Chromosome-wide, allele-specific analysis of the histone code on the human X chromosome

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Variation in the composition of chromatin has been proposed to generate a ‘histone code’ that epigenetically regulates gene expression in a variety of eukaryotic systems. As a result of the process of X chromosome inactivation, chromatin on the mammalian inactive X chromosome (Xi) is marked by several modifications, including histone hypoacetylation, trimethylation of lysine 9 on histone H3 (H3\textsuperscript{TrimK9}) and substitution of core histone H2A with the histone variant MacroH2A. H3\textsuperscript{TrimK9} is a well-studied marker for heterochromatin in many organisms, but the distribution and function of MacroH2A are less clear. Cytologically, the Xi in human cells comprises alternating and largely non-overlapping $\sim$10–15 Mb domains marked by MacroH2A and H3\textsuperscript{TrimK9}. To examine the genomic deposition of MacroH2A, H3\textsuperscript{TrimK9} and acetylated histone H4 modifications on the Xi at higher resolution, we used chromatin immunoprecipitation in combination with a SNP-based assay to distinguish the Xi and active X (Xa) in a diploid female cell line and to determine quantitatively the relative enrichment of these histone code elements on the Xi relative to the Xa. Although we found a majority of sites were enriched for either MacroH2A or H3\textsuperscript{TrimK9} in a manner consistent with the cytological appearance of the Xi, a range of different histone code types were detected at different sites along the X. These findings suggest that the nature of the heterochromatin histone code associated with X inactivation may be more heterogeneous than previously thought and imply that gene silencing can be achieved by a variety of different epigenetic mechanisms whose genomic, evolutionary or developmental basis is now amenable to investigation.

INTRODUCTION

The most fundamental unit of chromatin is the nucleosome, consisting of two molecules each of histones H2A, H2B, H3 and H4 encircled by 140–160 bp of DNA (1). Nucleosome placement or density can considerably influence gene expression; non-coding regions of the genome are characterized by evenly spaced nucleosomes and higher order chromatin, whereas transcriptionally active regions tend to be sparsely and irregularly populated with nucleosomes (2–6). Covalently modified nucleosomes, including methylated, phosphorylated and acetylated forms, have been proposed to comprise a combinatorial ‘histone code’ specifying functional attributes on the underlying genomic sequence (7).

The nucleosome can also be structurally modified through the substitution of histone variants for core histones. Several histone variants, including H2A.X, H2A.Z and the H3 variant CENP-A, have been described, each associated with specific functions and each with a distinct effect on chromatin structure (8). The histone variant MacroH2A has been implicated in mammalian X chromosome inactivation, because during interphase, it appears to be enriched on the Xi, coincident with the Barr body, where it forms the macro chromatin body (MCB) (9). Nucleosomes reconstituted with MacroH2A appear to resist SWI/SNF chromatin remodeling, inhibit both RNA polymerase II initiation and histone acetylation and show some evidence of higher order chromatin formation that is distinct from chromatin on the Xa (10,11). MacroH2A is characterized by a long C-terminal tail comprising roughly two-thirds of the total peptide; the tail has no significant homology to any known proteins, whereas the amino terminal third shares 65% amino acid identity to core H2A (12). MacroH2A deposition on the Xi occurs relatively late in the progression of X inactivation (13), suggesting that MacroH2A is involved in...
the maintenance of the inactive state rather than in its initiation. Although it has been shown that MacroH2A interacts with a ubiquitin E3 ligase complex (14) and requires Xist RNA to associate with the Xi (15), it is still unclear how MacroH2A, and heterochromatin in general, is distributed on the X chromosome.

Throughout the genome, gene expression levels can be quite variable, both between genes and between alleles (16,17), and genomic studies of chromatin in various organisms have begun to provide insights into how chromatin modifications are displayed over broad genomic regions, up to and including entire chromosomes or genomes. In yeast, the positions of histone acetyltransferases (HATs), histone deacetylases (HDACs) and RNA polymerase II across the entire genome have been mapped using a chromatin immunoprecipitation (ChIP) and microarray-based approach (18,19). Histone tail modifications for histones H3 and H4 have also been studied on a genome-wide scale in yeast and flies (20–25). In the human genome, chromatin on chromosomes 21 and 22 has been analyzed for several euchromatic histone tail modifications by hybridizing ChIP products to a BAC tiling microarray (26). On the basis of human–mouse comparisons, it was concluded that trimethylated lysine 4 on histone H3 was associated with promoter regions of actively expressing genes on these chromosomes, whereas dimethylated lysine 4 did not show as specific of an association.

