *PHKA1*, which contain these marks independently of their expression status. No obvious differences in active code patterns were observed between hybrid cells and lymphoblastoid cells, and no escape-specific patterns of active histone modifications were evident.

Heterochromatic histone modification patterns

To analyse histone modifications associated with heterochromatin, we carried out ChIP experiments for H3K9me2 and H3K9me3 and for H3K27me3. H3K9me2 has been associated with facultative heterochromatin and euchromatin (Peters *et al*, 2003; Rougeulle *et al*, 2004), H3K9me3 with constitutive heterochromatin, and H3K27me3 with the inactive X chromosome (Plath *et al*, 2003; Silva *et al*, 2003) and Polycomb-silenced genes (Cao *et al*, 2002; Kirmizis *et al*, 2004). The overall levels of H3K9me2 were low (Fig 2). Surprisingly, H3K9me3 was most prominent within coding regions of active alleles of *PIGA*, *PIR* (Fig 2A), *RPS4X* and *HDAC8* (Fig 2B). The highest levels of H3K9me3 were found within the highly expressed *RPS4X* gene (up to 50% relative to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase); see Fig 1B), suggesting a correlation between the level of H3K9me3 and transcription elongation activity (see also Fig 4B).

H3K27me3 covered intergenic, promoter and coding regions of inactive alleles, but was excluded from both promoter and coding regions of active alleles (Fig 2). However, such a pattern was not present at both the inactive and active *CITED1* alleles in the hybrid cells, which showed promoter-restricted exclusion of H3K27me3 but maintenance of H3K27me3 in the coding region (Fig 2B). The distribution of H3K27me3 at the active *PHKA1* allele in hybrid Xi cells resembled that of *CITED1*, although the *PHKA1* promoter was not completely devoid of H3K27me3.

Active *PIR* alleles were enriched for H3K9me3 in their coding regions, although in lymphoblastoid XX cells H3K27me3 was also detected. Although this probably indicates that both *PIR* alleles are differentially marked, it is not clear whether the H3K27me3-marked allele represents an active or inactive gene, as H3K27me3 was also found within active coding regions (*CITED1*; Fig 2B).

FIGF was unexpressed in all cell lines tested, but its histone modification pattern was different from other unexpressed genes: *FIGF* lacked abundant H3K27me3 in hybrid Xi, Xa cells and lymphoblastoid XY cells, and instead its histone modification pattern seemed to be an extension of the pattern observed in the *PIR* active coding region (H3K9me3). The *PIR*–*FIGF* head-to-tail distance is less than 500 bp, and although *PIR* contains a poly(A) signal and yields polyadenylated messenger RNA (Wendler *et al*, 1997), the similarity between the *PIR* and *FIGF* modification patterns could be related to transcriptional read-through. Bi-cistronic mRNAs containing both *PIR* and *FIGF* have been observed (Wendler *et al*, 1997; Rocchigiani *et al*, 1998), and a corresponding bicistronic expressed sequence tag is present in the current database release (<http://genome.ucsc.edu>, May 2004).

Fig 3 | ReChIP analysis of human XY lymphoblastoid cells. Two consecutive ChIP experiments were performed with antibodies against the indicated histone modifications. Error bars indicate standard deviation between ChIP experiments with at least three independent chromatin preparations. Ab, antibody; ChIP, chromatin immunoprecipitation.

In conclusion, whereas inactive alleles were marked by H3K27me3 in promoter and coding regions, H3K27me3 was excluded from these regions in active alleles. Instead, coding regions of active alleles contained H3K9me3 as the most prominent mark. H3K27me3 was maintained within coding regions of the active *CITED1* and *PHKA1* alleles. Furthermore, all intergenic regions were marked by H3K27me3.

