Histone Acetylation and X Inactivation

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ABSTRACT

In mammals, the levels of X-linked gene products in males and females are equalised by the silencing, early in development, of most of the genes on one of the two female X chromosomes. Once established, the silent state is stable from one cell generation to the next. In eutherian mammals, the inactive X chromosome (Xi) differs from its active homologue (Xa) in a number of ways, including increased methylation of selected CpG s, replication late in S phase, expression of the Xist gene with binding of Xist RNA and underacetylation of core histones. The latter is a common property of genetically inactive chromatin but, in the case of Xi, it is not clear whether it is an integral part of the silencing process or simply a consequence of some other property of Xi, such as late replication. The present review describes two approaches that address this problem. The first shows that Xi in marsupial mammals also contains underacetylated H4, even though its properties differ widely from those of the eutherian Xi. The continued presence of histone underacetylation on Xi in these evolutionarily distant mammals argues for its fundamental importance. The second approach uses mouse embryonic stem cells and places H4 deacetylation in a sequence of events leading to complete X inactivation. The results argue that histone underacetylation plays a role in the stabilisation of the inactive state, rather than in its initiation. Dev. Genet. 22:65–73, 1998.

Key words: histone underacetylation; mammals; X-chromosome; embryonic stem cells

DOSAGE COMPENSATION IN MAMMALS

Both mammals and flies of the genus Drosophila use chromosomal methods of sex determination. In each of these evolutionarily distant groups of organisms, males have one copy of each of two different sex chromosomes, designated X and Y, while females have two copies of the X. The Y is a gene-poor chromosome, containing just those few genes needed for initiating the male developmental pathway (mammals) or for fertility (Drosophila), while the X is relatively large and gene-rich. A twofold difference in copy number for such a chromosome would inevitably result in a twofold difference between the sexes in the intracellular concentrations of several hundred gene products. Given that almost all metabolic and developmental pathways are common to both sexes, such a difference, left unattended, would be expected to disrupt the metabolic balance in one sex or the other. Rather than trying to accommodate such major differences between the two sexes, evolution has developed ways of preventing them from occurring in the first place, i.e., through mechanisms of dosage compensation. Widely divergent mechanisms of dosage compensation have evolved independently in different species. For example, in Drosophila the rate of transcription of genes on the single male X is twice that of the same genes on the two female X chromosomes, while in the nematode worm C. elegans, transcription on each of the two female Xs is half that on the male X [Cline and Meyer, 1996]. This divergence attests to the importance of the end result, namely the equalisation of the products of X-linked genes in male and female cells.

In mammals, dosage compensation is achieved by the transcriptional silencing of genes on one of the two X chromosomes in female somatic cells [Lyon, 1961; Migeon, 1994; Riggs and Pfeifer, 1992]. Silencing begins early in development and the selected chromosome remains inactive through subsequent mitotic divisions. The process is thought to be controlled by a cis-acting gene switch, or X inactivation centre, XIC [Rastan and Brown, 1990]. In human cells XIC is located at Xq13.
of heterochromatin. It has been noted in the heterochromatin of the four-core histones) seems to be a general property of the mouse and human genes (Xist and XIST, respectively) and both map to the Xic/XI region. These genes do not code for a protein product, have no conserved open reading frames, and their transcripts are restricted to the cell nucleus [Brown et al., 1992]. In situ hybridization has shown that Xist RNA colocalizes with the Xi in interphase cells [Clemson et al., 1996]. Xist expression precedes X inactivation and appears to be an absolute requirement for it to occur [Brockdorff et al., 1992; Kay et al., 1993; Penny et al., 1996]. However, XIIC, and consequently XIST, seem not to be absolutely required for the maintenance of transcriptional silencing on Xi, at least in somatic cell hybrids in tissue culture [Brown and Willard, 1994].

The Xi chromosome shares several chemical and structural properties with constitutive heterochromatin, and is described as facultatively heterochromatic. Xi replicates in the latter part of S-phase in many cells [Takagi et al., 1982], has relatively high methylation of selected CpG residues [Miller et al., 1974], and shows distinctive histochemical staining in both interphase and metaphase cells [Kanda, 1973]. Like some types of constitutive heterochromatin, Xi remains visible through interphase, forming a distinctive structure, the Barr Body, often located adjacent to the nuclear envelope [Barr and Bertram, 1949]. The chromatin within this structure is often described as "condensed," but recent evidence suggests that the difference between Xi and its active homologue (Xa) is more a matter of conformation than of the amount of chromatin per unit volume [Eils et al., 1996].