Although such studies provide general information on chromatin composition, they do not allow assessment of heritable cis-acting effects that result in unequal expression of alleles. Such an allele-specific approach will be necessary to distinguish chromatin states between homologs in imprinted regions or between an increasing number of individual genes that show clear evidence of allelic imbalance (16,17,27–29). One study of chromatin from the imprinted Igf2r region used polymorphisms to distinguish maternal and paternal alleles and found promoter restricted acetylation and methylated lysine 4 on histone H3 (H3MeK4) on the active Air and Igf2r alleles and H3TrimK9 on the silenced alleles (30). Likewise, a study of the Prader–Willi region on human chromosome 15 found promoter restricted acetylation of H3 and H4 on only the expressed allele (31).

Analysis of chromatin on the inactive X chromosome may provide a useful model on a whole chromosome scale for such allele-specific studies. Previous efforts to examine histone modifications on the Xi have relied on mouse/human somatic cell hybrids containing the Xa or Xi or on male/female comparisons (32–37). To date, chromatin from a limited number of sites on the X chromosome has been studied in an allele-specific manner using microsatellite repeats (38). Although X inactivation shares many epigenetic modifications with other monochromatically expressed regions in the genome, there are specific features of X inactivation that imply the existence of novel chromatin arrangements at the level of large genomic regions. Several chromatin modifications distinguish the Xi, including hypoacetylation of all core histones (38,39), deposition of MacroH2A (9) and trimethylation of lysine 9 on histone H3 (40,41). Acetylation is believed to promote an open chromatin state by weakening the interaction of the histone tails with their targets. The nucleosome becomes partially destabilized because the positively charged, acetylated histone neutralizes electrostatic tension caused by the negative charge of the phosphate backbone of DNA. Acetylation of histone H4 (Ac-H4) occurs on actively expressing genes primarily near the promoter region on the Xa (36). H3TrimK9 is involved in the formation of heterochromatin through the recruitment of heterochromatin protein 1 in many organisms (42), whereas MacroH2A associates with the Polycomb group repression complex and, possibly, HDACs (43,44).

As a first step towards a detailed view of chromatin on the entire human X chromosome, we have determined the distribution and the relative level of enrichment of Ac-H4, MacroH2A and H3TrimK9 on the Xi versus the Xa. To distinguish alleles on the two X chromosomes in a female cell line, we used a series of SNPs along the length of the X at ~1.5 Mb resolution and determined the allele-specific genomic distribution of Ac-H4, H3TrimK9 and MacroH2A on the X chromosome using ChIP. Consistent with previous cytological observations, a majority of sites on the Xi are deficient in Ac-H4 (38,39) and enriched for either MacroH2A or H3TrimK9 (45). However, there are several sites with unusual patterns of enrichment that suggest a higher level of complexity to heterochromatin organization on the X chromosome.

RESULTS
The chromatin variants and modifications evaluated in this study show characteristic differences between the human Xa and Xi (Fig. 1). The antibodies used in this report were tested on a primary, non-transformed human female cell line, RPE1, and revealed staining patterns on both metaphase chromosomes and interphase nuclei, consistent with previously published cytological characterizations (39,45). In RPE1 and other primary cell lines we examined, acetylated histone H4 was largely devoid of the Xi at metaphase and interphase. MacroH2A and H3TrimK9 occupied largely non-overlapping territories of the Barr Body (Fig. 1A), and, at metaphase, they exhibited an alternating banded pattern on the Xi (Fig. 1B) (45). As expected, H3TrimK9 signals were particularly enriched at pericentric heterochromatin on all chromosomes (Fig. 1C). Although some autosomes possess regions of H3TrimK9 not associated with the centromere, the Xi chromosome is distinguishable because the banding pattern differs from its homolog and the size of the regions on the Xi is much greater than those seen on autosomes (Fig. 1D).

Allele-specific assay of X chromatin
The aforementioned antibodies were then tested in ChIP experiments on RPE1, using SNPs in a quantitative fluorescence assay to distinguish between the two X chromosomes. In total, we analyzed 112 X-linked SNPs and, as controls, eight autosomal SNPs that were heterozygous in RPE1. The SNaPshot assay (see Materials and Methods) quantitatively compares two alleles, thus permitting determination of the ratio of signal from the Xi allele to the Xa allele for all SNPs in each ChIP. The phase of each allele (e.g. which
allele was located on the Xa or Xi) was assigned for all informative X-linked SNPs, using a mouse/human somatic hybrid cell line. In addition, for expressed SNPs, we utilized RT-PCR to determine whether the gene was subject to or escaped from X inactivation, as described previously for a series of different cell lines (46,47). Overall, the set of 112 informative SNPs included 11 expressed SNPs within 10 genes on the X chromosome. Of these, eight were subject to inactivation in RPE1, whereas two escaped inactivation and showed biallelic expression. These expressed SNPs provide an opportunity to compare directly expression patterns with chromatin composition on the Xi.

