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X-linked genes in female embryonic stem cells carry an epigenetic mark prior to the onset of X inactivation

Laura P. O'Neill[1], Tamzin E. Randall[1], Jayne Lavender[1], Hugh T. Spotswood[1], Jeannie T. Lee[2,3] and Bryan M. Turner[1,*]

[1]Chromatin and Gene Expression Group, Anatomy Department, University of Birmingham Medical School, Birmingham B15 2TT, UK, [2]Howard Hughes Medical Institute, Department of Molecular Biology, Massachusetts General Hospital and [3]Department of Genetics, Harvard Medical School, Boston, MA 02115, USA

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We use chromatin immunoprecipitation to show that genes on the two active X chromosomes in undifferentiated, XX female embryonic stem cells (ES cells) are marked by hyperacetylation of all core histones, hyper(di)methylation of H3 lysine 4 and hypo(di)methylation of H3 lysine 9, compared with autosomal genes or genes on the single active X in XY male cells. The mark is found on both coding and promoter regions. On differentiation, and after the onset of X inactivation, the mark is reversed on the inactive X, whose genes show extreme hypoacetylation of all four core histones, hypo(di)methylation of H3K4 and hyper(di)methylation of H3K9. The mark is retained on the active X in female ES cells for at least several days of differentiation, but is not present in adult females. The selective marking of X-linked genes in female ES cells in a way that distinguishes them from the equivalent genes in males, is unprecedented. We suggest that the mark forms part of a chromatin-based mechanism that restricts X-inactivation to cells with more than one X chromosome.

INTRODUCTION

Genes on one of the two X chromosomes in female mammals are silenced early in development, a process of dosage compensation that equalizes levels of X-linked gene products in XX females and XY males (1,2). The inactive X chromosome (Xi) takes on properties associated with heterochromatin, including extreme underacetylation of its core histones (3–5), incorporation of a variant histone H2A (6) and characteristic changes in methylation of H3 lysines 4 and 9 (7–10). In addition, the facultative heterochromatin of Xi is coated with the non-coding RNA transcript of *Xist*, an X-linked gene that is, uniquely, expressed on Xi but silenced on Xa (reviewed in 2,10,11). Early in the process of X inactivation, *Xist* RNA increases in amount (12,13) and spreads in *cis* along Xi, coating the chromosome (14 and references therein). A functional *Xist* gene is essential for the initiation, but probably not for the maintenance, of gene silencing (15,16). An antisense transcript from the same locus, *Tsix*, is expressed from both X chromosomes early in development, but silenced on Xi at, or prior to, the onset of inactivation (17). Evidence suggests that down-regulation of *Tsix* is an integral part of the process that increases *Xist* RNA levels and leads to X inactivation (18).

X inactivation normally occurs only in cells with more than one X chromosome per diploid nucleus, implying the existence of a 'counting' mechanism that distinguishes such cells from those with only a single X and that triggers or permits dosage compensation (19). An alternative hypothesis proposes that a 'blocking factor' protects a single X, in all cells, from inactivation (20), leaving any additional Xs at the mercy of a ubiquitous inactivation process. In the experiments described here, we use mouse embryonic stem (ES) cells and chromatin immunoprecipitation (ChIP) to quantify acetylation and methylation of core histones on X-linked genes in male and female cells before and after X-inactivation. Surprisingly, we find that X-linked genes in XX female ES cells are distinguished from their counterparts in XY males, and from autosomal genes, by a characteristic pattern of histone acetylation and methylation. This epigenetic mark is present

*To whom correspondence should be addressed. Tel: +44 1214146824; Fax: +44 1214146815; Email: b.m.turner@bham.ac.uk

