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A developmental switch in H4 acetylation upstream of *Xist* plays a role in X chromosome inactivation

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We have investigated the role of histone acetylation in X chromosome inactivation, focusing on its possible involvement in the regulation of *Xist*, an essential gene expressed only from the inactive X (Xi). We have identified a region of H4 hyperacetylation extending up to 120 kb upstream from the *Xist* somatic promoter P_1. This domain includes the promoter P_0, which gives rise to the unstable *Xist* transcript in undifferentiated cells. The hyperacetylated domain was not seen in male cells or in female XT67E1 cells, a mutant cell line heterozygous for a partially deleted *Xist* allele and in which an increased number of cells fail to undergo X inactivation. The hyperacetylation upstream of *Xist* was lost by day 7 of differentiation, when X inactivation was essentially complete. Wild-type cells differentiated in the presence of the histone deacetylase inhibitor Trichostatin A were prevented from forming a normally inactivated X, as judged by the frequency of under-acetylated X chromosomes detected by immuno-fluorescence microscopy. Mutant XT67E1 cells, lacking hyperacetylation upstream of *Xist*, were less affected. We propose that (i) hyperacetylation of chromatin upstream of *Xist* facilitates the promoter switch that leads to stabilization of the *Xist* transcript and (ii) that the subsequent deacetylation of this region is essential for the further progression of X inactivation.

Keywords: dosage compensation/embryonic stem cells/gene silencing/histone acetylation/X-inactivation

Introduction

In eutherian mammals, most genes on one of the two X chromosomes in females are transcriptionally silenced at around the blastocyst stage of development. This process serves to equalize the levels of X-linked gene products in females with two X chromosomes, and males with only one (reviewed by Heard *et al*., 1997). Mutations that disrupt this dosage compensation mechanism are lethal early in embryonic development (Panning and Jaenisch, 1996; Marahrens *et al*., 1997). Apart from its relatively low overall level of transcriptional activity, the inactive X (Xi) differs from its active homologue (Xa) in various ways. These include replication late in S-phase (Takagi *et al*., 1982; Boggs and Chinault, 1994; Hansen *et al*., 1996a), underacetylation of core histones (Jeppesen and Turner, 1993; Belyaev *et al*., 1996a), enrichment in the histone variant macro-H2A (Constanzi and Pehrson, 1998) and increased methylation of CpG islands (Norris *et al*., 1991; Tribioli *et al*., 1992). Xi is often referred to as condensed and heterochromatic, although detailed analysis of its morphology by confocal microscopy revealed that Xi and Xa differ in shape rather than degree of compaction (Eils *et al*., 1996). Once X inactivation is complete, the process is difficult to reverse and transcriptional reactivation of genes on Xi is a rare event, even in the presence of inhibitors that disrupt one of the identified components of the inactivation process such as DNA methylation (Gartler and Goldman, 1994). It seems likely that there is redundancy built into X inactivation such that the loss of any single component is not sufficient, in itself, to cause reactivation.

While the properties of Xi have been well defined, the molecular mechanisms that convert just one of the two female X chromosomes into the mature Xi remain unclear, as do the genetic and developmental controls that act upon them. Studies of structurally altered X chromosomes have defined a single region in both mouse and human X chromosomes that is essential for the initiation of inactivation (Rastan, 1983; Brown *et al*., 1991). Designated the X inactivation centre (*Xic*), this region is several hundred kilobases in length and contains at least six genes (Heard *et al*., 1997). A diagram of the mouse *Xic* showing the locations of the five genes examined in the present report is shown in Figure 1. One of the *Xic* genes, designated *Xist* in mice and *XIST* in humans, is so far unique in being expressed only from Xi (Borsani *et al*., 1991; Brown *et al*., 1991, 1992; Brockdorff *et al*., 1992). The *Xist* transcript does not have any significant protein-coding potential, but remains in the nucleus where it co-localizes with Xi (Clemson *et al*., 1996). The role played by *Xist* in the process of X inactivation remains uncertain, but is clearly crucial. Deletions within *Xist* prevent X inactivation *in cis* (Penny *et al*., 1996; Marahrens *et al*., 1997, 1998) while *Xist* transgenes can induce at least some of the properties of inactive chromatin when inserted in multiple copies into autosomes (Lee *et al*., 1996; Lee and Jaenisch, 1997; Herzing *et al*., 1997; but see also Heard *et al*., 1999). An increase in *Xist* transcript levels coincides with the onset of X inactivation during development (Kay *et al*., 1993). This is not due to an increased rate of transcription, and in fact *Xist* is efficiently transcribed from both X

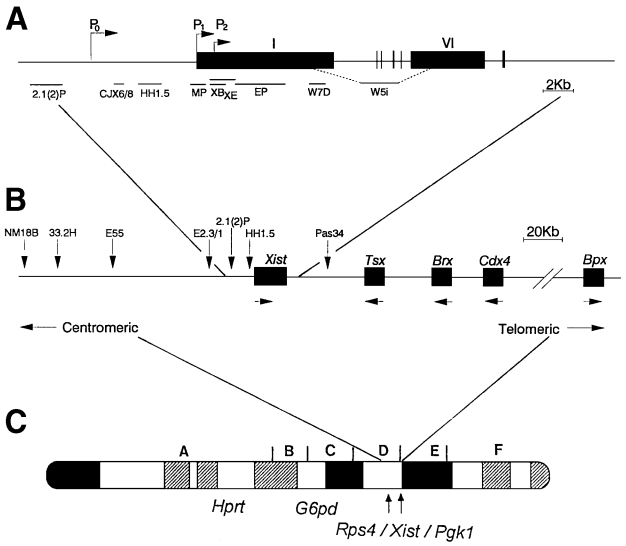

Fig. 1. Chromosome location and composition of the *Xic* region and the *Xist* gene. (**A**) The mouse *Xist* gene, showing the exons (dark boxes I and VI) and the three promoters, P_0, P_1 and P_2. The DNA probes used in this study and their approximate sizes are represented (horizontal lines). W5i is a cDNA probe incorporating DNA from exons I–VI as shown (dashed lines). (**B**) The mouse *Xic*, showing the genes examined in this study together with the directions in which they are transcribed (horizontal arrows). The locations of the DNA probes used to examine regions adjacent to *Xist* are shown by vertical arrows. Regions that are differentially methylated in male and female ES cells are found adjacent to *Xist* promoter P_1 and in the region identified by probe Pas34 (Courtier *et al*., 1995). (**C**) The mouse X chromosome, showing the major G-bands, regions A–F and some of the genes examined in the present report.

chromosomes prior to the onset of inactivation. Rather, a promoter switch from P_0 to P_1/P_2 (Figure 1) on one X chromosome only (Johnston *et al*., 1998) leads to stabilization of the *Xist* transcript and coating of the selected chromosome *in cis* (Panning *et al*., 1997; Sheardown *et al*., 1997).