A further parallel between Xi and constitutive heterochromatin has come from the use of indirect immunofluorescence microscopy to study the level of histone acetylation along metaphase chromosomes. In human and murine cells both constitutive, pericentric heterochromatin and the inactive X, labelled weakly with antibodies to the acetylated isoforms of H2A, H2B, H3, and H4, but not with antibodies to nonacetylated histones [Jepesen et al., 1992; Jepsen and Turner, 1993; Belyaev et al., 1996; and unpublished results]. Underacetylation of H4 (so far the most widely studied of the four-core histones) seems to be a general property of heterochromatin. It has been noted in the heterochromatic regions of both polytene and metaphase chromosomes in Drosophila [Lavender et al., 1994; Turner et al., 1992] and has also been associated with the long-term transcriptional silencing of mating type genes in the yeast S. cerevisiae [Braunstein et al., 1993]. However, the mechanism by which H4 underacetylation influences heterochromatin formation in general and X inactivation in particular remains unclear. We do not know whether it is (1) an early, possibly initiating event (such as Xist expression seems to be), (2) a later event (such as DNA methylation seems to be) with a possible role in the maintenance of transcriptional silence, or (3) just a consequence of one or other of the events associated with X inactivation and having no significant role in the process at all.

In an attempt to distinguish these possibilities, we have adopted two general approaches. The first considers the problem from an evolutionary viewpoint and asks to what extent characteristic patterns of histone acetylation are a characteristic of dosage-compensated chromosomes in widely divergent species. The second uses female mouse embryonic stem cells, in which X inactivation occurs in tissue culture as the cells differentiate and which provide a powerful experimental system with which the different elements of the inactivation process can be teased apart.

### X Inactivation in Eutherian and Marsupial Mammals

The lineages leading to the eutherian and marsupial mammals diverged about 120–150 million years ago (Graves, 1996, and references therein). Marsupials, like eutherians, use an XY (male) and XX (female) sex determination system and a dosage compensation mechanism in which one of the two female X chromosomes is inactivated [Cooper et al., 1993]. However, the properties of the inactivated X are radically different in the two groups. They are compared in Table 1. The inactive X in marsupials is always the paternally derived homologue (Xp), whereas in eutherians the X to be inacti-

### Table 1. Properties of Xi in Eutherian and Marsupial Mammals*

<table>
<thead>
<tr>
<th>Property</th>
<th>Eutherian</th>
<th>Marsupial</th>
</tr>
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<tbody>
<tr>
<td>Selective CpG methyl‘n</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Presence of XIST RNA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Homologue inactivated</td>
<td>Randomly chosen</td>
<td>Paternal</td>
</tr>
<tr>
<td>Stability of inactive state</td>
<td>Hyperstable</td>
<td>Tissue-dependent</td>
</tr>
<tr>
<td>Tissue dependence</td>
<td>Tissue-independent</td>
<td>Labile</td>
</tr>
<tr>
<td>Sex chromatin body</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Replication timing of Xi</td>
<td>Late</td>
<td>Late</td>
</tr>
<tr>
<td>H4 acetylation</td>
<td>Underacetylated</td>
<td>Underacetylated</td>
</tr>
</tbody>
</table>

*Based on Wakefield et al. [1997] and Graves [1996].
vated is chosen at random at around the blastocyst stage of development. An interesting exception is that in the trophoblastic cells of eutherian embryos, cells that will go on to form the extraembryonic tissues, the inactive X is always paternal [Takagi and Sasaki, 1975]. X inactivation in eutherians occurs in all somatic tissues, whereas in marsupials there is variability between tissues [Cooper et al., 1993]. Finally, a sex chromatin body is seen only occasionally in marsupial cells but is common in eutherians.

In view of the male lethality of mutations that disrupt dosage compensation in various organisms, including mammals, it is puzzling that incomplete dosage compensation is tolerated in marsupials. The reason may lie in the fact that the marsupial X carries rather fewer genes than its eutherian counterpart. Only those genes on the long arm of the eutherian X are also present on the marsupial X, with those on the short arm being distributed on the autosomes [Graves, 1996]. A large part of the marsupial X is gene poor and constitutively heterochromatic [Graves, 1996]. Perhaps this reduction in gene number has made it possible for some cell types to tolerate a relaxation of dosage compensation, while not being sufficiently great to allow the organism to dispense with dosage compensation altogether.