The eight autosomal SNPs served as controls for the ChIPs and SNaPshot. Notwithstanding the existence of imprinted genes, one would expect epigenetic modifications on chromatin at a majority of autosomal sites to be equally represented on both homologs and, consequently, gene expression to be essentially identical between the alleles. In accordance with this expectation, the six expressed autosomal SNPs in RPE1 were biallelically expressed at an equal level from both alleles. To provide control data to compare with X-linked SNPs, we assayed each autosomal SNP for the presence of Ac-H4, MacroH2A and H3TrimK9; an example of the SNaPshot data for one such SNP is contained in Figure 2A. After

Figure 1. Immunocytochemical distribution of heterochromatin on the human Xi. The arrow indicates the Barr body in each panel. (A) and (B) show a representative nucleus and Xi, respectively, from RPE1 double labeled for MacroH2A (green) and H3TrimK9 (red). (C) shows a representative RPE1 metaphase spread with the Xa and Xi chromosomes delineated, after staining with H3TrimK9 antibody. Several representative H3TrimK9-stained metaphase chromosomes from RPE1 are shown in (D). Importantly, the Xa and Xi represent the only instance of different staining patterns between homologs. Individual chromosomes were identified by DAPI staining.
normalization to the ratio between the heights of peaks in the input samples, it is apparent that both autosomal alleles were equally represented in the +RT sample and in the Ac-H4, MacroH2A and H3TrimK9 ChIPs. Although the assays used are quantitative between alleles for a given locus, they are not strictly quantitative on an absolute scale across loci or between different ChIPs; nonetheless, the peak heights in the Ac-H4 ChIP were very high relative to the MacroH2A and H3TrimK9 peak heights, as one would expect for an autosomal locus in euchromatin. Similar data were acquired for the seven other autosomal SNPs, and the ratios for all eight SNPs in each IP were used to determine experimental variation within each ChIP. On the basis of these data, we chose 2-fold enrichment or deficiency between the alleles as a very conservative cutoff for significant differences between the Xa and Xi.

Next, we tested SNPs on the X chromosome for the presence of Ac-H4, MacroH2A and H3TrimK9. Figure 2B shows SNaPshot data for some of the observed relative enrichment patterns. For the DJ473B4 and MID1 genes, only the Xa allele was preferentially precipitated in the Ac-H4 ChIP; conversely, the Xi allele was the only one present in the IP fractions from the MacroH2A ChIP for DJ473B4 and the H3TrimK9 ChIP for MID1. The TCTE1L gene was monoallelically expressed, and only the Xa allele was detected in the Ac-H4 ChIP. However, both MacroH2A and H3TrimK9 were strongly enriched (>6.8-fold) on the Xi at this site. It is possible that this SNP represents one of the overlapping regions of MacroH2A and H3TrimK9 (Fig. 1). The NRK gene represents a fourth pattern of chromatin uncovered by this analysis. H4 acetylation was found solely on the Xa allele, suggesting that this gene lies within a region of heterochromatin on the Xi; consistent with this, MacroH2A was enriched on the Xi allele by >10-fold. However, paradoxically, H3TrimK9 showed >10-fold relative enrichment on the Xa allele. We detected several SNPs with this oppositely enriched pattern, and, in four of five instances, H3TrimK9 was significantly enriched on the Xa...
allele, not (as expected) on the Xi allele. This pattern was seen in independent replicates and ChIPs, indicating that this is a consistent feature of chromatin at these loci on the X chromosome in RPE1.

**Genomic distributions of Ac-H4, MacroH2A and H3TrimK9 in Xi chromatin**

Hypocacylation of all core histones is a hallmark of Xi chromatin (39,48). We used this feature to demonstrate the success of our ChIPs and assays because it is the only epigenetic mark that has been examined cytochemically and, to some extent, by ChIP on the X chromosome (36,38). As such, it provided a control to which we could compare our SNaPshot data. Indeed, the majority of SNPs were deficient from the Xi for Ac-H4, as indicated in Figure 3A. We were unable to amplify a PCR product in our Ac-H4 ChIP fraction for 11 SNPs. These SNPs are included at the 0-fold enrichment mark on the graph in Figure 3A and represent loci that are poorly acetylated (or not acetylated at all) on both the Xa and Xi. It is possible that SNPs with equivalent levels of acetylation on Xa and Xi represent genes or regions that escape inactivation. There was a direct correlation between acetylation and expression status in our survey; 10/10 expressed SNPs that were subject to X inactivation (as determined by RT-PCR) showed at least 5-fold relative enrichment of the Xa allele in the Ac-H4 ChIP, whereas both alleles were detected equally in the Ac-H4 ChIP fraction for both SNPs in genes that escaped inactivation. These data validate the Ac-H4 ChIP and further indicate that the SNaPshot assay can accurately identify the relative enrichment of chromatin on Xa and Xi. We thus proceeded to examine all SNPs for the presence of MacroH2A and H3TrimK9.