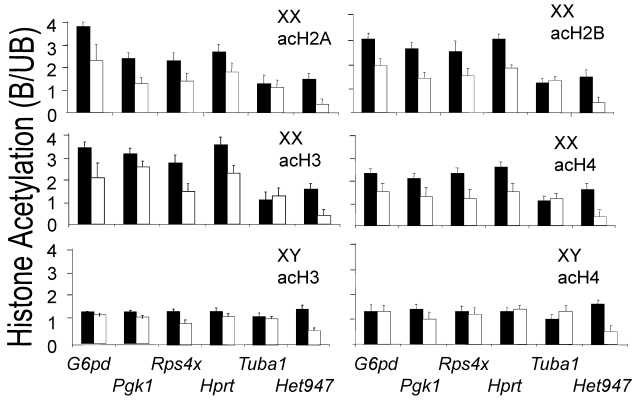


Figure 1. Acetylation of core histones on X-linked and autosomal genes in XX female and XY male ES cells before and after differentiation. Levels of acetylation (B/UB ratio) of core histones determined by immunoprecipitation of chromatin from XX female (PGK12.1) and XY male (CCE/R) mouse ES cells before (day 0, dark columns) and after (day 7, pale columns) differentiation and X-inactivation in culture. Experiments used affinity-purified antibodies to acetylated H2A (lysine 5), H2B (lysines 12 and/or 15), H3 (lysine 14) and H4 (lysine 16). DNA from the antibody-bound (B) and unbound (UB) chromatin fractions was serially diluted, applied to nylon filters, hybridized with ^{32}P-labelled DNA probes and quantified by PhosphorImaging. Each result is derived from at least three separate calculations of the B/UB ratio from successive serial dilutions (in duplicate) on the same filter. Bars are standard errors derived from these separate calculations. For XY male cells, illustrative results are shown for acetylated H3 and H4 only.

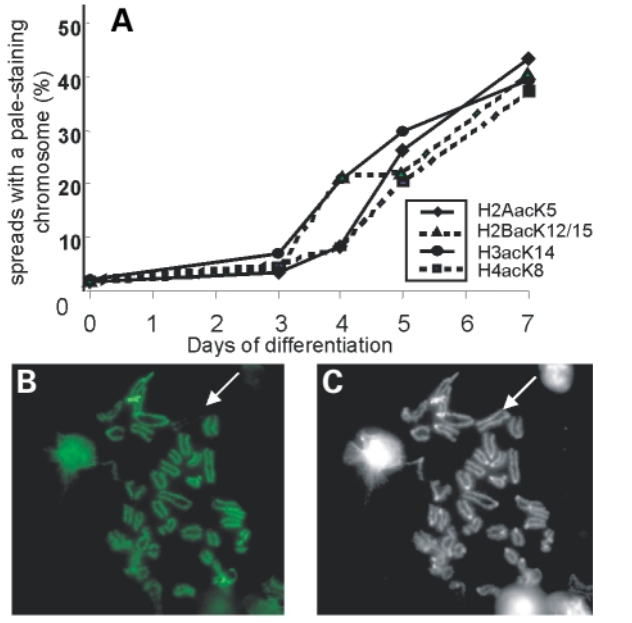


Figure 2. All four core histones are deacetylated at the same stage of ES cell differentiation. Metaphase chromosome spreads were prepared from female ES cells after differentiation for up to 7 days. Chromosomes were immunostained with antibodies to acetylated H2A, H2B, H3 and H4 and spreads with and without a clear, pale-staining chromosome were counted. (**A**) shows the results of such counts. (**B**, FITC, antibody stain) (**C**, DAPI counterstain) show an example of a spread with a pale-staining, hypoacetylated X (arrows).

on both the paternal and maternal X chromosomes in female ES cells prior to inactivation, but never in males. We suggest that it is closely linked to 'counting' and provides a chromatin-based mechanism that restricts X inactivation to cells with more than one X chromosome.

RESULTS

Patterns of histone acetylation on ES cell chromatin before and after differentiation

Chromatin was prepared from male and female ES cells before and after differentiation, and immunoprecipitated with antibodies to acetylated isoforms of each of the four core histones. DNA from antibody-bound (i.e. acetylated) and unbound (i.e. non-acetylated) fractions was assayed for sequences from X-linked and autosomal genes and centric heterochromatin. Levels of acetylation are expressed as the antibody-bound/unbound (B/UB) ratio and illustrative examples are shown in Figure 1. In female cells, acetylation of all X-linked genes tested fell ~2-fold by day 7 of differentiation. No such fall was seen for X-linked genes in male cells or for autosomal genes in cells of either sex (Fig. 1). In contrast, acetylation of all four core histones on centric heterochromatin (major satellite repeat) fell several fold on differentiation of both male and female cells, consistent with previous results (for H4 alone) by ChIP and immunofluorescence microscopy (5,21).

Unexpectedly, it was found that acetylation of X-linked genes in female cells is always higher than acetylation of either autosomal genes, or X-linked genes in male cells (Fig. 1). Throughout these experiments, we have found no significant differences between results obtained with antisera to acetylated isoforms of each of the four core histones. All antisera show 2–3-fold enhancement of acetylation of X-linked genes in female cells and an ~2-fold drop in acetylation of these genes by day 7 of differentiation. It is concluded that, for the genes and DNA regions tested so far, all four core histones are acetylated and deacetylated in a coordinated manner. This is consistent with microscopical studies of the global deacetylation of core histones on Xi in metaphase chromosome spreads from differentiating female ES cells. With antisera to acetylated isoforms of each of the four core histones, an increase in the frequency of spreads with a pale-staining chromosome is first apparent at day 3–4 of differentiation and increases thereafter (Fig. 2). The timing is consistent with that previously reported for acetylated H4 (21). In view of these results, we have pooled ChIP data obtained with antisera to individual core histones to give an overall picture of histone acetylation levels on various X-linked and autosomal genes and centric heterochromatin. Our findings are summarized in Table 1. (The complete data set from which the results in Table 1 are calculated is supplied as Supplementary Material.)