Two of the most significant differences in terms of understanding possible molecular mechanisms of X inactivation are that, as yet, no marsupial homolog of XIST has been identified [unpublished results cited in Wakefield et al., 1997] and the marsupial Xi shows no evidence of selective CpG methylation [Cooper et al., 1993]. Both XIST [Penny et al., 1996; Maherens et al., 1997] and CpG methylation [Li et al., 1992; Panning and J eanisch, 1996] have been shown to be crucial for complete X inactivation in eutherian mammals. The absence of an XIST homolog does not, of course, preclude the existence of a nonhomologous RNA that carries out the same function as an initiator of X inactivation. Human and mouse XIST are only about 70% homologous [Brockdorff et al., 1992; Brown et al., 1992], so divergence between eutherian and marsupial XIST equivalents may be so great that significant homology is no longer detectable. Selective CpG methylation is thought to play a role in the stabilisation of the inactive state of Xi and its absence from the marsupial Xi may contribute to its relative instability. In contrast to X inactivation in eutherian mammals, which is exceptionally difficult to reverse, reactivation of some genes on the marsupial Xi in tissue culture has been reported [Kaslow and Migeon, 1987]. DNA methylation may be a mechanism by which gene silencing is stabilised through many cell generations [Seigfried and Cedar, 1997].

Only two properties are unequivocally shared by the inactive X in eutherian and marsupial mammals. The first is that both replicates late in S-phase. The second is that both are marked by low levels of histone H4 acetylation [Wakefield et al., 1997].

**H4 Acetylation on a Marsupial X Chromosome**

To test the acetylation status of Xi in marsupials, metaphase chromosome spreads from male and female Tammar Wallabies (Macropus eugenii) were immunolabelled with antisera to acetylated H4. Chromosomes were prepared from cultured skin fibroblasts and immunolabelled as previously described [J epsen and Turner, 1993; Keohane et al., 1996]. The Tammar Wallaby is particularly appropriate for studies of this sort, having a relatively simple karyotype (2n = 16) and an easily recognisable X chromosome. The X is the only small submetacentric and has a prominent interstitial nucleolus organiser region (NOR) on the short arm. The short arm is consitutively heterochromatic and C-band positive and has a high proportion of sequences in common with the Y [Toder et al., 1997]. The NOR on the short arm is not silenced on Xi whereas genes on the long arm are subject to tissue-specific silencing [Vandenberg et al., 1986; Graves and Dawson, 1985; Cooper et al., 1993].

Examples of metaphase chromosome spreads from male and female wallabies immunostained with antibodies to acetylated H4 are shown in Figure 1. The following were consistently seen. First, the heterochromatic short arm of both X chromosomes in females (Fig. 1b) and the single male X (Fig. 1d) is underacetylated, consistent with the almost universal underacetylation of heterochromatin in many species and cell types. The Y chromosome is also underacetylated over its entire length (Fig. 1d). Second, conversely, the NOR, which is always transcriptionally active in interphase cells and is not dosage compensated, is brightly stained both in males and in females. The NOR is prominent on Xi (Fig. 1b), but less so on Xa (Fig. 1b,d), suggesting that its level of H4 acetylation is not much greater than that of other euchromatic regions. Third, the long arm of one female X (i.e., the region subject to dosage compensation) and of the single male X, is brightly labelled, while the long arm of the second female X is labelled only weakly. From these results we have concluded that underacetylation of H4 is a characteristic property of the inactive X chromosome in both marsupial and eutherian mammals [Wakefield et al., 1997].

It seems that H4 underacetylation is a highly conserved characteristic of Xi. But then so too is late replication (Table 1), raising the possibility that H4 underacetylation is just a consequence of late replication. To address this question, we have turned to a model system in which we can examine the relationship between different components of the X inactivation system, namely cultured mouse embryonic stem cells.

**X Inactivation in Mouse Embryonic Stem Cells**

Embryonic stem cells (ES cells) are derived from the inner cell mass of mouse embryos at the blastocyst
stage of development and, if allowed to differentiate in culture, form embryoid bodies containing a wide variety of cell types [Keller 1995]. Before differentiation, the cells have two active X chromosomes with one being inactivated as differentiation proceeds [Martin, 1978; Takagi and Martin, 1984]. In recent experiments we have used these cells as a model system in an attempt to place H4 deacetylation in a sequence of events leading to X inactivation.