Figures 3B and C contain the MacroH2A and H3TrimK9 ChIP data for all informative X chromosome SNPs in our survey. The MacroH2A data appear to correlate with the metaphase bands seen previously (45); in particular, regions 1 through 3 (bracketed in Fig. 3) generally fit a pattern of either MacroH2A or H3TrimK9 enrichment on the Xi. Region 2 identifies a MacroH2A band where, notably, the eight SNPs tested within this region were >2- to 10-fold enriched for the Xi allele in the MacroH2A ChIP, whereas only a single SNP showed enrichment on the Xi above background levels in the H3TrimK9 ChIP. However, the four SNPs immediately flanking this region were >4-fold enriched on the Xi for H3TrimK9, but <5-fold enriched on the Xi for MacroH2A. Region 3 may correspond to a smaller metaphase MacroH2A band on distal Xq; this is a region in which four SNPs were >3-fold enriched for MacroH2A on the Xi allele, whereas three of those four SNPs were >5-fold enriched for H3TrimK9 on the Xi allele. Thus, some regions on the Xi can be enriched for both MacroH2A and H3TrimK9, refining the apparently mutually exclusive nature of the two heterochromatin types noted previously on the basis of cytological assays (45).

Surprisingly, there were eight SNPs that showed >3-fold relative enrichment on the Xi (equivalent to a relative deficiency on the Xi) for either MacroH2A or H3TrimK9 (Figs. 3B and C). The most frequent pattern was one in which MacroH2A was enriched on the Xi allele and H3TrimK9 on the Xa allele (e.g. the NRK gene in Fig. 2B). Two such positions were located roughly 12 kb apart in the KIAA1202 gene at position 50 Mb in Xp and possessed a nearly identical level of enrichment on the Xa for H3TrimK9 and on the Xi for MacroH2A.

To determine if this particular histone code was a feature of a domain of chromatin that was more extensive than single localized sites, we identified additional SNPs across a region of >330 kb flanking the KIAA1202 gene (which is subject to inactivation (47)) and assayed those sites for MacroH2A and H3TrimK9. As shown in Figure 4, each of the SNPs showed >2-fold enrichment of MacroH2A on the Xa, but >2-fold enrichment for H3TrimK9 on the Xi, both within and flanking the KIAA1202 gene (Fig. 4), and two additional genes located proximal to KIAA1202 (data not shown). Thus, this novel pattern of chromatin composition is a feature of at least some regions on the X and reveals an unexpected type of heterochromatin on the Xi.

**Genic distributions of chromatin modifications**

A previous study reported high levels of H4 acetylation near the 5′ ends of genes (36). To address whether MacroH2A, H3TrimK9 or Ac-H4 was preferentially associated with chromatin near the 5′ or 3′ ends of X-linked genes, we compared the relative enrichment of these chromatin attributes on the Xa or Xi in different regions of genes (Table 1). A total of 56 SNPs in our survey were located within 5 kb upstream of exon 1 to 5 kb downstream of the last exon. Extending the previous report (36), we found that relative levels of chromatin features on the Xi were variable between genes but, when present, were associated throughout the length of a gene, with significant relative enrichment for SNPs located in the 5′, middle or 3′ regions of genes.

Recently, we reported an X inactivation profile of essentially all X-linked genes that are expressed in fibroblast cell lines (47). To examine the correlation between X inactivation status and chromatin composition, we compared the number of genes shown to be subject to or escaping inactivation with their relative levels of Ac-H4, MacroH2A and H3TrimK9 on the Xi. In total, there were 59 SNPs in our survey located within 34 genes whose X inactivation status had been reported (Table 2). There is a striking contrast in the level of MacroH2A and H3TrimK9 in genes that were subject to inactivation versus genes that escaped inactivation. Most (29/43) sites in genes subject to inactivation demonstrated >4-fold enrichment for MacroH2A and/or H3TrimK9 on the Xi. Conversely, only four of 16 sites in genes that escaped inactivation were similarly enriched for MacroH2A and/or H3TrimK9; most such sites showed no significant enrichment at either the Xa or Xi allele.