Xi is now just one of several examples of the association between histone underacetylation and transcriptionally quiescent chromatin. In some cases, transcriptional repression of specific promoters in transient transfection experiments has been shown to involve recruitment of histone deacetylases (HDACs) to the promoter. In mammals, for example, repression by Rb protein (Brehm *et al*., 1998; Luo *et al*., 1998; Magnaghi-Jaulin *et al*., 1998), unliganded retinoic acid receptors (Nagy *et al*., 1997) and mad–max heterodimers (Hassig *et al*., 1997; Laherty *et al*., 1997) involves, in all cases, the formation of complexes containing the repressor protein itself, one or more HDACs, and co-repressors such as N-Cor and SMRT (reviewed by Pazin and Kadonaga, 1997; Struhl, 1998). HDAC-1 is also involved in the mechanism by which genes are regulated in response to the Notch signalling pathway (Kao *et al*., 1998) which controls decisions about cell fate during development of many different organisms (Robey, 1997). Histone deacetylation has recently been linked to a second mechanism of genetic silencing, DNA methylation, by the demonstration of a functional association between histone deacetylases and the methyl-CpG-binding protein MeCP2 (Jones *et al*., 1998; Nan *et al*., 1998). It seems that the enzymatic activity of the HDAC component of these complexes is crucial. Deacetylase inhibitors such as Trapoxin and Trichostatin A (TSA) abrogate repressive activity, as does mutation of an HDAC-1 histidine residue presumed to be part of the active site (Hassig *et al*., 1998).

In an attempt to define the role of H4 acetylation in X inactivation, we have previously used cultured mouse embryonic stem cells (ES cells) as a model system to place H4 deacetylation into a sequence of events leading to the formation of a single Xi (Keohane *et al*., 1996). ES cells are derived from the inner cell mass of mouse blastocysts and can differentiate in culture to form embryoid bodies containing a wide variety of cell types (Keller, 1995). Undifferentiated female ES cells have two active X chromosomes, but within one week of the induction of differentiation, in the majority of cells, one of these chromosomes has taken on most of the properties associated with Xi. We found that whereas late replication, transcriptional silencing and increased levels of *Xist* RNA were all in place by day 2 of differentiation, overall deacetylation of Xi, detectable by immunofluorescence microscopy, was first detected only by day 4 (Keohane *et al*., 1996). Thus, if histone deacetylation is involved in the earlier stages of X inactivation, then it must be localized to specific genes or chromosome domains too small to be detected by immunocytochemistry.

Here we describe the use of a chromatin immunoprecipitation approach to assay the level of H4 acetylation along specific genes and gene subregions during ES cell differentiation. The results support the conclusion that changes in H4 acetylation along the *Xist* promoter region form an essential component of the developmental switch that leads to X inactivation.

Results

Pattern of histone acetylation in cultured ES cells resembles that of adult cell types

Histones extracted from ES cell nuclei were resolved by electrophoresis on acid/urea/Triton (AUT) gels. The frequency of acetylated H4 isoforms revealed by Coomassie Blue staining was similar to that seen in many other cultured cell types (Figure 2A, CB–). Growth of undifferentiated ES cells for up to 6 h in the presence of TSA, an inhibitor of histone deacetylases (Yoshida *et al*., 1990), leads to a dramatic increase in H4 acetylation such that the di-, tri- and tetra-acetylated isoforms ($H4Ac_{2–4}$), barely detectable in untreated cells, become the most prominent (Figure 2A, CB+). Thus, the great majority of H4 molecules in ES cells must go through one or more acetylation–deacetylation cycles within 6 h. We have shown previously that the rate of acetate turnover falls after the first few days of differentiation (Keohane *et al*., 1998). Western blotting from AUT gels and immunostaining with antisera to H4 acetylated at specific lysines show that the mono-acetylated isoform ($H4Ac_1$) is labelled strongly with antibodies to H4Ac16, but only weakly (relative to the most acetylated isoforms) with antibodies to H4 acetylated at lysines 12, 8 and 5. These lysines, along with lysine 16, are all acetylated in $H4Ac_{2–4}$ (Figure 2A). H4 acetylated at lysine 5 is barely detectable in untreated cells, appearing in the more highly acetylated isoforms only after TSA treatment (Figure 2A, H4Ac5). This pattern of H4 acetylation is essentially the same as that seen in adult mammalian cell types (Turner, 1991).

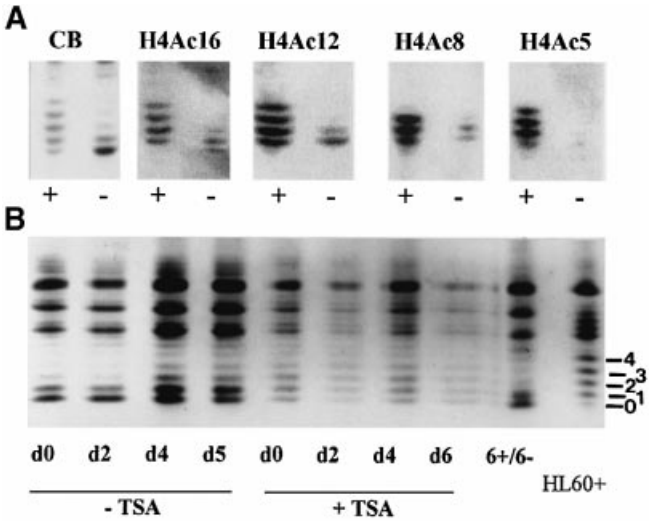

Fig. 2. The effect of TSA on patterns of histone H4 acetylation in ES cells. (**A**) Histones were extracted from undifferentiated female ES cells (line LF2) and resolved by electrophoresis on AUT gels. Gels were stained with Coomassie Blue (CB) or transferred to nylon filters and immunolabelled with antibodies to H4 acetylated at lysines 16 (H4Ac16), 12 (H4Ac12), 8 (H4Ac8) or 5 (H4Ac5). Cells were either untreated (–) or grown for 6 h in medium with 100 ng/ml TSA (+TSA) prior to extraction. Note that the antibodies to H4Ac8 label the tetra-acetylated (slowest migrating) H4 isoform inefficiently due to disruption of the epitope by acetylation of H4 lysine 5 (Turner *et al*., 1989). (**B**) Histones extracted from female ES cells (line LF2) differentiated for up to 6 days in the continuous presence or absence of 5 ng/ml TSA were resolved by electrophoresis on AUT gels and stained with Coomassie Blue. Undifferentiated cells (d0) were grown for 2 days with or without 5 ng/ml TSA prior to the initiation of differentiation. The inhibitor significantly increases the levels of the most highly acetylated isoforms, in relation to the non-acetylated and mono-acetylated isoforms, at all stages of differentiation tested. In cells grown for 6 days with and 6 days without TSA (6+/6–), the level of acetylation returned to that seen in untreated cells.

Chromatin upstream of Xist is hyperacetylated in female, but not male, ES cells

Chromatin fragments from mouse ES cell nuclei were immunoprecipitated with affinity-purified antibodies to acetylated H4. To confirm the specificity of immunoprecipitation, histones in the antibody-bound and unbound fractions were analysed by SDS–PAGE and Western blotting as described previously (O'Neill and Turner, 1995). In all experiments presented here, this analysis showed depletion of the unbound fraction in H4 acetylated at the appropriate lysine residue and a parallel enrichment in the bound fraction. To examine levels of acetylation along specific genes and gene subregions, equal amounts of DNA, based on ^{3}H-thymidine counts, from the bound (B) and unbound (UB) fractions, were applied as serial dilutions to nylon filters and hybridized with ^{32}P-labelled DNA probes. Hybridization levels were quantified by PhosphorImaging and levels of acetylation expressed as the B/UB ratio. Representative hybridizations are shown in Figure 3.

