Annu. Rev. Genet. 1997. 31:571–610

X-CHROMOSOME INACTIVATION IN MAMMALS

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KEY WORDS: X-inactivation, X-inactivation center (*Xic*), *Xist*, X controlling element (*Xce*), chromatin

ABSTRACT

The inactive X chromosome differs from the active X in a number of ways; some of these, such as allocyclic replication and altered histone acetylation, are associated with all types of epigenetic silencing, whereas others, such as DNA methylation, are of more restricted use. These features are acquired progressively by the inactive X after onset of initiation. Initiation of X-inactivation is controlled by the X-inactivation center (*Xic*) and influenced by the X chromosome controlling element (*Xce*), which causes primary nonrandom X-inactivation. Other examples of nonrandom X-inactivation are also presented in this review. The definition of a major role for *Xist*, a noncoding RNA, in X-inactivation has enabled investigation of the mechanism leading to establishment of the heterochromatinized X-chromosome and also of the interactions between X-inactivation and imprinting as well as between X-inactivation and developmental processes in the early embryo.

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0066-4197/97/1215-0571$08.00

INTRODUCTION

The presence of heteromorphic sex chromosomes, i.e. sex chromosomes clearly distinguishable in form and genetic content, has been accompanied by the development of mechanisms of dosage compensation. Dosage compensation ensures that the difference in copy number of genes on the sex chromosomes in males and females does not impair embryonic development and adult viability.

Various mechanisms have been adopted by different species to achieve this end. In mammals, inactivation of one of the two X chromosomes present in the homomorphic female ensures dosage compensation. In the fruit fly, *Drosophila melanogaster*, the single X chromosome in the heteromorphic male has twice the transcriptional activity of either of the two female X chromosomes, whereas in the nematode, *Caenorhabditis elegans*, normalization is achieved by lowering the level of X-linked transcription by half in hermaphrodite XX animals. Over and above the obvious differences between species, in each case regulation affects the chromosome as an entity. One distinctive feature of mammalian X-inactivation, compared to other dosage compensation strategies, is that the two X chromosomes present in the same nucleus are treated differently.

Historically, the X-inactivation process has been divided into three phases: initiation, spreading, and maintenance. Initiation is thought to involve the

choice of which of the two X chromosomes is to be inactivated and requires the presence of a unique locus on the X chromosome, the X-inactivation center (*Xic*). A developmental cue, present only at specific stages of early embryogenesis, is thought to trigger the X-inactivation process at the *Xic*. A counting mechanism ensures that only a single X chromosome remains active in a diploid cell, with all other X chromosomes inactivated. Such counting might be mediated by a "blocking signal" that is produced in limited quantity and that binds to the *Xic* of a single X chromosome per diploid cell, thus protecting it from inactivation. Spreading of an inactivation signal from the *Xic* ensues, resulting in the *cis*-limited inactivation of most of the several thousand genes on the X chromosome. Specific maintenance mechanisms ensure that the inactive X chromosome is clonally transmitted through successive cell divisions.

The *Xist* (X-inactive specific transcript) gene, a likely candidate for *Xic*, was discovered in 1991, more than 35 years after Mary Lyon (126) originally proposed a mechanism that supersedes control of individual X-linked gene activity in mammals. Its discovery has opened up X-inactivation to molecular analysis.

Since much of the original data concerning X-inactivation has been thoroughly reviewed, we have concentrated here on presenting the more recent advances in the molecular and genetic analysis of X-inactivation. Reviews have been cited where possible and early publications are cited only when essential.

X-INACTIVATION DURING DEVELOPMENT

X-Inactivation in the Early Embryo

The cycle of X chromosome activity during development of the female mouse is summarized in Figure 1. During the earliest stages of female embryogenesis in eutherian (or placental) mammals, both of the X chromosomes contributed by the sperm and the egg are active. Asynchronous replication of one of the two X chromosomes during the cell cycle, one of the earliest signs of X-inactivation, is observed only at the blastocyst stage (3.5–4.5 days post coitum, d.p.c.) in cells of the trophectoderm lineage. Further evidence for the presence of two active X chromosomes in the very early embryo was provided by biochemical studies of X-linked genes such as G6PD, HPRT, or α-galactosidase (see 69, 182, and references therein).