**Nucleosome density does not differ between the X chromosomes**

It has been proposed that the apparent enrichment of MacroH2A at metaphase may reflect a higher density of nucleosomes on the Xi relative to the other chromosomes (49). To test this hypothesis, we examined the distribution of histone H2B (a histone that does not differ between Xa
Figure 3. Chromosome-wide, allele-specific analysis of X chromosome chromatin. Data are shown for the Ac-H4 (A), MacroH2A (B) and H3TrimK9 (C) IPs. For each graph, the relative enrichment on the Xi for each SNP was plotted as a log base 2 value versus the SNP’s location on the X chromosome based on NCBI’s human genome sequence build 35 from the Ensembl genome browser. Datapoints above the X chromosome idiogram correspond to Xi enrichment, and datapoints below the idiogram indicate Xi deficiency. Levels of enrichment or deficiency are truncated at >10-fold (>3.3 on a log base 2 scale) for convenience of presentation. The dashed line in each panel represents a conservative level of error that is greater than ±2 standard deviation, based on autosomal controls. In (B) and (C), brackets indicate three regions discussed in the text.
and Xi) at interphase and metaphase and performed ChIP in RPE1, with antibodies directed against a stably transfected H2B construct (see Materials and Methods). A myc-tagged version of H2B was used to avoid the possibility that an antibody to core H2B may cross-react with covalently modified forms of H2B and influence the allele that is precipitated. Figure 5A shows an immunocytochemical analysis of cells or chromosomes double-labeled for Ac-H4 and H2B. We used the absence of Ac-H4 staining to demarcate the Xi territory (Barr body) at interphase and metaphase. The H2B-stained Xi was indistinguishable from other chromosomes in metaphase spreads (Figs 5A and B). We also counted 200 interphase nuclei in which the Xi territory was clearly defined by the absence of Ac-H4 for the presence or absence of cytological enrichment equivalent to the MCB observed with MacroH2A staining (Fig. 5B). Although 98% of nuclei showed cytological enrichment of MacroH2A on the Xi, we never observed any cytological enrichment on the Xi for H2B.

Next, we performed ChIP against histone H2B in RPE1 followed by SNaPshot analysis. If nucleosome density differs between the Xa and Xi, with the Xi containing more nucleosomes that contribute to the apparent cytological enrichment of MacroH2A, then one would expect a core histone ChIP to preferentially precipitate the Xi allele. Representative SNaPshot data for one of 10 X-linked SNPs tested are shown in Figure 5C. Unlike the MacroH2A ChIP, where only the Xi allele was detected, both alleles were equally represented in the H2B ChIP. The Xi and Xa alleles were indistinguishable with regard to H2B at all X-linked SNPs tested, similar to the five autosomal SNPs. The X chromosome SNPs had a mean Xi-to-Xa ratio of $1.00 \pm 0.23$, compared with $0.94 \pm 0.13$ for the autosomal SNPs. We conclude that the bands of MacroH2A and H3TrimK9 enrichment on the Xi are not a consequence of increased nucleosome density, but rather indicate that more MacroH2A and H3TrimK9 are associated with Xi nucleosomes relative to the composition of chromatin on the Xa.

**DISCUSSION**

There is increasing interest in the composition of chromatin throughout the human genome (50), and a number of papers have begun to examine the distribution of histone modifications in various chromosomal regions (26,51). However, such global analyses cannot distinguish between chromatin on homologous chromosomes, as is necessary to examine...
Ac-H4 deficiency on the Xi relative to the Xa (Fig. 3). In general agreement with these studies, we detected marked hypoacetylation along the length of the Xi at a majority of informative sites (Fig. 3A); however, on the Xa, some hybrid cell lines (36) have shown that Ac-H4 is largely confined to the promoter region of genes on the Xa. The SNaPshot ChIP assay measures the ratio of Xi to Xa alleles for a given SNP, but has a more limited ability to quantify the amount of signal present in an absolute sense between loci. Nonetheless, the global deficiency of Ac-H4 along the Xi may reflect the fact that unlike imprinted or other differentially expressed loci in the genome, X inactivation is a chromosome-wide event. Thus, it is plausible that chromatin on the Xi may be organized to maintain the silenced state over large distances. Whereas imprinting at the H19/Igf2r or Prader–Willi loci extends 1–2 Mb (53) and reflects a single domain of monoallelically expressed genes, the Xi is interspersed with larger domains of genes subject to and escaping from X inactivation along the entire length of the chromosome (47).

### MacroH2A and H3TrimK9

MacroH2A and H3TrimK9 were enriched on the Xi at a majority of informative sites in a manner consistent with cytological observations (Fig. 1) (45). As predicted, we also noted a correlation between expression status and the presence of MacroH2A and H3TrimK9. Monoallelically expressed genes, as determined directly by expression analysis in this study and/or in a comprehensive Xi expression study in a series of human or mouse/human hybrid cell lines (47), were more likely to have relative enrichment at the Xi allele for either MacroH2A or H3TrimK9 (Table 2), consistent with the role of these alternative chromatin modifications in gene silencing in different regions of the Xi (45). However, we also uncovered several SNPs in which both MacroH2A and H3TrimK9 were preferentially enriched on the Xi allele. A higher resolution study will be required to determine whether these SNPs represent transition zones between larger domains marked by MacroH2A or by H3TrimK9, as suggested by immunocytochemistry (45); alternatively, the presence of both chromatin signatures may reveal an element of the Xi histone code that is distinct from chromatin enriched for only one of these marks. As with Ac-H4, we found that MacroH2A and H3TrimK9 were not confined to the 5′ regions of genes, but rather showed significant enrichment throughout the body of genes (Table 1). This phenomenon likely reflects the

### Table 1. Chromatin signatures in genes on the inactive X chromosome

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### Table 2. X inactivation status and chromatin signatures on the inactive X chromosome

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<td>&gt;4 24</td>
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aData are expressed as fold deficiency or enrichment on the Xi relative to the Xa (Fig. 3).

bFrom Carrel and Willard (47).