In undifferentiated female ES cells, the four X-linked housekeeping genes examined have overall levels of acetylation, that are two to three times higher than those found on autosomal genes or X-linked genes in male cells (Table 1). All six probes to X-linked housekeeping genes detect this increased acetylation, whereas none of the probes to autosomal genes or heterochromatin show such an effect. This argues that hyperacetylation of X-linked genes in female ES cells is a general phenomenon. Even *Xist* (transcribed region) and *Smcx* genes that escape inactivation (22,23) are more highly

Table 1. Core histone acetylation on X-linked genes, autosomal genes and heterochromatin in female and male ES cells before (day 0) and after (day 7) differentiation

Genes		Core histone acetylation (B/UB ratio)[a]			
		Day 0	Day 7	Day 0	Day 7
		PGK12.1 (female)		CCE/R (male)	
X-linked (transcribed)	*G6pdx*	3.2 ± 0.65	2.0 ± 0.34	1.3 ± 0.05	1.2 ± 0.05
	Hprt	3.0 ± 0.63	1.9 ± 0.25	1.4 ± 0.05	1.4 ± 0.03
	Pgk1	2.6 ± 0.46	1.7 ± 0.64	1.3 ± 0.12	1.1 ± 0.05
	Rps4x	2.5 ± 0.24	1.4 ± 0.14	1.4 ± 0.06	1.1 ± 0.17
	Smcx[b]	2.0 ± 0.10	1.4 ± 0.23	1.2 ± 0.10	1.1 ± 0.11
	Xist	1.9 ± 0.21	1.4 ± 0.23	0.9 ± 0.32	0.9 ± 0.25
X-linked (promoter)	*Hprt*[b]	2.5 ± 0.17	1.1 ± 0.12	1.3 ± 0.11	1.2 ± 0.18
	Pgk1[b]	2.7 ± 0.35	1.3 ± 0.20	1.4 ± 0.05	1.3 ± 0.08
Autosomal	*Tuba1*	1.2 ± 0.10	1.2 ± 0.10	1.2 ± 0.13	1.3 ± 0.17
	Gapd	1.2 ± 0.08	1.1 ± 0.10	1.1 ± 0.10	1.0 ± 0.20
	Actb	1.3 ± 0.06	1.2 ± 0.04	1.1 ± 0.18	1.2 ± 0.14
Heterochromatin (major satellite)	*Het947*	1.5 ± 0.07	0.4 ± 0.03	1.4 ± 0.10	0.6 ± 0.05
		16.7 (female, WT)		3F1 (female, *Tsix* KO)	
X-linked (transcribed)	*G6pdx*	2.0 ± 0.26[c]	0.8[d]	2.3 ± 0.82	1.0
	Pgk-1	2.8 ± 0.37[c]	1.0[d]	2.5 ± 0.61	1.3
	Rps4x	2.6 ± 0.50[c]	0.9[d]	2.8 ± 0.32	1.2
Autosomal	*Tuba*	1.2 ± 0.04[c]	1.1[d]	1.0 ± 0.19	1.2

[a]Average of four independent immunoprecipitations with antibodies to each of the core histones, except where indicated; ±SD.
[b]Average of immunoprecipitations with antibodies to three of the four core histones; ±SD.
[c]Average, ±SD, of four or five separate immunoprecipitations with different antisera to acetylated H3 and H4.
[d]Average of duplicate immunoprecipitations, with a range of no more than ±20%.

acetylated than autosomal genes, although their acetylation levels are below those of female X-linked genes subject to inactivation (Table 1). Importantly, the data from PGK12.1 (female) and CCE/R (male) ES cells presented in Table 1 is entirely consistent with results from independently established ES cell lines, grown either on fibroblast feeder layers or gelatin-coated flasks (lines 16.7, 3F1 in Table 1; L.P.O., B.M.T., E. Heard, E. Dubrand, P. Avner, unpublished results). Thus, the findings are not attributable to differences between ES cell lines or the culture conditions used.