Antibody-bound and unbound fractions from undifferentiated (d0) and differentiated (d7) female ES cells were tested with DNA probes to coding and non-coding regions within the *Xic*, particularly within and around the *Xist* gene. The locations of the genes and chromosome regions tested are shown in Figure 1. Results for antibody R232/8 are presented in Figure 4A. These results, like those shown

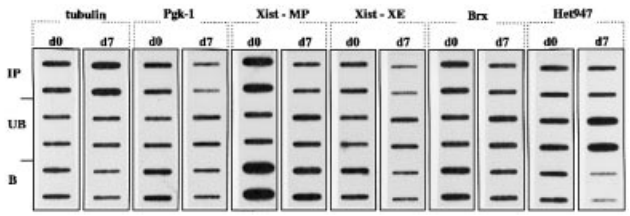

Fig. 3. Slot blots of acetylated (B) and non-acetylated (UB) chromatin fractions from undifferentiated (d0) and differentiated (d7) ES cells. Equal amounts of DNA from input (IP), unbound (UB) and antibody-bound (B) chromatin fractions were applied as duplicate serial dilutions (only one dilution is shown) to nylon filters and hybridized with ^{32}P-labelled DNA probes as indicated. All the d0 hybridizations were done on the same filter, as were all the d7 hybridizations. The images shown are autoradiographs.

in Figures 4B, 4C, 5A and 5B, are each derived from a single immunoprecipitation experiment and values for each probe are thus directly comparable. All experiments shown here were carried out at least twice with consistent results. The *Xic* genes *Tsx*, *Brx*, *Bpx* and *Xist* exons I–VI (Figures 1 and 4A) all consistently gave B/UB ratios in the range 1.5–3.0, as did genes that map outside the *Xic*, such as *Hprt*, *Pgk-1* (Figure 5A) and *Rps4* (Figure 6). This level of acetylation was comparable to that of the autosomal genes tested, namely α-*tubulin*, β-*actin* and *Aprt*, (Figure 5A, Figure 6 and results not shown). The only possible exception to the consistently high level of H4 acetylation on X-linked genes is *Cdx4*, which shows a lower level of H4 acetylation in undifferentiated cells than other X-linked genes, although the very low level in Figure 4A is exceptional (c.f. results in Figures 5A and 6). *Cdx4* also showed a less pronounced drop in acetylation on differentiation than other X-linked genes (see below). It may be significant that *Cdx4* is not expressed in ES cells (P.Avner and C.Chureau, unpublished).

The most striking feature of H4 acetylation across the *Xic* was the very high level consistently present in the region between the *Xist* promoter P_1 and sequences detected by probe NM18B, 120 kb 5′ of the transcription start site. A sharp drop in acetylation levels occurs between the *Xist* promoter P_1 (probe MP) and the 3′ region of exon I (probes XB, XE and EP, see Figure 1). The results in Figure 5A show this particularly well. B/UB ratios in this *Xist* upstream region were consistently around 5 with chromatin from undifferentiated female cells. We have not detected levels of H4 acetylation as high as this for any genes in any other cell type. H4 hyperacetylation at this level was not detected in the promoter regions of the *Bpx* (Figure 4A) or *Hprt* genes (not shown), and thus is not a general property of X-linked gene promoters in female cells. This localized hyperacetylation was seen consistently with both LF2 and PGK12.1 cell lines (Figures 4A and 5A, respectively), as was the sharp drop in acetylation between the P_1 promoter region and exon I. Results from four independent experiments with LF2 cells are summarized in Table I. Absolute values for B/UB ratios varied up to 2-fold between experiments, but relative values for different regions were much less variable and were always consistent with the distribution of acetylation levels shown in Figures 4A and 5A.

Immunoprecipitations with antisera to H4 acetylated at lysines 5 (R41/5) and 12 (R101/12) showed that the

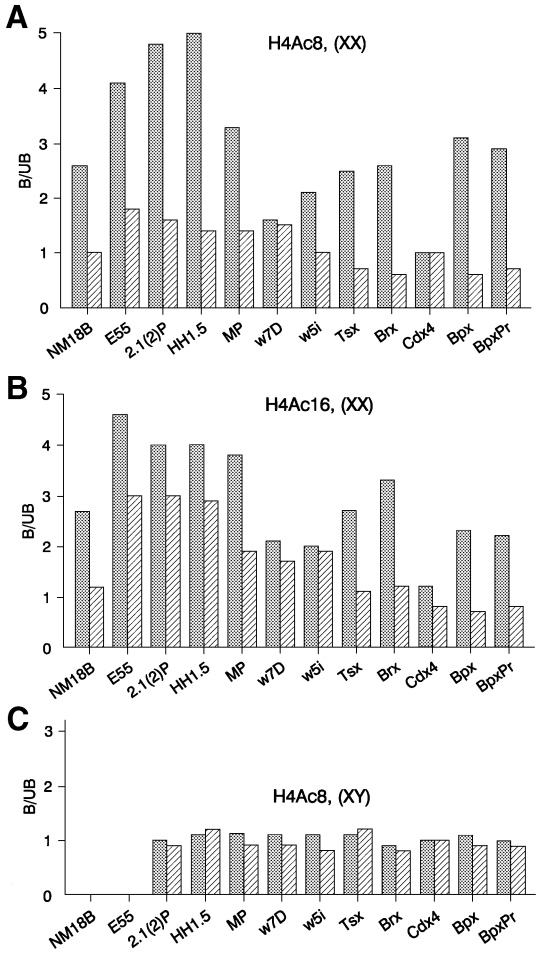

Fig. 4. Levels of H4 acetylation along *Xist* and other genes within the *Xic* of mouse ES cells. Chromatin fragments from XX female [(**A** and **B**) cell line LF2] and XY male [(**C**) cell line EFC-1] ES cells were immunoprecipitated with antibodies to histone H4 acetylated at lysine 8 [R232/8, (A and C)] or 16 [R14/16, (B)]. Cells were undifferentiated (shaded bars) or differentiated for 7 days (hatched bars) prior to chromatin preparation. The level of H4 acetylation across the *Xic* is expressed as the distribution of specific DNA sequences (assayed by hybridization with the indicated probes) between the antibody bound (i.e. acetylated) and unbound (i.e. non-acetylated) chromatin fractions (B/UB ratio). Probes Tsx, Brx, Cdx4 and Bpx are all to the coding regions of the corresponding genes. Probe BpxPr is to the *Bpx* promoter. The locations of all probes are shown in Figure 1.

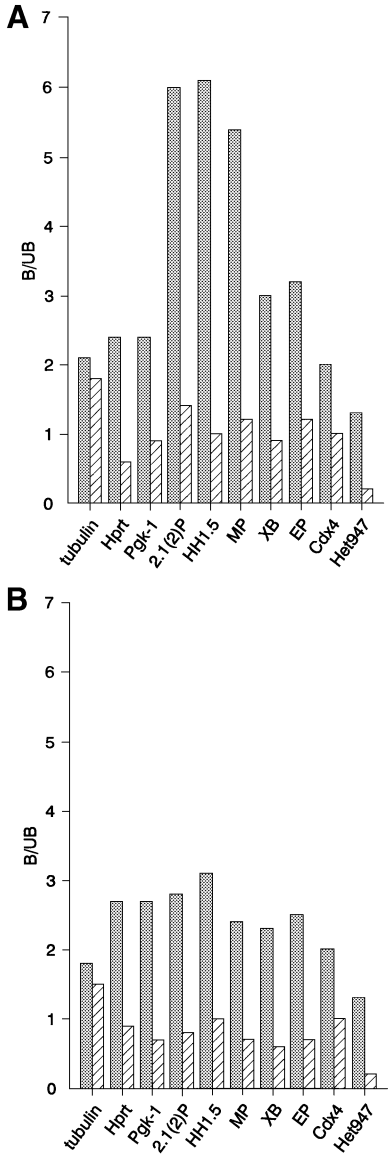

Fig. 5. Levels of H4 acetylation along *Xist* and other X-linked genes in (**A**) PGK12.1 (wild type) and (**B**) XT67E1 (mutant) female ES cells. Levels of H4 acetylation (B/UB ratio) are shown for the coding regions of an autosomal gene (α-*tubulin*), the X-linked genes *Hprt*, *Pgk-1* and *Cdx4*, for upstream [2.1(2)P, HH1.5], promoter (MP) and transcribed (XB, EP) regions of *Xist*, and for centric heterochromatin (Het947). Acetylation levels were determined by immunoprecipitation, with antibodies to acetylated H4 (R232/8), of chromatin fragments prepared from undifferentiated cells (shaded bars) and cells allowed to differentiate for 7 days (hatched bars).