Cytologically, most of the Xi shows little, if any, staining for Ac-H4 (39), and previous ChIP experiments on X chromosome hybrid cell lines (36) have shown that Ac-H4 is largely confined to the promoter region of genes on the Xa. In general agreement with these studies, we detected marked histone H4 hypoacetylation along the length of the Xi at a majority of informative sites (Fig. 3A); however, on the Xa, we found significant acetylation at the 3′ end of some genes, as well as near the promoter (Table 1). This extends a previous report of substantial acetylation on the Xa in the 3′ region of a few genes, using polymorphic microsatellite repeats to distinguish the X’s in a human female cell line (38). In contrast, other studies have observed promoter restricted acetylation of histone H4 in autosomes (19,26,30,31,52).

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<td></td>
<td>&gt;4 19</td>
<td>2</td>
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<tr>
<td>H3TrimK9 enrichment</td>
<td>n.s. 19</td>
<td>10</td>
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<tr>
<td></td>
<td>2–4 6</td>
<td>1</td>
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<td></td>
<td>&gt;4 18</td>
<td>5</td>
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aData are expressed as fold deficiency or enrichment on the Xi relative to the Xa (Fig. 3).

bFrom Carrel and Willard (47).
long-range spreading of heterochromatin complexes in cis on the Xi chromosome.

In a previous report (49), core histones H2B and H3 were shown to exhibit an MCB-like enrichment on the Xi in a primary human dermal cell line. Here we have examined directly the distribution of H2B in RPE1 cells and found no enrichment of H2B was cytologically or molecularly associated with the Barr Body (Fig. 5). We examined the distribution of H2B in several hundred nuclei as well as in metaphase spreads and never detected MCB-like enrichment for H2B in RPE1. One explanation for the discrepancy is that the antibodies used in the previous study (49) may not have been specific to core H2B or H3 and recognized covalently modified versions of the peptides as well (such as H3TrimK9).

Further, in ChIP experiments, all of the selected X chromosome SNPs showed 4-fold enrichment for either MacroH2A and/or H3TrimK9; yet, these SNPs were not preferentially enriched for either allele in the H2B ChIP and had a distribution similar to the autosomal control SNPs. Thus, the patterns of enrichment we observed in RPE1 do not appear to be a consequence of nucleosome density, but rather reflect specific and characteristic differences in chromatin composition between the Xa and the Xi. Our findings are also consistent with a recent paper in which a MacroH2A ChIP in mouse found no basis for different nucleosome densities at the sites tested on the X chromosome (54).

**Novel chromatin patterns**

The most unexpected finding of our survey was the demonstration of a subset of SNPs that were equally but oppositely enriched on the Xa and Xi for MacroH2A and H3TrimK9. The most common form of this pattern was one in which H3TrimK9 and Ac-H4 were relatively enriched on the Xa, whereas MacroH2A was relatively enriched on the Xi (Figs 3A and B and 4). The relative enrichment of a heterochromatic marker on the Xa was unanticipated, and it is unclear what purpose such a chromatin conformation could play, if at all, in the X inactivation process. One possible model to explain these data is transvection; this process has been proposed to contribute to X inactivation (55), suggesting that the pairing of the two X chromosomes might be involved in setting up chromatin domains on the Xa and the Xi. Recently, two independent groups associated the pairing of a portion of the two X chromosomes as part of the counting/choice step of X inactivation (56,57). It is possible that a similar type of pairing may be involved in the maintenance of the inactive state or serve to propagate the inactivation signal in the domains of Xa H3TrimK9 enrichment. Alternatively, genes located in these domains of oppositely enriched heterochromatin might be developmentally restricted or cell type-specific genes that are not expressed in RPE1. Acetylation has been seen in some forms of methylated lysine 9
in H3 heterochromatin (58–60). It has been suggested that H3TrimK9 is associated with transcription elongation (61). Although the observed patterns in Figure 4 are consistent with this hypothesis, it is notable that this pattern is not seen in other areas of the chromosome, particularly in regions where the acetylation pattern suggests active transcription; this suggests that this novel pattern likely reveals a mark other than transcription. More work will be necessary to determine the epigenetic marks that define these domains of chromatin on the Xa and to explore if they are conserved in other mammalian species. Of course, it is conceivable that this pattern is not specific to X inactivation at all and instead reflects a broader role of chromatin modification in distinguishing alleles throughout the genome. A previous analysis of chromatin modifications on human chromosomes 21 and 22 could not have detected differences between homologs, as allele-specific assays were not used in that study (26).