Undifferentiated ES cells can be grown for many weeks in culture in gelatin-coated flasks in medium containing the growth factor leukemia inhibitory factor (LIF) [Williams et al., 1988]. Differentiation is initiated by detachment of the cells with trypsin and transfer to microbiological-grade Petri dishes in medium without LIF. Over the next few days, the cells continue to divide but form floating clumps of cells, embryoid bodies, in which cells are progressing down various differentiation pathways. Cells were taken at various times from day 0 to day 12 of differentiation and tested for features diagnostic for X inactivation: (1) the presence of an underacetylated X chromosome, (2) the presence of a late-replicating X chromosome, (3) expression of Xist, and (4) a reduction in the level of mRNAs from X-linked genes. The results (summarised in Fig. 2) showed quite clearly that these events occur sequentially over a

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**Fig. 1.** Histone H4 acetylation in metaphase chromosomes from male and female marsupials. Metaphase chromosome spreads were prepared from cultured fibroblasts of the Tammar wallaby and immunolabelled with antibodies to acetylated histone H4. a: Chromosomes from a female cell stained with Hoechst 33342. b: The same spread as in (a) immunolabelled with rabbit antibody (R12/8) to acetylated H4 and FITC-conjugated goat anti-rabbit IgG. The two X chromosomes are indicated. The constitutively heterochromatic short arms on both Xs are pale staining. The long-arm on one X (labelled Xa) is brightly stained while on the other (labelled Xi) it is dim. Nuclear Organiser Regions stand out as brightly labelled on Xi. c: Chromosomes from a male cell stained with Hoechst 33342. d: The same spread as in (c) immunolabelled as above with antibody to acetylated H4. The single X (labelled Xa) has a brightly stained long arm and a dim short arm. The Y chromosome is very weakly labelled. For further details, see Wakefield et al. [1997]. Bar (d) ~ 10 µm.
A period of several days (Keohane et al., 1996). The earliest detectable changes were the appearance of a late-replicating (X) chromosome and increased expression of Xist, a gene expressed exclusively from the inactive X chromosome, or from that X destined to become inactive. Both were detected two days after removal of LIF. Levels of mRNA from the X-linked genes g6pd, hprt, pgk1, and rps4 were reduced by 40–50% by days 2–4. An X marked by low levels of acetylation of histone H4 was first detected only after 4 days (reaching its maximum frequency by day 6). Collectively, these results confirm that X inactivation occurs by a series of discrete steps, and show that each of the properties characteristic of Xi is put in place at a defined stage of the differentiation process.

The finding that underacetylation of an X chromosome follows the onset of late replication is, at first sight, consistent with the possibility that underacetylation is simply a consequence of the shift in replication timing. However, the time lag between the first appearance of a late-replicating X (day 2) and an underacetylated X (day 4) argues that the two events are not necessarily causally related. This has been confirmed by double-labelling experiments in which cells were tested for the presence of both a late-replicating and an underacetylated X chromosome. At early stages of differentiation (days 2–3) the late-replicating (i.e. BrdU-labelled) X was never underacetylated [Keohane et al., 1996]. Thus, while a shift to a late replicating compartment of S-phase may be a necessary prelude to underacetylation of the X, underacetylation is not an immediate or inevitable consequence of late replication.

The results obtained with ES cells are generally consistent with studies of X inactivation in transformed cultured cells. These have also shown that symptoms of inactivation are detected only after several days of differentiation. In EK cells (stem cell lines with a normal karyotype derived from parthenogenetic embryos) a chromosome with characteristically dark staining using the Kanda staining method [Kanda, 1973] appears after four days in culture [Rastan and Robert-son, 1985]. Cultured EC cells, that are of teratocarcinoma origin but considered to be similar to normal embryonic cells at a stage prior to X inactivation, showed non-synchronous replication of X chromosomes after four to six days of differentiation, though the timing varied with the method used to induce differentiation [Takagi and Martin, 1984].

**Turnover of Histone Acetate Groups During ES Cell Differentiation**

The extent to which histone acetylation increases in cells grown for a fixed time in the presence of deacetylase inhibitors such as sodium butyrate and the fungal
antibiotic Trichostatin A (TSA) [Yoshida et al., 1990] provides a measure of the rate at which histone acetylases are turning over. Growth of undifferentiated LF2 cells in the presence of 100 ng/ml TSA for 6 hr resulted in a major increase in acetylation of histones H2B, H3, and H4, measured by electrophoresis on acid/urea/Triton gels and staining with Coomassie Blue (Fig. 3). In the case of H4, the di-, tri-, and tetra-acetylated isoforms, all very weak in untreated cells, were the most prominent after TSA treatment (compare d0- with d0+ in Fig. 3). Cells that had been allowed to differentiate for 2 days behaved in the same way, indicating that the overall rate of acetate turnover had not significantly changed. After 4 and 6 days of differentiation, there was a slight decrease in turnover, with all five H4 isoforms now being present in approximately equal amounts in treated cells. After 8 days of differentiation, the mono-acetylated isoform was the most prominent in both untreated and treated cultures, with the latter showing only low levels of the more acetylated isoforms.