With probes to the coding regions of X-linked genes, the overall fall in core histone acetylation on differentiation is at least 40%. Similar results were obtained with probes to the *Hprt* and *Pgk1* promoter regions, although the loss of acetylation on differentiation may be greater than for coding DNA (Table 1). Our findings show that histone deacetylation on Xi is not *limited* to promoter regions, as suggested for the human Xi in somatic cell hybrids (24). The results also tell us that the female active X (Xa) must remain 2–3-fold hyperacetylated until at least day 7 of differentiation. If genes on Xa were acetylated to the same extent as autosomal genes in differentiated cells, then the B/UB ratio for these genes in differentiated female cells (with each gene having one acetylated and one non-acetylated allele) should be half that for autosomal genes (with two acetylated alleles). This is clearly not the case. However, ChIP experiments with chromatin from adult mouse tissues show that the overall level of histone acetylation on X-linked genes is about half that on autosomal genes (results not shown), consistent with X-linked genes having one deacetylated allele and one allele acetylated to the same level as the two alleles of each autosomal gene. Thus, a degree of hyperacetylation of the female Xa is retained for at least several days after X inactivation, but is lost as development proceeds.

Female X-linked genes are marked by a distinctive pattern of H3 methylation

Immunofluorescence microscopy has been used to show that Xi labels weakly, compared with autosomes, with antibodies to H3 di-methylated at lysine 4 (H3me2K4) and relatively strongly with antibodies to H3 di-methylated or tri-methylated at lysine 9 (H3me2K9, H3me3K9) (7–9,25). In light of these results, we were interested to use ChIP to examine the levels of H3K4 and H3K9 methylation on individual X-linked genes in female ES cells before and after X inactivation.

In undifferentiated female ES cells, levels of H3me2K4 are consistently at least 2-fold higher than levels on autosomal genes or on the equivalent X-linked genes in male ES cells (Fig. 3). After 7 days of differentiation levels fall, becoming comparable to those on autosomal genes (which show no significant change). This is true of genes that are inactivated and those that escape inactivation (i.e. *Xist* and *Smcx*). Changes in H3me2K4 on X inactivation closely parallel, in extent and distribution, those in histone acetylation (Fig. 1). In complete contrast, H3me2K9 levels on X-linked genes in female cells are always lower than levels on autosomal genes or on X-linked genes in male cells, but are dramatically increased after X-inactivation, reaching in some cases levels more than 2-fold greater than those on autosomal genes (Fig. 3).

The interpretation of results with antibodies to H3 methylated at K9 is complicated by cross-reaction of at least some of these antisera with H3 peptides methylated at K27 (26) (B.M.T., L.P.O., unpublished results). The two epitopes share the

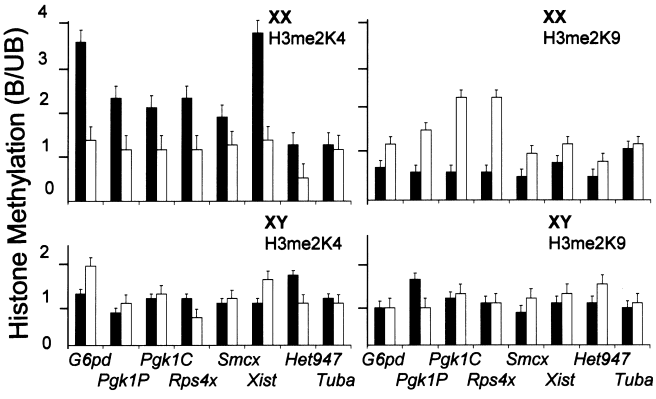


Figure 3. Dimethylation of H3K4 and H3K9 on X-linked and autosomal genes in XX female and XY male ES cells before and after differentiation. The experimental approach was the same as in Figure 1, but ChIP was carried out with antibodies specific for H3(di)methylated at lysine 4 or lysine 9. Promoter and coding regions of *Pgk1* are labelled as *Pgk1P* and *Pgk1C*, respectively.

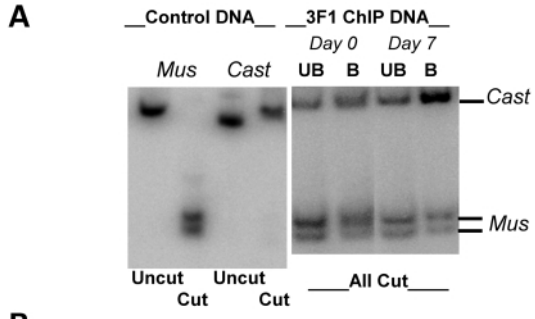
tetrapeptide motif ARKS. We tested the batch of commercially available (Upstate) antiserum to H3me2K9 used for the experiments below by inhibition ELISA, and found no significant cross-reaction with H3me2K27 peptides (results not shown but see Supplementary Material). Using the same peptide inhibition approach it was also found that the acetylation status of H3K14 has no effect on the ability of the antibody to bind to H3me2K9 (results not shown). Thus, the elevated binding of anti-H3me2K9 antibodies following X inactivation cannot be attributed to deacetylation of H3K14. It is important to note that all the experimental approaches employed here (i.e. ELISA, ChIP and immunofluorescence microscopy) use *unfixed* material (histones, chromatin or metaphase chromosomes). Because of this, antibody specificities defined in one approach are likely to hold true for the others.