distribution across the *Xic* of H4 acetylated at these lysines reflected that of H4Ac8 (results not shown). As all three antibodies recognize the more highly acetylated H4 isoforms, $H4Ac_{2–4}$ (Figure 2A), the distribution shown in Figure 4A is likely to reflect the distribution of highly acetylated H4 rather than of H4 isoforms acetylated independently at individual lysines. Immunoprecipitation with antibodies to H4Ac16, which recognize primarily the most common, mono-acetylated isoform (Figure 2A), shows that mono-acetylated H4 has the same distribution pattern as the other acetylated isoforms (Figure 4B). Thus, chromatin upstream of *Xist* is enriched in all acetylated H4 isoforms.

In undifferentiated male ES cells, levels of H4 acetylation across the *Xic* never showed this variation. In male cells, X-linked genes within and outside the *Xic* and autosomal genes all had comparable levels of H4 acetylation, with B/UB ratios ranging from 1.0 to 1.5 (Figure 4C and results not shown). Whether the lower overall level of H4 acetylation associated with X-linked regions in male ES cells is a consistent characteristic remains to be established, although it is interesting to note that H4 acetylation along the autosomal genes tested so far is comparable in male and female cells (L.P.O'Neill and B.M.Turner, unpublished). The important point here is that the region upstream of *Xist* in male ES cells does not show any relative increase in H4 acetylation.

Levels of acetylated H4 associated with X-linked genes and regions within the *Xic* fell 2- to 4-fold over 7 days of differentiation in female, but not male, cells (compare day 0 and day 7 values in Figure 4A and C with the mean

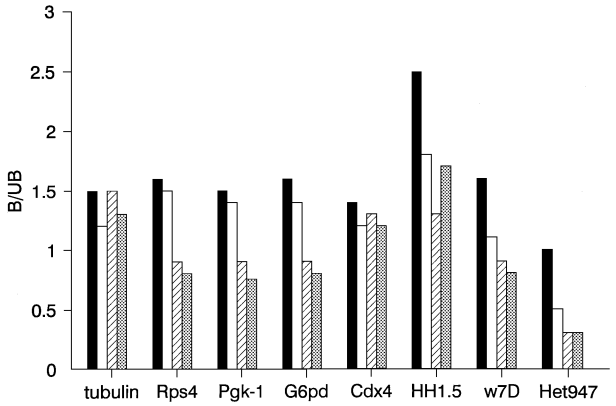

Fig. 6. The timing of H4 deacetylation in differentiating female ES cells. Levels of H4 acetylation (B/UB ratio) are shown for the coding regions of an autosomal gene (α-*tubulin*), the X-linked genes *Rps4*, *Pgk-1*, *G6pd* and *Cdx4*, for upstream (HH1.5) and transcribed (W7D) regions of *Xist*, and for centric heterochromatin (Het947). Acetylation levels were determined by immunoprecipitation with antibodies to acetylated H4 (R101/12) of chromatin fragments prepared from undifferentiated cells (solid bars) and cells allowed to differentiate for 1 day (white bars), 4 days (hatched bars) and 7 days (grey bars).

Table I. Levels of H4 acetylation (B/UB ratios) at selected chromosome regions calculated from four independent immunoprecipitation experiments with antibody to H4Ac8 and chromatin from LF2 cells

Gene (probe)	H4 acetylation (B/UB ratio)[a]	
	Day 0	Day 7
Xist, promoter (MP)	4.6 ± 1.0	1.6 ± 0.68
Xist, exon I (XE)	2.2 ± 0.54	1.1 ± 0.48
Pgk-1, coding	2.3 ± 0.74	0.9 ± 0.41
α-*tubulin*, coding	1.4 ± 0.69	1.2 ± 0.54
	Relative acetylation[b]	
MP/XE	2.1 ± 0.25	1.5 ± 0.17
Pgk-1/XE	1.0 ± 0.10	0.9 ± 0.18

[a]Mean ± standard deviation.
[b]For each of four experiments, levels of acetylation of the *Xist* promoter (probe MP) and the *Pgk-1* gene were calculated relative to the acetylation of *Xist* exon I (probe XE) by dividing the B/UB ratio for each region by the B/UB ratio for XE. Values are the mean ± standard deviation.

values in Table I). In female cells, the fall in acetylation was similar for genes within and outside the *Xic* (Figure 5A) but considerably greater for the hyperacetylated domain upstream of *Xist* (Table I). By day 7, acetylation in this region was only slightly above that along *Xist* itself and other X-linked genes. In contrast, levels of H4 acetylation along autosomal genes changed little on differentiation of male or female cells (Figure 5, Table I and results not shown). These findings indicate that the changes in H4 acetylation along X-linked genes in female cells are due to X-inactivation and the associated deacetylation of genes of one of the two X chromosomes, rather than to ES cell differentiation per se.

Levels of H4 acetylated at lysine 16 (primarily the mono-acetylated isoform) on the *Tsx*, *Brx* and *Bpx* genes showed the same 2- to 4-fold drop after 7 days of differentiation as the more highly acetylated isoforms, whereas levels across the *Xist* upstream and coding regions fell less (Figure 4B). This may be because levels of the mono-acetylated isoform decline more slowly in this particular region during differentiation. This difference shows that the pattern of H4 acetylation across the *Xist* domain does not simply reflect the differentiation-related changes in other regions of the X chromosome, but is independently regulated.

Deacetylation of X-linked genes in female cells begins early in differentiation

To define the timecourse of deacetylation of specific X-linked genes, we immunoprecipitated chromatin from female ES cells harvested after 1, 4 and 7 days of differentiation. Illustrative results (antibody R101/12) are shown in Figure 6. Acetylation of X-linked genes such as *Rps4* and *Pgk-1* and of the coding and upstream region of *Xist* had fallen little by day 1, but fell progressively thereafter, reaching about half their initial levels by days 4–7 (Figure 6). Acetylation along autosomal genes (α-*tubulin* is shown) did not change. Male ES cells tested in parallel after 3 and 7 days of differentiation showed no significant deacetylation of X-linked genes or the *Xist* upstream region (not shown, but see Figure 4C). We conclude that deacetylation of the coding regions of X-linked genes and of the upstream region of *Xist* is unlikely to precede the increase in *Xist* RNA levels, late replication and gene silencing, all of which have begun by day 2 (Keohane *et al*., 1996; Sheardown *et al*., 1997). It may be concurrent with these events. It certainly occurs before the overall deacetylation of Xi, which is first detected in a small proportion of cells only by day 4 (Keohane *et al*., 1996).