Fig. 3. Turnover of histone acetates in embryonic stem cells differentiating in culture. Mouse embryonic stem cells were induced to differentiate by trypsinization and replating in nonadherent dishes in medium lacking the growth factor LIF. Undifferentiated cells (d0) and cells at different times (in days) since initiation of differentiation (d2–d12) were grown in medium supplemented with 100 ng/ml Trichostatin A (TSA) for 6 hr before harvesting. Histones were extracted, resolved by electrophoresis on acid/urea/Triton gels, and stained with Coomassie Blue as previously described [Turner and Fellows, 1989]. Note that as differentiation proceeds, growth in TSA induces a progressively smaller shift towards the more highly acetylated H4 isoforms, indicative of reduced turnover of acetate groups. From day 8 onwards the effect of TSA is small. Although the acetylated isoforms of H3 and H2B are less well resolved than those of H4, a similar down-regulation of acetate turnover seems to occur with these histones as well. All cell pellets were extracted, electrophoresed, and stained in parallel.

In general, H2B and H3 behaved similarly to H4, with acetate turnover declining at the later stages of differentiation. Thus, turnover of histone acetate groups is at its most rapid when developmental decisions are being made most frequently (i.e., during the first few days of differentiation). These results provide an interesting parallel with those of Dmitrov et al. [1993], which show that during development of the amphibian X. laevis, turnover of histone acetate groups begins during or just before the mid-blastula transition, the developmental stage at which zygotic transcription is initiated and formation of cell lineages begins.

Differentiation of ES Cells in the Presence of TSA Prevents Underacetylation of Xi

Culture of ES cells in medium containing 100 ng/ml TSA (the concentration used to induce H4 hyperacetylation in the turnover experiments outlined above) results in progressive cessation of growth. This is in line with previous results with other cultured cell types, in which TSA was shown to cause arrest of cells in the G1 and G2 phases of the cell cycle [Yoshida and Beppu, 1988]. In order to test the effect of TSA on X inactivation in differentiating ES cells, it was necessary to find a
TSA concentration that would induce an increased steady-state level of acetylation while permitting the cells to grow. A series of preliminary tests established that a concentration of 5 ng/ml caused a readily detectable increase in H4 acetylation (measured by electrophoresis on AUT gels) and allowed continued cell growth at rates not significantly different to normal with concomitant formation of morphologically normal embryoid bodies. Initial experiments with cells differentiated under these conditions have shown that the appearance of an underacetylated X chromosome, as detected by immunofluorescent labelling of metaphase chromosomes, was delayed for at least six days (i.e., was not seen by day 10 of differentiation, the longest time point tested). In contrast, a late replicating chromosome was first detected at day 3 (i.e., only slightly later than normal) and reached control levels by day 6. These experiments will be presented in detail elsewhere, but these initial findings confirm that H4 underacetylation is not mechanistically linked to replication timing. The fact that underacetylation of Xi can be prevented (or at least delayed by several days) by TSA also provides a means for testing the linkage between H4 acetylation levels and expression of X-linked genes.

CAUSE AND EFFECT IN DOSAGE COMPENSATION

It is possible to list a series of tasks that must be performed before the process of X inactivation is complete. These are:

1. Counting: All Xs but one are inactivated so the cell must have some mechanism for determining how many there are.
2. Choice: The X chromosome(s) to be inactivated must be selected.
3. Initiation: The inactivation process must be started, possibly at a specific point, on the chosen chromosome.
4. Spreading: Inactivation must spread along selected regions of the chromosome; if initiation occurs at multiple points then spreading, and particularly the fact that inactivation can skip over some regions, becomes less problematic.
5. Stabilisation: The inactive state must be maintained from one cell generation to the next.

In differentiating female ES cells, three events associated with X inactivation occur at about the same time, i.e., between 1 and 3 days of differentiation. These are (1) increased levels of Xist RNA, (2) down-regulation of expression of X-linked genes, and (3) replication of a single X late in S-phase. Thus, stages 1–4 have all occurred within 3 days of initiation of differentiation. In contrast, reduced acetylation of histone H4 occurs later (days 4–6), while methylation of specific CpG residues in the promoter of the hprt gene is seen only after 14–21 days [Kehane et al., 1996, A.M.K., unpublished observations]. These results suggest that H4 acetylation plays a role in stabilisation of the inactive state of Xi, rather than in the initiation of the silencing process. DNA methylation is also likely to play a role in stabilisation, perhaps by further securing the inactive state and rendering its reversal even more unlikely [Lock et al., 1987].