Recently, quantitation of allele-specific RNA (197) using single nucleotide primer extension (SNuPE) has enabled the direct measurement of transcript levels. Transcripts derived from both X chromosomes (e.g. for the *Hprt* and *Pgk1* genes) have been detected from the 2-cell embryo stage through to the blastocyst (113, 197). The presence of two active X chromosomes in the very early stages of development is thought to be tolerated because of the relatively

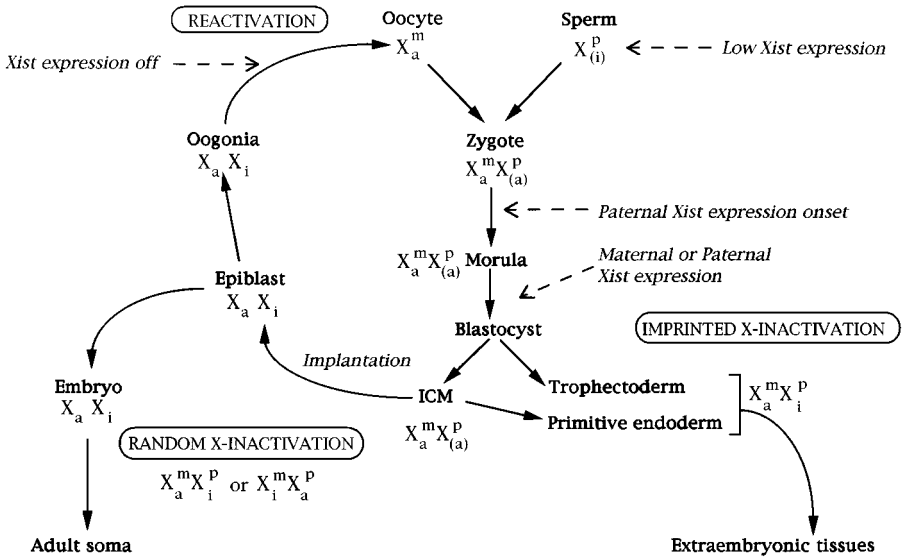

Figure 1 X chromosome activity in the different cell lineages of the female mouse. ICM: inner cell mass; X^m: maternal X chromosome; X^p: paternal X chromosome; X_a: active X chromosome; X_i: inactive X chromosome; the active or inactive state of the paternally provided X chromosome is given in brackets as these activity states appear to be partial (see text).

small number of X-linked genes active. Later in development, an absence of dosage compensation is assumed to be detrimental because of transcription of larger numbers of genes, or genes that are particularly dosage sensitive. Takagi & Abe (210) have shown, using a mouse strain with an X chromosome that cannot be inactivated, that the presence of two active X chromosomes is lethal prior to 10 d.p.c. in female embryos.

X-inactivation takes place in a developmentally regulated manner, which seems to coincide with cellular differentiation (147). In the mouse, X-inactivation is nonrandom in the earliest lineages to differentiate (the trophectoderm and primitive endoderm, which will both contribute to the extraembryonic tissues): the paternal X chromosome is preferentially inactivated (212, 234). X-inactivation in cells that give rise to the embryo proper occurs at the late blastocyst stage at 5.5–6.5 d.p.c., i.e. about the time of placental implantation (132). Here, the paternal imprint is no longer operative. The paternal or maternal X chromosome is inactivated at random, though the probability of inactivation is affected by the *Xce* locus (see below).

X-inactivation has been assumed, based on biochemical studies (147), to be complete in all cells of female mice by the onset of gastrulation (6.5 d.p.c.).