In undifferentiated ES cells, even centric heterochromatin showed a significant level of acetylation (probe Het947 in Figures 5 and 6), consistent with the results of indirect immunofluorescence microscopy on ES cell metaphase chromosomes (Keohane *et al*., 1996). We have not seen comparable acetylation of centric heterochromatin in any adult cell type and it would seem to be a characteristic of embryonic cells. A 3- to 5-fold drop in the acetylation of centric heterochromatin occurred on differentiation of both male (not shown) and female cells (Figures 5 and 6), and by day 4 it had reached the low level characteristic of adult cells (O'Neill and Turner, 1995; Johnson *et al*., 1998).

Chromatin upstream of Xist is not hyperacetylated in cells heterozygous for a partial deletion of Xist exon I

In female ES cells, the immunoprecipitation results must reflect the combined levels of H4 acetylation along the two *Xist* alleles. As a first step towards distinguishing between H4 acetylation along these two alleles, we used the female mutant ES cell line XT67E1. These cells were derived from the female PGK12.1 cell line by targeted deletion, and one of their two *Xist* alleles lacks promoters P_1 and P_2 and most of exon I (Penny *et al*., 1996). On the deleted chromosome, promoter P_0 is intact and active prior to the onset of X inactivation (Johnston *et al*., 1998), but only the X carrying the wild-type *Xist* allele can undergo inactivation (Penny *et al*., 1996). By immunoprecipitating chromatin from XT67E1 cells and hybridizing with probes to the deleted region, one can measure levels

of H4 acetylation specifically on Xi (i.e. on the homologue carrying the active *Xist* allele).

With chromatin from undifferentiated and differentiated XT67E1 cells, levels of H4 acetylation associated with X-linked genes, autosomal genes and centric heterochromatin were essentially the same as those found with chromatin from PGK12.1 cells, as was the fall in acetylation after 7 days of differentiation (compare Figure 5A and B). The level of H4 acetylation along the single copy of *Xist* exon I in XT67E1 cells was not significantly different to that on the coding regions of other X-linked genes, either before or after differentiation. It seems that *Xist* exon I on Xi (i.e. the active allele) retains a moderate level of acetylation. Whether, conversely, the inactive *Xist* allele on Xa becomes deacetylated in wild-type female cells remains to be established. However, the most striking difference between PGK12.1 and XT67E1 mutant cells was in the level of H4 acetylation upstream of *Xist*. In XT67E1 cells there was no sign of the hyperacetylation of this region invariably seen in normal female ES cells [compare the results with the two upsteam probes 2.1(2)P and HH1.5 in Figure 5A and B]. This was true for both H4Ac8 (Figure 5B) and H4Ac16 (not shown). The absence of H4 hyperacetylation in this region had been seen previously only in male ES cells (Figure 4C). The fact that, in XT67E1 cells, neither the normal X nor the X carrying the exon I deletion was hyperacetylated upstream of *Xist* shows that such hyperacetylation is not essential for an X chromosome to become inactivated. The results also show that hyperacetylation of the region upstream of *Xist* does not occur in cells that possess only one, intact *Xist* gene. We return to these conclusions and their possible interpretation in the Discussion.

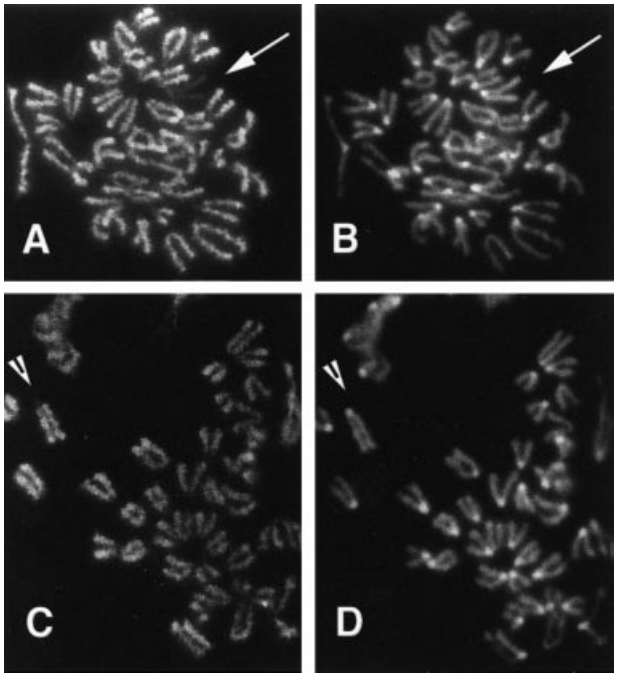

Fig. 7. Immunofluorescence labelling showing the effects of TSA on H4 acetylation along metaphase chromosomes. Metaphase chromosome spreads were prepared from ES cells differentiated for 6 days in the absence of TSA (**A** and **B**) or 9 days in the presence of 5 ng/ml TSA (**C** and **D**). Spreads were immunostained with antibodies to H4 acetylated at lysine 8 (antiserum R232/8, A and C) and counterstained with DAPI (B and D). A pale-staining (underacetylated) X chromosome (shown by arrows in A and B) was not seen in spreads from TSA-treated cells (C), but deacetylation of centric heterochromatin occurred as usual (shown by arrowheads in C and D).

ES cells continue to grow and differentiate in low concentrations of the deacetylase inhibitor TSA

In order to address the functional significance of the differentiation-related deacetylation of the *Xist* upstream region in female cells, we examined the effect on X inactivation of treatment with inhibitors of histone deacetylation. The fungal antibiotic TSA is an effective inhibitor of histone deacetylases at nanomolar concentrations and induces a rapid increase in overall levels of histone acetylation when added to cell cultures (Yoshida *et al*., 1990). It is also causes growing cells to arrest in G_1 and G_2 (Yoshida and Beppu, 1988). Preliminary experiments to find a concentration of TSA that increased histone acetylation levels, while having a minimal effect on the growth of cultured ES cells, showed 5 ng/ml to be the best compromise. Cells grown in the continuous presence of 5 ng/ml TSA showed a clear increase in the overall level of acetylated H4 that was maintained over at least the first few days of differentiation (Figure 2B, compare lanes d0–d5, –TSA, with lanes d0–d6, +TSA). After removal of TSA, levels of acetylation fell to those found in untreated cells (Figure 2B, track labelled 6+/6–). Cells grown in TSA at 5 ng/ml had only a slightly reduced growth rate, as measured by the cell-doubling time, and formed embryoid bodies with very similar morphologies (they tended to be slightly smaller) to those in control cells.

TSA prevents global H4 deacetylation on Xi in wild-type ES cells but not XT67E1 mutants

In PGK12.1 and LF2 female ES cells, an underacetylated X was first detected in metaphase cells by indirect immunofluorescence after 4 days of differentiation, with the maximum frequency of underacetylated chromosomes being reached by day 7 (Figures 7A and B, and 8A). In cells differentiated in the presence of 5 ng/ml TSA, an underacetylated chromosome was never detected, even by day 12 of differentiation, which was the latest time point tested (Figures 7C and D, and 8A). If the TSA concentration was reduced to 2 ng/ml, occasional chromosome spreads with a single, pale-staining chromosome were seen from day 4 onwards (Figure 8A), but such spreads never reached the frequency seen in cells grown in TSA-free medium. This is consistent with the finding that TSA at 2 ng/ml had only a small effect on bulk H4 acetylation, as measured by electrophoresis on AUT gels.