The fact that Xist RNA levels are upregulated just before X inactivation in early embryos suggests that it plays a role in one or all of the choice, initiation, and spreading stages. Xist is also not required for the maintenance of the inactive state in hybrid cell lines [Brown and Willard, 1994]. Recent experiments have shown that multiple copies of a 450-kb YAC containing Xist and surrounding sequences, when inserted into an autosome, can bring about, on that autosome, many of the changes associated with X inactivation, including a reduction in H4 acetylation [Lee et al., 1997; Lee and J. aenisch, 1997]. A much smaller construct, containing only the Xist gene and 9 kb of 5′ and 6 kb of 3′ flanking sequences, when transfected into ES cells, was shown to be strongly expressed when the cells were induced to differentiate [Herzing et al., 1997]. The Xist RNA was localised close to the autosome into which it was inserted and a β-gal reporter gene incorporated into the Xist construct was inactivated. These results suggest that the Xist gene itself is sufficient to initiate at least some aspects of X inactivation and that Xist, or very closely linked sequences, forms part of the chromosome counting mechanism.

The experiments with Xist-containing constructs show, at the very least, the fundamental importance of Xist in the early stages of X inactivation. However, there are some discrepancies between these findings and experiments with cells containing only the endogenous Xist gene. Work on early mouse embryos has indicated that increased Xist RNA levels are not involved in the counting mechanism, nor are they dependent on it [Kay et al., 1994], while work on X:autosome translocations in both human and mouse cells has shown that the translocated autosomal fragment retains its own pattern of H4 acetylation when attached to Xi. The latter finding [A.M.K., unpublished observations] shows that a single, endogenous Xist gene, even when active, cannot cause complete deacetylation of an autosomal fragment in cis.

How can we reconcile the delaying effects of TSA on the onset of X inactivation in ES cells with the conclusion that deacetylation is most likely to be part of a stabilisation mechanism? Perhaps local deacetylation is important, resulting in altered transcription of selected genes. Such changes are not detectable by immunofluorescent labelling of metaphase chromosomes but can be detected by immunoprecipitation of chromatin fragments from undifferentiated and differentiated cells [O’Neill and Turner, 1995; Kehane et al., 1996]. Such an approach can be used to search for changes in the acetylation status of the Xist gene itself. However, recent results demonstrating that the up-regulation of
Xist RNA levels are regulated through changes in the stability of Xist transcripts, rather than any increase in the rate of transcription, moves the focus of attention, a least temporarily, away from chromatin and transcriptional control and adds an extra level of complexity to an already difficult mechanistic problem [Panning et al., 1997; Sheardown et al., 1997].

**SUMMARY AND FUTURE DIRECTIONS**

It seems likely that attention will continue to focus on the role of Xist RNA in silencing and, specifically, on attempts to define the mechanism by which it operates. The fact that Xist RNA coats the pseudoautosomal region on Xi, a region whose genes escape inactivation, shows that Xist coating is not sufficient, by itself, for silencing. What else is required? Does Xist RNA interact with silencing proteins, or is it part of a silencing complex? Comparisons with the RNA product of the Xist gene, implicated in the dosage compensation system interact. With regard to histone acetylation, we should ask what are the relationships, if any, between histone acetylation and replication timing, or between histone acetylation and DNA methylation. It will also be important to distinguish between overall deacetylation of Xi and local changes. The description of changes in histone acetylation at defined locations along Xa and Xi by immunoprecipitation should help clarify its role in silencing. In view of the recent findings implicating changes in the stability of Xist RNA in the X inactivation process, we must now ask what mechanisms could lead to a chromosome-specific shift in RNA stability at a defined stage of development. Whether the answer is to be found in the Xist gene itself or elsewhere remains to be seen.

The problem of X inactivation is a shining example of the generally accepted fact that research throws up more questions than answers. It is humbling to realise that after almost 40 years of experimentation, fundamental questions relating to the mechanisms involved in the various stages of X inactivation remain unanswered. With this in mind, perhaps the most important question to ask now is whether our present foci of attention are likely to provide these answers. Are we really asking the right questions?

**REFERENCES**