Concluding remarks

We have provided a 1.5 Mb resolution map of chromatin on the X chromosomes in a human female cell line. This analysis sets the stage for a higher resolution map that will be required to determine precisely how MacroH2A and H3TrimK9 are distributed in various regions. There were several genes in which we examined multiple SNPs, and it is evident that there can be some variability in the relative level(s) of MacroH2A or H3TrimK9 even between regions that are quite close on the chromosome. This initial allele-specific chromatin map has revealed new complexities of X heterochromatin, complementing and extending previous cytological analyses (45). It is likely that a higher resolution study of X chromatin will address the specific nature of a possible histone code(s) at these sites and thus delineate the apparently heterogeneous epigenetic signatures along the length of the X chromosome.

It would be of particular interest to examine the boundary regions between the MacroH2A and H3TrimK9 bands evident along the chromosome. It seems likely, given the proposed histone code and previous work, that there are two major types of heterochromatin complexes on the Xi, one containing XIST RNA, MacroH2A and Polycomb group proteins and the other containing H3TrimK9 and HP1 (45,62). However, what exactly prevents the encroachment of one complex on the territory of the other complex is unknown. Further examination of the boundaries where the transitions between H3TrimK9 and MacroH2A heterochromatin occur will provide insight into the mechanism of how these chromatin states are established and maintained. In addition, if there are heterochromatin boundaries on the Xi, it will be interesting to see whether those boundaries are stable and identical within an individual’s collection of cell types and between females, as patterns of X inactivation can vary quite substantially (47). Metaphase spreads stained for MacroH2A clearly indicate its presence on autosomes (45), suggesting possible relevance beyond the X chromosome. Moreover, there may be autosomal regions with unusual chromatin conformations similar to the ones documented here on the X. Our data underscore the importance of an allele-specific approach to study epigenetic modifications on the X chromosome or other differentially expressed regions of the genome. Given increasing evidence of allelic differences in gene expression across the genome (16,63), an extension of this approach to examine allele-specific chromatin composition genome-wide may be highly informative.

MATERIALS AND METHODS

Cell culture and transfection

RPE1 is a human telomerase-immortalized female cell line derived from retinal pigment epithelial cells. It exhibits non-random X inactivation with the same X inactive in all cells. As recommended by Clonetech, RPE1 was maintained as a monolayer in D-MEM/F12 (Gibco), supplemented with 10% (v/v) FBS (Gibco), 0.348% (v/v) sodium bicarbonate, 0.1 mg/ml penicillin and streptomycin and 2 mM l-glutamine. Cells were grown at 37°C in a 5% CO2 environment.

To detect MacroH2A or H2B by indirect immunofluorescence or ChIP experiments (see what follows), RPE1 cells were transfected with either a myc-tagged MacroH2A construct or a myc-tagged H2B construct cloned into pcDNA3.1 vector, as described (45). Stably transfected clones were isolated and maintained in medium containing 1.41 mg/ml geneticin (Gibco).

Active X hybrid cell formation

To allow assignment of alleles to either the Xa or Xi, mouse/human somatic-cell hybrid lines containing the Xa chromosome from RPE1 were made using the method of microcell-mediated chromosome transfer (64,65). Briefly, RPE1 cells were treated with colcemid for 2 days to form micronuclei. The micronuclei were purified by ultracentrifugation and fused with mouse A9 recipient cells by treatment with PEG-1500 (NBS Biochemicals). Fused cells were placed on selective media containing hypoxanthine–aminopterin–thymidine (HAT) to select the human Xa, and HAT-resistant single-cell colonies were screened for the presence of a single X chromosome by genotyping and by chromosome analysis.

Antibodies

Anti-trimethyl K9 on H3 and anti-acetyl H4 antibodies were obtained from Upstate Biotechnology (07-442 and 06-866, respectively). H3TrimK9 and H3TrimK27 have very similar epitopes, which often results in cross-reactivity. The manufacturer claims specificity of the 07-442 antibody for H3TrimK9, and we have confirmed this specificity by slot blot (data not shown). Both immunocytochemistry and ChIP experiments were repeated with a different H3TrimK9 antibody from Upstate Biotechnology (07-523); we found significant agreement between the two antibodies for the vast majority of loci tested. Variation was detected at several sites that are also enriched for MacroH2A, however, likely reflecting slight cross-reactivity with H3TrimK27, which co-localizes with MacroH2A (45). Anti-myc antibodies were obtained from Invitrogen (46-0603). Patterns observed with
anti-myc-MacroH2A were indistinguishable from those previously detected with antibodies against the endogenous protein (45).