An epigenetic mark is found on both X chromosomes in undifferentiated female ES cells

The results so far do not allow us to distinguish levels of acetylation on each of the two Xa chromosomes in undifferentiated female ES cells. It could be that hyperacetylation is limited to just one of these two chromosomes. It is formally possible that our results reflect selective hyperacetylation (and parallel H3 methylation changes) on the paternal X (Xp). This would explain the overall hyperacetylation of X-linked genes in females (XpXm) compared with males (XmY). In this regard, it is interesting that, in mice, selective hyperacetylation of the paternal genome (27) and hypermethylation of H3K9 on the maternal genome (28) have both been detected shortly after fertilization. Although these differences are lost, at least at the light microscope level, by the first zygotic mitosis, it might be that local hyperacetylation associated with specific (X-linked) genes persists through later stages of development. To test this directly, we carried out ChIP analysis of X-linked genes in ES cells derived from a parthenogenetic (XmXm) embryo and found exactly the same degree of hyperacetylation as that found in bi-parental lines (results not shown).

An alternative possibility that would lead to differential marking of the two female Xs prior to inactivation is that the future active and inactive Xs have already been chosen in undifferentiated ES cells and that selective histone hyperacetylation (in female cells only) reflects this choice. To test this, we have studied two hybrid ES cell lines showing extreme skewing of X inactivation and in which alleles of selected X-linked genes could be distinguished (17). Line 16.7 carries two stable X chromosomes, one of *Mus m. castaneus* origin and one of *Mus m. musculus* (129/Sv) origin in a 129 × (*castaneus* × 129) hybrid background (17). On differentiation, the *musculus* X is inactivated in 80% of cells, due to the relatively strong *Xce* allele (Xce^c) carried by the *castaneus* X chromosome (2,10). Line 3F1 is a clone derived from 16.7 in which the *musculus Tsix* allele has been disrupted by targeted deletion, a mutation that results in inactivation of the *musculus* X in close to 100% of cells (18). If hyperacetylation of X-linked genes is a pre-emptive marker for the future active X, then in 16.7 and 3F1 cells it should be found predominantly or exclusively on the *castaneus* X chromosome.

To test this prediction, we immunoprecipitated chromatin from both cell lines with antibodies to acetylated H4. Slot blotting of immunoprecipitated DNA showed the same 2–3-fold hyperacetylation of X-linked, but not autosomal, genes as seen in other undifferentiated ES cell lines (Table 1). To distinguish *castaneus* and *musculus* alleles in immunoprecipitated chromatin, we used PCR to amplify a region of the *Pgk1* gene that contains a TthIII1 cutting site in *musculus*, but not in *castaneus.* The two alleles can be separated by electrophoresis of cut DNA (Fig. 4A). As an autosomal control gene, we used *Atp1b2*, a gene containing a PCR polymorphism distinguishing *musculus* and *castaneus* alleles (MGD; www.informatics.jax.org) and for which both alleles were detected in 16.7 and 3F1 cells. ^{32}PαdCTP was incorporated in the last cycle of PCR to allow quantitative analysis by PhosphorImaging. In undifferentiated cells, both *musculus* and *castaneus* alleles of *Pgk1* were present in immunoprecipitated chromatin (Fig. 4A). Quantitation of the relevant bands allowed us to calculate levels of acetylation as B/UB ratios, as before. In undifferentiated (day 0) cells, levels of acetylation on the *castaneus* and *musculus* alleles of *Pgk1* were equal (i.e. the *castaneus–musculus* ratios are around 1, Fig. 4B). As expected, levels of acetylation were also the same on the *castaneus* and *musculus* alleles of the autosomal *Atp1b2* gene (Fig. 4B). Because of the skewing of X inactivation in 16.7 and 3F1 cells, it would be predicted that, after 7 days of differentiation, immunoprecipitated (i.e. acetylated) chromatin should contain predominantly the *castaneus Pgk1* allele. This is exactly what is found. In differentiated 16.7 cells (where Xa will be 80% *castaneus*), the *castaneus–musculus* ratio in immunoprecipitated chromatin is just over 3, while in 3F1 cells (where Xa will be close to 100% *castaneus*), it approaches 4 (Fig. 4B). For the autosomal *Atpb1* gene, the *castaneus–musculus* ratio remains close to 1 on differentiation. These results confirm the accuracy and consistency of the assays used to measure allelic acetylation levels, and demonstrate that genes on both female X chromosomes are equally hyperacetylated prior to ES cell differentiation.