Recently, however, differences in the timing of X-inactivation in different tissues have been suggested, using a mouse line with an X-linked *LacZ* transgene subject to inactivation. Loss of β-galactosidase activity provides a direct visualization of X-inactivation in individual cells of the intact embryo. In female embryos hemizygous for the transgene, β-galactosidase activity was found as late as 10.5 d.p.c. in some lineages, suggesting differences in the timing of X-inactivation in different tissues (213, 215). No such tissue-specific delay in X-inactivation could be detected for the endogenous X-linked genes, *Pgk1* and *Hprt*, in 9.5-day female embryos of the same strain using the sensitive quantitative RT-PCR SNuPE assay (114). The discrepancy between the endogenous and transgenic inactivation profiles may be linked to the particular nature of the *LacZ* transgene, or to differences in the half-life of β-galactosidase mRNA and protein in different tissues.

X-Inactivation in the Germline

Although in female somatic cells the inactive state is stable and heritable throughout the lifetime of the organism (for review see 69), in the germline the inactive X is reactivated at the onset of meiosis at around 12.5–13.5 d.p.c. (45). It has been proposed that this reactivation is linked to the requirement for a euchromatic, active state for normal meiotic chromosome pairing (89, 141). The molecular basis of this developmentally regulated reversal of X-inactivation is unknown. Tam et al (214) have recently shown, using the X-linked *LacZ* transgene, that primordial germ cells (PGCs) must be located in the vicinity of the genital ridge for X chromosome reactivation to occur: The absence of reactivation, even by 15.5 d.p.c, in small populations of PGCs left outside the genital ridges suggests that local signals may be essential for reactivation. The reactivated X chromosome remains active in oocytes throughout ovulation and fertilization until inactivation occurs during preimplantation development.

In male germ cells, the single X chromosome becomes condensed and transcriptionally inactive at or just before the onset of meiosis. It replicates late during S phase and associates with its pairing partner, the Y chromosome, which also becomes transcriptionally inactive at this stage, to form the cytologically identifiable sex vesicle (146). Inactivation of the single X chromosome during male meiosis may prevent the initiation of damaging recombination events that might occur as a result of the presence of unpaired sites on the single X chromosome (89, 141). The X chromosome was thought to remain inactive in meiotic spermatocytes, postmeiotic spermatids, and spermatozoa, and only to become reactivated in embryos shortly after fertilization. However, the recent finding that a number of X-linked genes show postmeiotic transcription in spermatids (81) suggests that transcriptional down-regulation of the X chromosome is actually restricted to the period of meiosis itself, as it is for the Y chromosome.

The transient X-inactivation of somatic cells in the male urogenital ridge at 10.5–11.5 d.p.c., observed by Jamieson et al (91) using the X-linked *LacZ* transgenic mouse strain, has been suggested to have a role in male sex determination. It may serve to down-regulate loci such as the dosage-sensitive sex reversal locus, whose repression at this stage may be critical for *Sry*, the testis-determining gene, to exert its full effect on testis differentiation.

X-Inactivation in ES and EC Cells

A major difficulty inherent to the study of X-inactivation is the small size of the embryo and the limited amount of material available at the peri-implantation stages when X-inactivation occurs. In vitro model systems, such as embryonic stem (ES) cells (derived from the inner cell mass of blastocysts) and embryonic carcinoma (EC) cells (derived from gonadal tumors or embryonic tissues), provide a valuable alternative. In ES and some EC cell lines with two intact X chromosomes, both X chromosomes are active while the cells are maintained in a totipotent state, but if the cells are allowed to differentiate, X-inactivation occurs (129, 177). Such cell lines have facilitated investigation of the different steps in X-inactivation and reactivation and have been particularly useful in the genetic dissection of the *Xic*, as is discussed below.

FEATURES OF THE INACTIVE X CHROMOSOME

The inactive X chromosome was originally identified by its late replication and its condensed heterochromatic nature, which is cytologically recognizable as the Barr body in many species (14). Other specific properties of the inactive X chromosome include differences in methylation and in the acetylation state of histones with respect to its active counterpart.