H3K4me3 and H3K27me3 colocalization

Although we detected both the active mark H3K4me3 and the inactive mark H3K27me3 at promoter regions of unexpressed *CITED1* and *PHKA1* alleles, the detection of these marks could be caused by clonal differences in the cell populations rather than colocalization of these two opposing marks. To distinguish between these possibilities, we performed reChIP experiments in which different modifications were analysed consecutively on the same chromatin sample. Crosslinked chromatin from XY lymphoblastoid cells was subjected to a first-round ChIP, eluted and then subjected to a second-round ChIP. As expected, a first-round ChIP using anti-H3K9me3 or anti-H3K27me3 showed that *CITED1* 0.0 and *PHKA1* 0.0 contained H3K27me3 but not H3K9me3, whereas *RPS4X* +0.5 and *PIGA* +0.5 lacked both modifications (Fig 3). Next, anti-H3K9me3 and anti-H3K27me3 were used in the first-round ChIP, followed by a second-round ChIP using anti-H3K4me3. Although all of the analysed positions contained high H3K4me3 (Fig 2), this mark could be recovered only at *CITED1* 0.0 and *PHKA1* 0.0, and only after a first-round ChIP using anti-H3K27me3. *RPS4X* +0.5 and *PIGA* +0.5, which also have high H3K4me3, could not be recovered because these positions were devoid of H3K9me3 and H3K27me3. These results provide strong evidence for the simultaneous presence of H3K4me3 and H3K27me3 at *CITED1* 0.0 and *PHKA1* 0.0, and exclude the

Fig 2 | ChIP analysis of histone modification patterns associated with X-linked genes of hamster–human hybrid cells (Xi and Xa) and human lymphoblastoid cells (XX and XY). (A) Xp22.22. (B) Xq13.1. Error bars represent standard deviation between at least three different ChIPs with independent chromatin preparations. Genes are depicted on top (not drawn to scale), with positions relative to their transcription start sites indicated (kb). Gene coding regions are indicated by a grey background. The expression status of the depicted genes is indicated by horizontal coloured bars between x axes (green, active; red, inactive; striped, biallelic). ChIP, chromatin immunoprecipitation.

Fig 4 | Chromatin immunoprecipitation analysis of histone modification patterns associated with autosomal genes of human lymphoblastoid cells (XY). Legend as for Fig 2. Chromosome numbers are indicated. (A) Unexpressed genes (BDNF, brain-derived neuronal factor). Arrows indicate transcription start sites. (B) Active genes (GAPDH, glyceraldehyde-3-phosphate dehydrogenase).

possibility that clonal differences within the cell population caused the detection of H3K4me3 and H3K27me3 at the same position.

Autosomal-linked histone modifications

Our analysis of two X-linked chromosomal regions showed that H3K9me3 is present within active coding regions, whereas H3K27me3 is an abundant mark within intergenic, promoter and coding regions of inactive genes. To determine whether such patterns are X specific, we analysed several unexpressed or active autosomal loci in XY lymphoblastoid cells. Within unexpressed genes (myoglobin and BDNF (brain-derived neuronal factor)), H3K27me3 was predominant throughout intergenic, coding and promoter regions (Fig 4A). Coding regions of active genes (GAPDH and β -actin) contained H3K9me3 and lacked H3K27me3 (Fig 4B). At these active loci, intergenic regions were devoid of H3K27me3. Thus, apart from a less pronounced H3K27me3 within intergenic regions that flank active loci, essentially similar patterns of modification were observed. Active histone marks colocalized at promoter regions, but seemed to be less localized than X-linked genes.

DISCUSSION

Our analysis shows that intergenic, coding and promoter regions of two regions of the X chromosome are segregated into differentially marked chromatin: H3K27me3 marks intergenic

regions, silent genes, but also some active genes; H3K9me3 is predominant in coding regions of active genes; that is, chromatin that is not generally regarded as heterochromatic. None of these features is specific for (escape from) X inactivation, although widespread intergenic H3K27me3 may be more prominent on the X chromosome. Possibly, X-inactivation-specific features, if present, could involve the association of XIST, MacroH2A or histone modifications not analysed in this study.

The presence of H3K27me3 at unexpressed autosomal genes suggests that this mark may be a more global (facultative) heterochromatic mark rather than an Xi-specific mark as anticipated. The reported interphase Xi-specific immunostaining of H3K27me3 (Plath *et al*, 2003; Silva *et al*, 2003; Kohlmaier *et al*, 2004) could be attributed to the higher density of inactive genes and/or the highly condensed structure of the Xi. Chromatin of the Xi has been reported to be enriched for H3K9me2 (Peters *et al*, 2003), but within the genes analysed here, the overall levels are low. H3K9me2 has been detected in a 'hotspot' upstream of mouse XIST (Heard *et al*, 2001; Rougeulle *et al*, 2004), and it is possible that local enrichment of H3K9me2 in such hotspots is characteristic for this mark and was therefore not detected in our analysis.