Cell line	Day	H4 Acetylation (B/UB ratio)					
		Autosomal (*Atp1b2*)[1]			X-linked (*Pgk1*)[2]		
		cast	*mus*	*cast/mus*	*cast*	*mus*	*cast/mus*
16.7	0	5.24	4.49	1.17	0.40	0.50	0.80
(H4Ac16)	7	1.17	1.01	1.16	1.13	0.35	3.23
3F1	0	0.72	0.91	0.79	0.51	0.57	0.89
(H4Ac16)	7	0.73	0.62	1.18	1.24	0.32	3.88
3F1	0	0.74	0.94	0.79	0.99	0.79	1.25
(H4Ac8)	7	0.54	0.55	0.98	0.86	0.22	3.91

Figure 4. TthIII1 cutting distinguishes the *Mus m. musculus* and *Mus m. castaneous* alleles of the X-linked *Pgk1* gene. (**A**) A 180 bp *Pgk1* sequence in *Mus m. musculus* and *Mus m. castaneus* control DNA (left panel) and DNA from immunoprecipitated chromatin (right panel), was amplified by PCR and digested with TthIII1. This cut the *musculus* fragment into two similar sized pieces, but left the *castaneus* fragment uncut (left panel). Band identity was verified by DNA sequencing. Immunoprecipitated DNA (bound and unbound fractions, antibody to H4acK16) from undifferentiated (day 0) and differentiated (day 7) 3F1 cells was PCR-amplified, ^{32}P-labelled, digested with TthIII1 and resolved into *castaneus* (*cast*) and *musculus* (*mus*) fragments (right panel). Note that the *castaneus* allele is relatively enriched in bound (i.e. acetylated) chromatin from differentiated cells. (**B**) Chromatin from 3F1 and 16.7 hybrid ES cells was immunoprecipitated with antibodies to H4acK8 and H4acK16 as indicated. The *musculus* and *castaneous* alleles of the autosomal *Atp1b2* gene were resolved by PCR amplification across a region that differs in size between the two sub-species (see text). The *castaneous* and *musculus* alleles of the X-linked *Pgk1* gene were distinguished by PCR and TthIII1 cutting (see text and A above). ^{32}P-labelled PCR fragments were resolved by electrophoresis and quantified by Phosphorimaging. Levels of acetylation are expressed as B/UB ratios. Note that, because the PCR amplification used in each analysis is non-linear, the absolute B/UB ratios will vary between samples and cannot be compared across different experiments in the same way as ratios derived from slot-blotting (Table 1). However, within a single sample the *musculus* and *castaneous* fragments are amplified to the same extent from the same set of primers; the *mus*/*cast* ratio should not vary for experimental reasons and should be comparable between experiments. This expectation is confirmed by the results.

DISCUSSION

Our results show that X-linked genes in XX female ES cells carry an epigenetic mark that distinguishes them both from autosomal genes and from X-linked genes in XY male cells. The mark is found on both female X chromosomes and consists of hyperacetylation of all four core histones, hyper(di)methylation of H3 lysine 4 and hypo(di)methylation of H3 lysine 9. Following 7 days of differentiation and X inactivation, acetylation of all four core histones and (di)methylation of H3K4 fall by at least 80% on Xi while genes on Xa remain hyperacetylated and hyper(di)methylated at H3K4. Hyperacetylation of the female Xa is lost in adult tissues, and initial evidence suggests that the loss of this mark may have already begun by day 7 of ES cell differentiation. For example, in the 16.7 ES cell line, acetylation of *Pgk1* fell by 65% by day 7 of differentiation (Table 1), a fall that requires some deacetylation of Xa, even in the presence of complete deacetylation of Xi. In contrast, immunoprecipitation of X-linked genes with antibodies to H3me2K9 increases dramatically following differentiation of female (but not male) cells. We have shown that the antibody to H3me2K9 used for these ChIP experiments does not cross react with H3me2K27, a site that is also preferentially methylated on Xi (29), nor was its binding found to be influenced by the acetylation status of H3K14. It is proposed that these results reflect selective, localized hypermethylation of H3K9 on Xi. An increased, overall level of H3K9 methylation has been detected on Xi by immunofluorescence microscopy (7–9,25).