Replication Timing

The inactive X chromosome replicates asynchronously with respect to its active homologue in cells of the female in man, mouse (reviewed in 65, 69), and marsupials (181). In adult somatic cells and in most tissues of the embryo, the inactive X chromosome replicates in the late S phase, with the exception of initially early replication in extraembryonic tissues (207). Late replication timing is frequently used as a criterion for identifying the inactive X and for determining the inactive status of autosomal regions translocated onto the X chromosome. It is one of the most precocious characteristics of the inactive X; the appearance of a late replicating chromosome is observed during embryogenesis (211), at day 4.5 in extraembryonic territories, and at day 6 in the embryonic tissues. In female ES cells, a late replicating X chromosome is already detectable two

days after the onset of differentiation, a period concomitant with the induction of *Xist* expression and the silencing of X-linked genes (105).

X chromosome replication has been examined at the single-gene level in man for the *FMR1*, *HPRT*, *PHKA1*, *XPCT*, *PGK*, *F8*, *F9*, *IDS*, and *G6PD* genes (20, 72, 73, 100, 191, 218, 219), all subject to inactivation. In all cases, the genes on the active X chromosome were found to replicate earlier than their homologue on the inactive X. FISH analysis of the replication patterns of *RPS4X*, *ZFX*, *ANT3*, and *STS*, which all escape X-inactivation, revealed that the gene on the inactive X chromosome and the gene on the active X chromosome replicate synchronously (20). Replication timing of individual X-linked genes therefore appears to reflect their transcriptional activity rather than the global inactivation status of the chromosome on which they lie.

The replication status of *XIST* is more controversial. A study involving chromatin fractionation after BrdU incorporation into female human fibroblasts suggests that *XIST* replicates on the active X earlier than on the inactive X (73). A similar result was found for the *F9* gene, which is specifically expressed in hematopoietic lineages and thus not expressed in the cells used in this study. It was concluded that chromosomal environment and not transcriptional activity per se dictates the timing of replication of these genes (73). Data from FISH analysis, on the contrary, suggest that the expressed copy of *XIST* on the inactive X replicates earlier than the silent copy on the active X (20, 218, 219). These conflicting results may well reflect shortcomings in the use of FISH for assessing replication timing. The ability to visualize replicated regions as a doublet FISH signal may require several additional steps, including the physical separation of the replicated region.

Since replication timing domains may range in size from 1 Mb to 3 Mb (for example see 192), they could well include multiple genes of varying transcriptional status and chromatin properties. In this case, occasional exceptions to the general correlation between active transcription and early replication timing, such as for the *XIST* gene, would not be surprising. An instructive example of the potentially complex interactions between different chromosomal features, including replication timing, is provided by the effect of 5-azacytidine, which leads not only to a depletion in methyl-cytosines, but also to a decondensation of inactive X chromatin at the cytogenetic level (71) and to an increase in the Dnase I sensitivity of the inactive X chromosome (88). 5-Azacytidine treatment also advances replication of the inactive X chromosome, as observed at the cytogenetic level in *Gerbillus gerbillus* fibroblasts (88) and at the single-gene level in somatic cell hybrids retaining an inactive human X chromosome (72). Such results raise the possibility of a control of replication timing through DNA methylation.

Chromosome Conformation

Higher-order changes in the conformation of the inactive X chromosome are suggested by the historic observation that the inactive X chromosome forms a Barr body usually located at the periphery of the nucleus (14), by the presence of a bend in the proximal long arm of human inactive X chromosomes in metaphase (61, 227), and by a possibly looped structure in interphase (230a). Although recent experiments using chromosome painting and optical sectioning have failed to observe significant differences in the volume of the inactive X chromosome compared to the active X, differences in the topology of the chromosome surface have been observed in human amniotic cells (57).

Histone Acetylation

Dosage compensation in *Drosophila* (222), man, mouse (93), and marsupials (J Graves, personal communication) is associated with modifications of the acetylation status of lysine residues present in the amino-terminal tails of core histones. This evolutionary conservation reflects the fundamental role of histone acetylation in the dynamics of chromatin properties (224, 239).