If cells were grown in the presence of TSA for several days before being transferred to TSA-free medium, within 4 days the frequency of cells with a pale-staining chromosome approached that seen in control cells (Figure 8A). Thus, the prevention of the global deacetylation of Xi by TSA is dependent on the continued presence of the inhibitor. It was interesting to note that TSA did not prevent the differentiation-dependent deacetylation of H4 in centric heterochromatin. Metaphase chromosome spreads from ES cells grown in 5 ng/ml TSA for up to 4 days and labelled with antisera to acetylated H4 showed the same progressive reduction in labelling of the centromeric regions as spreads from control cells (compare Figure 7A and C). The mechanisms governing deacetylation of H4 in centric heterochromatin and Xi are clearly different.

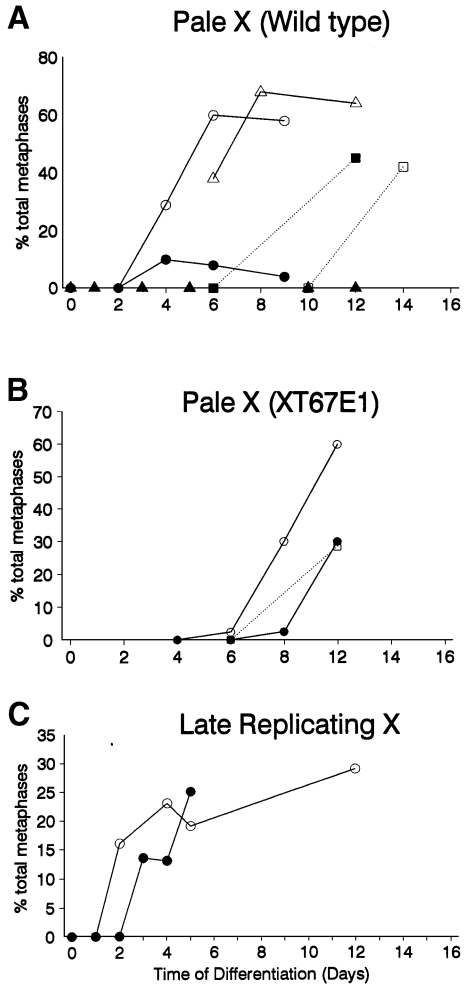

Fig. 8. Effect of TSA on the timing of events involved in X inactivation in female ES cells. (**A**) Frequency of metaphase chromosome spreads with a pale-staining (i.e. underacetylated) X chromosome after immunostaining with antibodies to acetylated H4. ○, △ wild-type cells (LF2 and PGK12.1, respectively) grown in the absence of TSA, ● cells grown in medium containing 2 ng/ml TSA, ▲ cells grown in medium containing 5 ng/ml TSA, ■ cells grown for 6 days with 5 ng/ml TSA then for 6 days without TSA, and □ cells grown for 10 days with 5 ng/ml TSA then for 4 days without TSA. (**B**) Frequency of metaphase chromosome spreads from XT67E1 cells with a pale-staining (i.e. underacetylated) X chromosome after immunostaining with antibodies to acetylated H4. ○ cells grown in the absence of TSA, ● cells grown in medium containing 5 ng/ml TSA, and □ cells grown in medium containing 5 ng/ml TSA for 6 days then for 6 days without TSA. Note that the appearance of an underacetylated X chromosome is delayed in these cells compared with the wild-type PGK12.1 cells shown in (A). (**C**) Frequency of metaphase chromosome spreads from LF2 female ES cells with a late-replicating (BrdU-labelled) X chromosome. ○ control cells, ● cells grown in medium containing 5 ng/ml TSA.

The effect of 5 ng/ml TSA on underacetylation of Xi is much less pronounced in XT67E1 mutant cells lacking the region of H4 hyperacetylation upstream of *Xist*. In these cells, a pale-staining (underacetylated) X chromosome is first detected only after 6–8 days of differentiation (Figure 8B), i.e. later than in wild-type LF2 and PGK12.1 cells. In addition, the timing of the first appearance of the underacetylated X and its frequency were much less sensitive to TSA in XT67E1 than in wild-type cells (compare Figure 8A and B).

In contrast to its complete inhibition of the deacetylation of Xi in wild-type female ES cells, 5 ng/ml TSA slightly delayed, but did not prevent, the appearance of a late replicating X chromosome (Figure 8C). In untreated cells, a significant number of metaphase spreads with a late replicating X (as measured by BrdU incorporation) was detected by day 2 of differentiation. In the presence of TSA, no such spreads were detectable until day 3, but by days 5 and 6 the frequency of spreads with a late replicating X was equal to or greater than that seen in untreated cells.

Discussion

We have used immunoprecipitation of chromatin from cultured mouse embryonic stem cells to examine levels of H4 acetylation along *Xist* and other X-linked genes both before and after the onset of X inactivation. We find that in undifferentiated female ES cells, a region extending up to 120 kb upstream of the *Xist* somatic promoters is hyperacetylated. Levels of H4 acetylation in this region are higher than we have observed previously in genes and chromosome domains from a variety of cell types (O'Neill and Turner, 1995; Johnson *et al*., 1998). The acetylated domain upstream of *Xist* in female cells has a sharp 3′ boundary, beginning at and including the somatic promoter P_1. The 5′ boundary has not yet been defined exactly. We find no evidence of H4 hyperacetylation upstream of *Xist* in male ES cells or in the mutant female cell line XT67E1, heterozygous for a partial deletion of *Xist* exon I and the somatic promoter region. Thus, hyperacetylation upstream of *Xist* is seen only in cells carrying two functional copies of *Xist*. Differentiation of female ES cells is accompanied by loss of H4 hyperacetylation upstream of *Xist*. This finding is consistent with the pattern of H4 acetylation around Xist in adult mouse tissues (McCabe *et al*., 1999).

The functional significance of the hyperacetylated domain

Using the same chromatin immunoprecipitation approach as used here, Hebbes *et al*. (1994) defined a hyperacetylated chromatin domain extending across 33 kb of the chicken β-globin locus and whose boundaries corresponded closely to those defined by DNase I sensitivity. Hyperacetylation extended across the coding regions of the four genes within the locus and the non-transcribed DNA in between. Comparable levels of acetylation across the domain were seen in erythrocytes from chick embryos at 5 and 15 days of development, and did not relate to the different patterns of globin gene expression at these two developmental stages. However, the domain was not hyperacetylated in those cell lineages in which the globin genes would not be expressed. It was suggested that the function of histone hyperacetylation across the β-globin domain was to maintain an open chromatin conformation (detectable as a region of DNase I sensitivity) and thereby facilitate binding of the various factors necessary for gene switching through development. As discussed below, a similar function can be proposed for the H4 hyperacetylation upstream of *Xist*. However, whereas in the case of the β-globin domain the hyperacetylation facilitates the developmentally regulated switching between promoters of different genes, in the *Xist* upstream domain only one gene, *Xist* itself, so far appears to be involved. No other functional genes have yet been identified within the hyperacetylated

domain, although sequencing is not yet complete (our unpublished work).