Previous examples in which entire chromosomes, or sets of chromosomes, were found to be marked by distinctive patterns of histone modification, always showed parallel differences in transcriptional activity. For example, the heterochromatin-like pattern of modifications on Xi is consistent with its generally transcriptionally silent state, while the presence of H4acK16 in the single male X chromosome in *Drosophila* (30–32) is associated with a 2-fold up-regulation of transcription and may be involved in targeting chromatin remodelling complexes that mediate chromatin decondensation (33). Similarly, the relatively increased histone acetylation and decreased H3K9 methylation on *paternal* chromatin in one-cell mouse embryos shortly after fertilization is correlated with increased transcription of the paternal genome (27,28). Whether the pattern of histone modifications associated with X-linked genes in female ES cells is also associated with increased transcription is unknown, but there is no *a priori* reason to assume that it is. It has been hypothesized, primarily as a result of evolutionary considerations, that genes on the single Xa in both male and female mammals should be transcribed twice as fast as autosomal genes, in order to balance the levels of their transcripts (34). Evidence that this is indeed the case has come from quantitative analysis of transcription from *Ccl4*, a gene which, unusually, is X-linked in *Mus spretus* but autosomal in laboratory mouse strains (35). If the epigenetic mark that we detect is a reflection of this (possibly) altered transcriptional activity, then it should be present also on the male X, which it clearly is not.

Our experiments with hybrid female ES cells carrying one X chromosome of *M. musculus* origin and one of *M. castaneus* origin, show that the *Pgk1* gene is equally hyperacetylated on each X, indicating that the epigenetic mark detected is present on both X chromosomes in undifferentiated female cells. In view of this, the mark cannot be a pre-emptive marker for Xa or Xi and is therefore unlikely to be a determining factor in the *choice* of which X to inactivate. Nor is the mark dependent on whether the X chromosome is of paternal (Xp) or maternal (Xm) origin. The mark is present at equal levels in wild-type (XmXp) and parthenogenetic (XmXm) female ES cells, but is absent in normal males (XmY). In so far as the mark is found only in XX female ES cells, and never in XY males, it may instead depend on the number of X chromosomes present, and may therefore be linked to *counting*, a mechanism that distinguishes cells with one X chromosome from those with more than one and allows X inactivation only in the latter. As

failure of X inactivation in XX female cells, or inappropriate inactivation in XY males, are both lethal, this mechanism is essential (2,19). The robustness of the epigenetic mark we detect and its presence in a variety of normal and mutant female (but never male) ES cell lines, is consistent with a role in allowing X-inactivation only in cells with more than one X chromosome.

It is interesting to ask at what stage of development the epigenetic mark present on X-linked genes in female ES cells might be put in place. Does it arise early in development, or is it put in place only at the blastocyst stage? Although we have no evidence that directly addresses this point, the behaviour of the two X chromosomes through female development suggests the latter possibility. There are epigenetic differences in the maternal and paternal X chromosomes from the one-cell stage through to the pre-implantation blastocyst (2,19,36). As noted earlier, the maternal and paternal genomes develop overall differences in histone acetylation and methylation shortly after fertilization (27,28). These differences are lost during the earliest cell divisions, but more detailed differences in the maternal and paternal X chromosomes then become apparent. From as early as the four-cell stage, *Xist* RNA is produced at low levels from Xp, but not from Xm (2,37,38). This *Xist* RNA does not cover the chromosome in *cis*, as happens during X-inactivation in the blastocyst. However, a degree of spreading is suggested by the finding that *Pgk1* is selectively inactivated on Xp, while genes located further from the X inactivation centre are not (38,39). The nature of the imprint that leads to this selective, partial inactivation of Xp remains unknown, but it is presumably also responsible for the selective, complete inactivation of Xp in cells of the trophectoderm (cells that go on to form the extra-embryonic tissues). In the epiblast, whose cells go on to form the embryo itself and from which ES cell lines are derived, X inactivation occurs slightly later and either Xp or Xm can be inactivated, i.e. the imprint that leads to selective inactivation of Xp earlier in development has been either over-ridden or erased. This is consistent with the low-level expression of *Xist* RNA from *both* Xp and Xm in undifferentiated female ES cells (reviewed in 36). Whether the mark we detect is involved in erasing or over-riding pre-existing imprints on Xp or Xm to allow such dual expression remains unclear, although it can be concluded that it is not *necessary* for *Xist* expression. Male ES cells, in which the mark is absent, also show low-level expression of *Xist* RNA prior to differentiation (36).

We propose that the distinctive pattern of chromatin modifications on female X-linked genes is a transcription-independent mark that is put in place at the pre-implantation blastocyst stage of development. It is dependent upon, or part of, the counting mechanism that limits dosage compensation to cells with more than one X chromosome. It is suggested that it provides the two X chromosomes with comparable chromatin structures, and thereby facilitates the subsequent stages that lead ultimately to selective X inactivation. It is interesting to note that the mark detected, namely hyper-acetylation of all four core histones, hyper-methylation of H3K4 and hypo-methylation of H3K9, is one that has been associated with 'open' (usually transcriptionally active) chromatin. Such an open structure may facilitate access of factors necessary for the choice, initiation and/or spreading stages of X-inactivation. Choice and initiation could be associated with more local chromatin marks such as the extreme hyper-acetylation of core histones found within a 100 kb region upstream of *Xist* (5) (L.P.O., unpublished data), but the fact that the mark described here is distributed across the X, is consistent with its involvement in a chromosome-wide event, such as spreading of the inactive state.