In mouse and man, both the inactive X chromosome and constitutive pericentric heterochromatin of all chromosomes are depleted of acetylated isoforms of histones H2A, H3, and H4 (18, 93). This was shown by examining the profile of acetylation of histones of metaphase chromosomes by indirect immunofluorescence (18, 93), using antisera specific for individual acetylated lysine residues of these histones (18, 223, 225). Pale staining of the inactive X chromosome, as well as of the centric heterochromatin of all chromosomes, was observed while the arms of the other chromosomes, including the active X, appeared R-banded (18, 93). This might suggest a general role for long-range histone underacetylation in heterochromatinization, although some differences also occur in the patterns of acetylation of histones of the inactive X. For instance, three regions of residual histone H4 hyperacetylation have been noted on the inactive X chromosome of human and mouse cells grown in the presence of deacetylase inhibitors (93); of these only the pseudoautosomal region is hyperacetylated for histones H2A and H3 (18). Two of the regions associated with residual H4 hyperacetylation correspond in human to the pseudoautosomal region and to Xp11.2–Xp11.3, which are known to contain genes escaping X-inactivation (93). These results are consistent with the reported association of histone H4 hyperacetylation with inducible or transcribed chromatin (76, 158).

The underacetylation of histone H4 associated with the mouse inactive X chromosome probably results from a reduction in acetyltransferase activity, since the deacetylase inhibitor sodium butyrate does not lead to any increase of acetylation of histone H4 on the inactive X chromosome (93). The recent cloning and characterization of several yeast and human acetyltransferases

(reviewed in 183, 239) may provide insights into how acetyltransferase activity is excluded from the inactive X chromosome.

A possible role for histone acetylation during the initiation of inactivation was evaluated in a recent study of the chronological appearance of several features of the inactive X chromosome after in vitro differentiation of female ES cells (105). All the chromosomes of undifferentiated female ES cells, including the two active Xs, are stained with an antiserum against acetylated histone H4. The appearance of a late-replicating chromosome and an increase in *Xist* transcript level were detected as early as day 2 of differentiation, the silencing of four X-linked genes (*Hprt, G6pd, Rps4X*, and *Pgk1*) between days 2 and 4, while underacetylation of an X chromosome was found from day 4 onward (105). Therefore, global histone underacetylation of the X chromosome, as detected by indirect immunofluorescence, is probably neither a triggering signal nor an early effector in the initiation and spreading of X-chromosome inactivation. This does not, however, preclude a role for histone underacetylation of particular domains in the initiation of X-inactivation that might only be detectable using techniques such as chromatin fractionation.

DNA Methylation

DNA methylation has long been considered to be an important component of X-inactivation, particularly as a mechanism for stably maintaining the inactive state. Methylation patterns can be inherited from cell to cell, thanks to a DNA methyltransferase activity that recognizes and methylates hemimethylated CpG sites after DNA replication. Recent strong evidence suggests that methylation of key elements may play a more primary role in establishing X-inactivation.

GLOBAL METHYLATION LEVELS OF THE ACTIVE AND INACTIVE X CHROMOSOME

The overall methylation status of the inactive versus the active X chromosome is controversial. When in situ restriction enzyme-directed nick translation was used on human metaphase chromosomes, either no difference between the two X chromosomes (2), global hypomethylation of the inactive X chromosome (230), or hypomethylation of the active X chromosome (170) was observed. Using antibodies against 5-methylcytosine, Bernardino et al (19) found globally comparable methylation levels of the inactive X and the active X in human female cells.

METHYLATION OF THE INACTIVE X CHROMOSOME

CpG islands, which have a higher than average density of CpG dinucleotides and are often associated with the 5′ end of genes, tend to be heavily methylated on the inactive X in somatic cells, and completely unmethylated on the active X. For example, in the mouse, 11 of 13 mouse X-linked CpG islands (156) and 28 of 28 human X-linked CpG islands (220) were found to be methylated exclusively on the inactive X. CpG

islands associated with genes that escape X-inactivation remain unmethylated on both the inactive X and active X.