It may be significant that the 3′ boundary of the hyperacetylated domain falls at or close to the *Xist* somatic promoters. Hyperacetylation of the *Xist* coding region is not seen and is clearly not necessary for its efficient transcription. It is interesting that in undifferentiated female ES cells, the *Xist* somatic promoter region has both a high level of H4 acetylation and a low level of CpG methylation, relative to male cells (Norris *et al*., 1994; Penny *et al*., 1996). This raises the possibility that proteins that bind selectively to methylated CpG residues may be involved in targeting deacetylases to the promoter region during differentiation (Nan *et al*., 1998).

If the differentiation-dependent deacetylation of the *Xist* promoter region, and of other X-linked genes, is functionally significant for X inactivation, one would predict that inhibitors of histone deacetylation such as TSA would prevent the appearance of at least some of the changes associated with X inactivation. This is exactly what is observed. TSA, at a concentration that induces increased H4 acetylation while having only a minimal effect on cell growth, reversibly inhibits global H4 deacetylation. Initial experiments suggest that it also inhibits other aspects of X inactivation, namely co-localization of Xi and *Xist* RNA and silencing of X-linked genes (A.M.Keohane, A.L.Barlow and S.M.Duthie, unpublished). Significantly, it does not prevent late replication. It has been noted by others that late replication is not an essential component of the X inactivation process, although allocyclic replication may be (Yoshida *et al*., 1993). We have shown previously that in ES cells, late replication and deacetylation of Xi are not mechanistically linked (Keohane *et al*., 1996). TSA did not prevent the differentiation-dependent deacetylation of centric heterochromatin, providing further evidence that different mechanisms regulate the deacetylation of constitutive and facultative heterochromatin during development.

A role for H4 hyperacetylation in X chromosome counting and Xist promoter switching

The increased levels of H4 acetylation upstream of *Xist* are not related to the transcriptional activity of the P_0 promoter. This promoter is equally active in male cells, where the region in which it is located is not highly acetylated, and in female cells, where it is (Johnston *et al*., 1998). The fact that the hyperacetylation upstream of *Xist* is seen neither in male cells nor in female XT67E1 cells heterozygous for a deletion of *Xist* exon I shows that hyperacetylation occurs only in cells with at least two complete copies of *Xist*; in other words, it is dependent upon a counting mechanism that senses the number of *Xist* genes. Evidence has been presented previously (Herzing, 1997) that the *Xist* gene is an integral component of an X chromosome counting mechanism, although recent findings indicate that it is not, in itself, sufficient (Heard *et al*., 1999).

We suggest that hyperacetylation of the region 5′ of *Xist* facilitates the promoter switch that leads to stabilization of the *Xist* transcript. In general terms, it could do this simply by maintaining a more open chromatin structure that would facilitate binding of the factors necessary for the promoter switch to occur. The fact that hyperacetylation occurs, in normal circumstances, only in those cells in which the promoter switch will occur provides a biological justification for its proposed function. In male cells, lack of hyperacetylation may help guard against inappropriate and lethal inactivation of the single X chromosome. The need for such protection is illustrated by the properties of the *Xist* deletion mutant described by Clerc and Avner (1998). This mutant chromosome, which carries a 65 kb deletion of *Xist* 3′ of exon VI, is inactivated even in cells in which it is the only X present. Thus, even in XO and XY cells, the factors necessary for promoter switching and stabilization of *Xist* RNA must be present.

Results with the mutant ES cell line XT67E1 show that hyperacetylation 5′ of *Xist* facilitates, but is not essential for, X inactivation. In these cells, inactivation of the wild-type chromosome does occur, despite the fact that the region 5′ of *Xist* is no more highly acetylated than other regions on the X chromosome. However, two lines of evidence show that X inactivation is less efficient in these mutant cells. First, differentiating XT67E1 cells have an increased frequency of cells with two active Xs compared with their wild-type counterparts (Penny *et al*., 1996). This may be due to a proportion of cells 'choosing' to inactivate the mutant X, which is unable to form Xi because of the lack of a functional *Xist* gene (Penny *et al*., 1996), but it may also reflect a general reduction in the frequency of promoter switching on both wild-type and mutant chromosomes. Secondly, there is a delay of several days in the first appearance of an underacetylated X chromosome (presumably Xi) in XT67E1 cells compared with the PGK2.1 parental cells. These results are consistent with the proposal that histone hyperacetylation facilitates the *Xist* promoter switch, and hence X inactivation, but is not essential for it to occur.

In contrast, the effects of the histone deacetylase inhibitor TSA indicate that histone deacetylation is an essential component of the early stages of X inactivation. Cells differentiated in the presence of 5 ng/ml TSA failed to form a normal Xi. This effect could be due to inhibition of deacetylation of the region upstream of *Xist*, of X-linked genes in general or of some as yet unidentified genomic region. These possibilities are not mutually exclusive and we cannot yet distinguish between them. However, results with XT67E1 cells show that deacetylation of the region upstream of *Xist* makes a major contribution to the X inactivation process. In XT67E1 cells, the region upstream of *Xist* is not hyperacetylated prior to differentiation, but X-linked genes in general show the same level of acetylation as those in wild-type female cells. If the effect of TSA were due to inhibition of deacetylation of X-linked genes in general, then its effects on XT67E1 and wild-type cells should be the same; in fact they are not. X inactivation in XT67E1 cells, at least as measured by the appearance of an underacetylated X chromosome, is relatively resistant to the inhibitory effects of TSA. Collectively, the results suggest that histone acetylation upstream of *Xist* has a dual role in X inactivation. First, hyperacetylation of this region facilitates initiation of the *Xist* promoter switch. Secondly, deacetylation is essential for completion of the switch and progression to the subsequent stages of the inactivation process. It is possible that deacetylation upstream of *Xist* is necessary for silencing of the P_0 promoter.

Xist is just one of several genes whose regulation involves the use of alternative promoters (Ayoubi and Van de Ven, 1996). The change in promoter can result in changes in the stability of the transcript, as in the case of *Xist*, or the efficiency with which it is translated. Alternatively, the different promoters may differ in the efficiency with which they are transcribed in different tissues (Ayoubi and Ven de Ven, 1996). In no case have the factors responsible for bringing about the promoter switch been defined. They may be transcription factors that bind selectively to one promoter or the other, or factors that bring about rearrangements in higher order chromatin structure and thereby enhance or restrict promoter accessibility. The extent of the hyperacetylated domain upstream of *Xist* suggests that it may be more involved in regulating higher-order chromatin conformation than in local changes in transcription factor binding. It will be interesting to determine whether H4 hyperacetylation is part of the mechanism of promoter switching in other systems.

Materials and methods

Cells and cell culture

The mouse embryonic stem (ES) cell lines LF2 (XX) and EFC-1 (XY) were derived from 129J mice and were provided by Dr A.G.Smith (Nichols *et al*., 1990). The two XX female lines PGK12.1 and XT67E1 (heterozygous for a partial deletion of *Xist* exon I) have been described previously (Norris *et al*., 1994; Penny *et al*., 1996). All cells were grown at 37°C in 5% CO_2 in air and maintained as undifferentiated monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin, L-glutamine, non-essential amino acids, 20% fetal calf serum and 10^3 U/ml murine Leukemia Inhibitory Factor (LIF, all reagents from Gibco-BRL) (Williams *et al*., 1988; Nichols *et al*., 1990). Flasks were coated in 0.1% gelatin (20 min at room temperature) prior to plating cells and cultures were split 1:4 every 2–3 days. Cells were induced to differentiate into non-attached embryoid bodies by trypsinization (1× Trypsin–EDTA solution, Gibco-BRL) and transfer to non-adherent Petri dishes in medium lacking LIF. Differentiating cultures were fed daily by replacing half the medium. For some experiments, media were supplemented with the histone deacetylase inhibitor TSA (generously provided by Dr Minoru Yoshida) at 100 ng/ml (to induce histone hyperacetylation within 6 h) or at 5 ng/ml to study longer-term effects.