MATERIALS AND METHODS

Cells

Female (40XX) ES cell line PGK12.1 has been described previously (5,40). Male (40XY) ES cell line CCE/R was a kind gift by Dr Graham Anderson (University of Birmingham) of a line originally derived by Dr Klaus Eichmann (Max Plank Institute for Immunobiology, Freiburg, Germany). Previous publications have described the derivation and properties of the *Mus musculus* × *Mus castaneus* hybrid ES cell lines 16.7 and 3F1 (17,18) and the parthenogenetic line PR8 (41). All cells were grown at 37°C, 5% CO_2 in air, on gelatin-coated flasks in Dulbecco's modified eagle medium (DMEM) supplemented with penicillin/streptomycin, non-essential amino acids, 20% fetal calf serum and 103 Units/ml LIF (all reagents from Gibco-BRL). Cells were induced to differentiate and harvested as described (5).

Antibodies and chromatin immunoprecipitation

Chromatin was prepared, by micrococcal nuclease digestion, from ES cells grown overnight in medium supplemented with ^{3}H-thymidine (Amersham, 0.25 μCi/ml), and immunoprecipitated with affinity-purified antibodies to histones H2A, H2B, H3 and H4 acetylated at defined lysine residues. ChIP procedures and the derivation and specificities of the antibodies to acetylated histones have been described previously (5,42). The antibody to H3 di-methylated at lysine 4 (H3me2K4) was prepared from rabbits immunized with the peptide ARTme2KQTARKSTGGC using the procedures previously described (42). Specificity was confirmed by inhibition ELISA and western blotting. The rabbit antibody to H3 di-methylated at lysine 9 (H3me2K9) was from Upstate.

DNA was isolated from antibody-bound (B) and unbound (UB) fractions and equal amounts (based on ^{3}H-thymidine counts) were serially diluted and applied in duplicate to nylon filters (Hybond N+, Amersham) by slot blotting as described (5). Specific DNA sequences were detected by hybridization with ^{32}P-labelled DNA probes and quantified on a PhosphorImager (Molecular Dynamics).

Immunofluorescence microscopy

Immunostaining of metaphase chromosome spreads was carried out exactly as described by Keohane *et al.* (21).

DNA probes and PCR

The *Pgk1* promoter probe was a 281 bp PCR product: forward primer-5′ GGGAGCTGAGAAAGCAGAGG 3′; reverse primer 5′ CAGAAAGCGAAGGAACAAAGC 3′. The *Gapd* probe is

a 304 bp PCR product: forward primer, 5′ CGTAGACAAAA-TGGTGAAGG 3′; reverse primer, 5′ TCCACGACATACTC-AGCAC 3′.

Probes for the *Hprt* promoter (43), *Smcx* (22) and for *G6pdx, Pgk1* (coding), *Rps4x, Xist* (W7D), *Tuba, Actb* and *Het947* (heterochromatin, major satellite repeat) were as previously described (5).

PCR analysis was performed on 50 ng immunoprecipitated DNA using 1.1 × ReddyMix PCR mix (ABgene) and 0.5 μM primers. Denaturation was performed at 95°C for 90 s followed by 30 cycles of PCR (1 min at 95°C, 45 s at 60°C and 45 s at 72°C). The final cycle was performed in the presence of [^{32}P]αdCTP (1% of total dCTP). *Pgk1* PCR products were purified using Qiaquick columns (Qiagen) and digested with TthIII1 at 65°C for 3 h to distinguish *Mus musculus* and *Mus castaneus* alleles. Digested products were analysed by electrophoresis on 5% polyacrylamide gels, visualized with ethidium bromide, dried down and analysed using a PhosphorImager. *Atp1b2* PCR products were analysed on 5% gels as above or on denaturing gels containing 8 M urea. The relative band intensities for *Pgk1* and *Atp1b2* were calculated using Molecular Dynamics software. Primer sequences for *Pgk1* and *Atp1b2* were as follows: *Pgk1*—forward primer, 5′ CCCA-TCACCTTAAGTTGCAC 3′, reverse primer, 5′ CCTTT-ATCAGTGGATCAACTCC 3′; and *Atp1b2*—forward primer, 5′TGGTGTTCCTGCCACAGAAA 3′, reverse primer, 5′ TCT-GAAGACAGCTACAGTGT 3′.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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