Although H3K9 methylation and binding of HP1 to H3K9 methylated histones have been widely correlated with heterochromatin formation, it was reported recently that H3K9 methylation and HP1 γ are enriched in transcribed regions of

active mammalian genes (Vakoc *et al*, 2005). The data presented here support the idea that this may be a general phenomenon and indicate that these marks are not exclusive for heterochromatin.

Transitions between H3K27me3 and H3K9me3 occur at positions containing local enrichment of H3K4me3 and H3/H4ac, suggesting a possible role for these active marks in the partitioning of differentially marked chromatin. Colocalization of H3K4 methylation and H3/H4 acetylation at promoter regions of genes escaping X inactivation has been reported previously (Boggs *et al*, 2002; Goto *et al*, 2002) but seems to be a general phenomenon rather than X specific, as it has been described for yeast (Ng *et al*, 2003; Roh *et al*, 2004; Pokholok *et al*, 2005), *Drosophila* (Schubeler *et al*, 2004), chicken (Schneider *et al*, 2004), human and mouse (Liang *et al*, 2004; Bernstein *et al*, 2005). The presence of such colocalized modifications around transcription start sites positively correlates with gene activity, but strictly taken, such active code is not exclusive for actively transcribed genes, as similar levels of these modifications also colocalize at the silent CITED1 and PHKA1 genes in lymphoblastoid cells. The maintenance of the active code at these genes may be related to their facultative expression patterns.

Microscopic analysis suggested that human Xi chromatin is segregated into non-overlapping types of heterochromatin, dominated by either H3K9me3 or H3K27me3 (Chadwick & Willard, 2004). Most of the human Xi was stained by anti-H3K9me3, whereas two chromosomal bands (Xp11 and Xq23) were stained by anti-H3K27me3. The chromosomal regions analysed in our study (Xp22.22 and Xq13.1) correspond to H3K9me3-staining chromatin; nevertheless, we find abundant H3K27me3. Because of structural determinants such as chromosome condensation, immunofluorescence microscopic analysis may not be directly comparable with gene-by-gene ChIP analysis.

Although it is clear that differentially marked chromatin can be distinguished at intergenic, coding and promoter regions, it is unknown how these different types of chromatin are established, and whether this differential marking is the cause or the consequence of continuing transcription or subnuclear localization. The results shown here disagree with the concept that heterochromatin and euchromatin can be defined on the basis of individual histone modifications. Instead, composite patterns consisting of interdependent or mutually exclusive histone modifications signal the transcriptional status of chromatin.

METHODS

Cell culture. Hamster–human hybrid cell lines 578 (Xa, Wg3H hamster background) and 789 (Xi, hamster A3 background) have been described (Wieacker *et al*, 1984), and were cultured in DMEM medium and 10% FCS. Human lymphoblastoid cells were cultured in RPMI medium and 10% FCS.

Antibodies. The following antibodies were used for ChIPs: H3ac, Upstate (Lake Placid, NY, USA) #06-599; H4ac, Upstate #06-866; H3K4me3, Abcam (Cambridge, UK); H3K9me2, Upstate #07-212; H3K9me3 #4861 (Peters *et al*, 2003); H3K27me3 #6523 (Peters *et al*, 2003). Specific details on these antibodies are described in the supplementary information online.

Chromatin immunoprecipitations. A detailed protocol for ChIPs and reChIPs is described in supplementary information online.

Quantitative real-time PCR. Quantitative real-time PCR was performed using iQ SYBR green Supermix and a single-colour

detection MylQ iCycler (Bio-Rad, Hercules, CA, USA). Primers used for quantitative real-time PCR are listed in supplementary Table S2 online. To show that these primers selectively amplify human sequences from hamster–human hybrid cells, amplicon melting curves are provided for amplification of hamster–human hybrid DNA, human lymphoblastoid DNA and parental hamster DNA lacking the human X chromosome.

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

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