Immunocytochemistry

Interphase RPE1 cells were grown on microscope slides, and metaphase spreads were obtained after a 1 h colcemid treatment followed by incubation in a hypotonic solution and cytopspinning. We used a 1:200 dilution of each primary antibody—anti-Ac-H4 (Upstate Biotechnology), anti-H3TrimK9 (Upstate Biotechnology) or anti-myc (Invitrogen)—and a 1:200 dilution of secondary goat anti-rabbit IgG (Jackson ImmunoResearch) labeled with rhodamine (for Ac-H4 and H3TrimK9) or goat anti-mouse IgG (Jackson ImmunoResearch) labeled with FITC (for myc). Labeled slides were fixed in 4% formaldehyde and counterstained with DAPI (Vector).

Chromatin immunoprecipitation

ChIP was performed essentially as described (66). Briefly, ~5 × 10⁷ cells were harvested and treated with a hypotonic solution. Swollen cells were lysed with a 2 ml Dounce homogenizer under conditions that adequately separated nuclei from the cytoplasm (as judged by phase contrast microscopy). Nuclei were digested with 60 U of micrococcal nuclease (Worthington) for 15 min. Under these conditions, the majority of DNA fragments were less than 1 kb in length (data not shown). Cell debris was separated from the crude chromatin extract by centrifugation.

Non-specific immunoprecipitation (IP) of chromatin was reduced by a clearing step consisting of the addition of 100 µl of a 50% Protein A (for the Ac-H4 and H3TrimK9 ChIPs) or Protein G (for the myc-MacroH2A or myc-H2B ChIPs) slurry and either rabbit or mouse serum. The chromatin extract was pre-cleared for 30 min at 4°C on a rotating drum. The IP was performed on cleared chromatin by the addition of 5 µl of primary antibody, as described earlier. A mock IP was performed in parallel with either rabbit or mouse serum. After 2 h at 4°C on a rotating drum, 100 µl of 50% Protein A or G slurry was added. Binding was allowed to proceed overnight at 4°C with gentle rocking. Bound immune complexes were washed three times and eluted by Proteinase K (Roche) digestion. DNA fragments were precipitated and quantitated by conventional methods.

SNP identification and SNaPshot assay

Approximately 300 reported SNPs with a reported minor allele frequency greater than 0.45 were acquired from the Celera Discovery System (http://www.celera.com), The Cancer Genome Anatomy Project (CGAP, http://cgap.nci.nih.gov) and NCBI’s SNP database (dbSNP, http://www.ncbi.nlm.nih.gov/SNP). SNPs heterozygous in RPE1 were identified using a quantitative SNaPshot (ABI) (47) assay resulting in >100 SNPs with high-quality assays.

PCR was performed using an appropriate dilution of ChIP DNA for the Ac-H4, H3TrimK9 and MacroH2A ChIPs. Each SNP was analyzed in duplicate for Ac-H4 and H3TrimK9 and in quadruplicate for MacroH2A. We also replicated a representative subset of the data multiple times with independent ChIPs. Individual SNPs were amplified by conventional PCR, and PCR products were purified using the Roche Highpure PCR purification kit according to the manufacturer’s specifications. Purified PCR products were used in combination with a conventional primer that abuts the SNP in the amplified PCR product. The primer becomes labeled with a fluorescently tagged dideoxynucleotide through a single base pair extension (SNaPshot kit from ABI). Labeled primer was separated on an ABI 3100 capillary sequencer, and peak heights were logged using GeneScan software (ABI). To determine conditions under which the SNaPshot assays were quantitative, male DNAs with different genotypes were mixed in a range of ratios and analyzed by SNaPshot using X chromosome primer sets for a representative subset of SNPs. Conditions were then selected for SNaPshot under which the ratio of peak heights was quantitative.

RNA isolation and RT–PCR

To determine the expression status of genic SNPs, RT–PCR was performed, essentially as described (47). An ~85% confluent T-175 was harvested, and the cell pellet was incubated in 10 ml of TRIzol (Invitrogen) for 5 min at room temperature. Nucleic acids were separated through the addition of 2 ml of chloroform. The aqueous phase was collected, and the RNA was precipitated with isopropanol. RNA was resuspended in water and stored at −80°C until needed.

In preparation for reverse transcription, 5 µg of RNA was digested with 1 U of DNase I (Invitrogen) for 15 min at room temperature. RNA was reverse transcribed by the addition of random hexamers (Amersham Pharmacia), RNASEOUT (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). The reaction was allowed to proceed for 2 h at 42°C and then heat inactivated. cDNAs were stored at −20°C. For expression analysis, 1 µl of cDNA was used and PCR products were processed for SNaPshot, as described previously.

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Conflict of Interest statement. The authors declare no conflicts of interest in this work.

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