Chromatin immunoprecipitation

Prior to preparation of chromatin, cells were grown overnight in medium supplemented with ^{3}H-thymidine (Amersham) at 0.25 μCi/ml. Undifferentiated cells were detached by incubation for a few min with Trypsin-EDTA solution (Gibco-BRL). Embryoid bodies were washed in phosphate-buffered saline (PBS) containing 5 mM sodium butyrate (PBS/butyrate) and allowed to settle. The supernatant was removed and the bodies resuspended in ~1 ml of collagenase/dispase (Sigma; made up as a 5 mg/ml stock solution in PBS and diluted ×1000 in PBS before use). Bodies were disrupted by pipetting, left for 5 min at 37°C and disrupted again. This was repeated as necessary until a single-cell suspension was formed. Cells were washed in cold PBS/butyrate before isolation of nuclei and digestion with micrococcal nuclease (Pharmacia) to release chromatin, exactly as described previously (O'Neill and Turner, 1995). Digestion conditions were adjusted so that the chromatin to be used for immunoprecipitation was rich in mononucleosomes and the smaller oligonucleosomes, typically 2- to 5-mers. A typical immunoprecipitation experiment used eight to ten 25 cm^2 flasks of undifferentiated cells and a similar number of 9-cm-diameter Petri dishes containing embryoid bodies. Chromatin was immunoprecipitated with affinity-purified antibodies to acetylated H4 exactly as described previously (O'Neill and Turner, 1995; Johnson *et al*., 1998). DNA was isolated from the antibody-bound (i.e. highly acetylated), unbound (i.e. underacetylated) and input chromatin fractions by extraction in phenol/chloroform (×2), chloroform (×1) and ethanol precipitation. All DNA samples were analysed by electrophoresis in 1.2% agarose gels. ^{3}H-thymidine incorporation was determined by scintillation counting. Equal amounts of DNA from each fraction, based on ^{3}H-thymidine content, were serially diluted and applied to nylon filters (Hybond N^+, Amersham) by slot blotting. Specific DNA sequences were detected by hybridization with ^{32}P-labelled DNA probes and quantified on a PhosphorImager (Molecular Dynamics) as described previously (O'Neill and Turner, 1995; Johnson *et al*., 1998).

DNA probes

DNA probes used were as follows. The approximate locations of all the X-linked probes are shown in Figure 1.

Autosomal coding regions. α-*tubulin*, 1.5 kb *Pst* fragment (Promega); *Aprt*, 167 bp PCR product (upper primer, ccagcagcactaggaactgctt; lower primer, agggtgtgtgggacgtgtacaa) (Singer-Sam *et al*., 1990); β-*actin*, 460 bp PCR product, a gift from Dr Nel C.Moore (Moore *et al*., 1993).

X-linked coding regions outside the Xic. All of the following are PCR products: *G6pd*, 150 bp (upper primer, actcgccccatttttcaaggc; lower primer, agctgctagtttggcttcgg) (Zucotti *et al*., 1993); *Hprt*, 172 bp (upper primer, cgaggagtcctgttgatgttgc; lower primer, ctggcctataggctcatagtgc) (Singer-Sam *et al*., 1990); *Pgk1*, 166 bp (upper primer, tagtggctgagatgtggcacag; lower primer, gctcacttccttttctcaggcag) (Singer-Sam *et al*., 1990); *Rps4*, 160 bp, upper primer agggtcgctttgctggttca, lower primer agtttcaccatgctgttta (Penny *et al*., 1996).

Xic, 3′ region. Brx, 570 bp PCR fragment covering the last exon (Simmler *et al*., 1996); *Bpx*, 652 bp cDNA (Rougeulle and Avner, 1996; Simmler *et al*., 1996); *Tsx*, 400 bp PCR fragment covering 315 bp 5′ of the coding region and 85 bp of exon I (Simmler *et al*., 1996); *Cdx4*, 700 bp *Eco*R1 fragment (Horn and Ashworth, 1995).

Xic, 5′ region. *NM18B*, 1 kb *Bam*H1 fragment; *p33.2H*, 2 kb *Hin*dIII fragment; *E55*, 2 kb *Eco*R1 fragment; *2.1(2)P*, 2.1 kb *Eco*R1 fragment; *HH1.5* 1.5 kb *Hin*dIII fragment.

Xist. *MP*, 0.5 kb PCR product, upper primer: catggctggagcaag, lower primer: tatggagtcaccaggttcccag; the following are all derived from *Xist* exon I; *XE*, 1.4 kb *Xho*1–*Eco*R1 (913–2319); *XB*, 1 kb *Xho*1–*Bam*H1 (913–1877); *EP*, 3.2 kb *Eco*R1–*Pvu*II (2379–5613); *XP*, 4.7 kb *Xho*1–*Pvu*II (913–5613). *W7D* (exon I) and *W5i* (exons I–VI) are cDNA probes (Brockdorff *et al*., 1992).

Probes were ^{32}P-labelled by random priming with an oligolabelling kit (Pharmacia) used according to the manufacturer's instructions.

Antibodies and immunofluorescence labelling

The preparation and characterization of polyclonal antisera against acetylated isoforms of H4 have been described previously (Turner and Fellows, 1989; Turner *et al*., 1989). For the experiments described here we used antisera R41/5 (to H4Ac5), R12/8 and R232/8 (to H4Ac8), R101/12 (to H4Ac12), and R14/16 and R252/16 (to H4Ac16). All antibodies were raised in rabbits against synthetic peptides conjugated to ovalbumin and affinity purified on peptide–Sepharose columns prior to use (White *et al*., 1999).

For immunolabelling of metaphase chromosomes, mitotic cells were collected following growth for 3 h in medium containing Colcemid (Gibco-BRL, 0.1 μg/ml). Cells were harvested (by mitotic shake-off for undifferentiated cells or by dissociating embryoid bodies for differentiated samples) and washed twice in PBS. Cells were swollen in 0.1 M KCl for 10 min at room temperature, then spun onto washed glass slides using a cytocentrifuge (Shandon Cytospin). Approximately 5×10^4 cells were spun down at 1800 r.p.m. for 8 min. Immunolabelling was carried out using the method described previously (Jeppesen *et al*., 1991) with minor modifications (Keohane *et al*., 1996). Slides were viewed using a Zeiss Axioplan epifluorescence microscope.

Detection of late-replicating chromatin

To detect a late-replicating X chromosome, the thymidine analogue BrdU was added to growing cultures at 5 μg/ml together with Colcemid (0.1 μg/ml). Four hours later, chromosomes were prepared and labelled with FITC-conjugated mouse anti-BrdU antibodies as described previously (Belyaev *et al*., 1996b). Preliminary experiments showed that in ES cell cultures, only those chromosome regions replicating at the very end of S-phase are incorporated into metaphase chromosomes within 4 h.

Histone extraction, AUT gels and Western blotting

Histones were extracted with 0.2 N HCl, resolved by electrophoresis on AUT gels and Western blotted as described previously (Turner and Fellows, 1989). Immunostaining with peroxidase-conjugated second

antibodies (Sigma) was carried out by enhanced chemiluminescence as specified by the reagent suppliers (Amersham).

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