In extraembryonic tissues, the methylation status of X-linked genes is less clear. Classic DNA transfection studies involving the mouse *Hprt* gene have suggested that they might be relatively unmethylated in extraembryonic tissues (109). This suggestion is consistent with results obtained for three X-linked genes examined in human extraembryonic tissue (135; see below), but conflicts with the observation that critical CpG sites associated with the mouse *Pgk1* and *G6pd* genes are methylated in murine extraembryonic tissues (68).

The best studied CpG islands are those of the *Pgk1* and *Hprt* genes (86, 87, 166, 162, 120, 217), which have been studied using high-resolution techniques such as LMPCR (164, 165) and bisulphite sequencing (63). Their methylation patterns on the inactive X appear to be heterogeneous, with fully methylated CpGs interspersed with unmethylated CpG sites. For the human *PGK1* promoter, inactive X-specific methylation at 60 of 61 CpG sites was found within an 800-bp region (165). In the mouse *Pgk1* promoter, on the other hand, only a single site was found to be consistently methylated (217). No obvious conserved methylation pattern in the 5′ regions of different X-linked genes nor between the same gene in different species has been observed. Thus the broad methylation pattern of the CpG island and the promoter region may be more significant than any particular site-specific methylation. Alternatively, CpG sites whose methylation profile is strictly conserved may be located outside the immediate promoter regions so far examined.

ROLE OF METHYLATION AND MECHANISMS IN X-INACTIVATION CpG island hypermethylation on the inactive X chromosome seems to be involved in the stabilization of the inactive state of some, but not all, genes in eutherian mammals. The relative instability of X-inactivated genes in the marsupial and the tendency for cells of the human chorionic villus to reactivate X-inactivated genes in culture have both been linked to hypomethylation of CpG islands of X-linked genes (51, 139). The timing during development of CpG island methylation on the inactive X is consistent with a role in maintaining rather than initiating transcriptional silencing, although few examples have been examined. Lock et al (123) found methylation of sites in the first intron of the *Hprt* gene to be a relatively late event in X-inactivation, whereas more recently sites in the CpG islands of the *G6pd* and *Pgk1* genes were found to be methylated closer to the time of X-inactivation (68, 199). The X chromosome can, however, clearly adopt an inactive state in the absence of CpG island methylation. Inactivation can also apparently be accomplished without methylation of CpG islands in both human (56) and mouse (68, 198) germ cells, and in the somatic tissues of marsupials (51).

By what mechanism might CpG island methylation repress X-linked genes? The hypothesis that methylation directly prevents binding of transcription

factors is argued against by the fact that Sp1, which is excluded from the inactive X, can bind to both methylated and unmethylated DNA (82, 166). Alternatively, methylation could repress transcription through the binding of proteins such as MeCP1 and MeCP2, which bind without sequence specificity to methylated DNA (23, 154). It has been suggested that binding of proteins of this type could be responsible for differential accessibility of the active and inactive X chromosomes to nucleases (5). In vivo footprinting of the mouse and human *Hprt* promoters (86, 120) and the human PGK-1 promoter (166) has revealed multiple DNA-protein interaction sites associated with the active X chromosome but no such protein binding on the inactive X. The absence of obvious footprints on the inactive X in the CpG islands examined so far can possibly be explained by the heterogeneous binding of proteins such as MeCP1 and MeCP2. It has been shown that transcriptional repression mediated by MeCP2 binding requires dense but not full occupancy of all methyl-CpG sites in a CpG cluster (154).

Ackerman et al (1) have proposed that GC-rich promoters may form stem loop structures around major transcription start sites that are implicated in increasing chromatin accessibility and basal promoter function. Such structures could exist in the region flanking the major initiation sites of the HPRT and PGK genes in man and mouse (162; see 94 for review). Methylation might disrupt or inhibit the intrastrand base pairing of such regions and thus stabilize DNA and prevent formation of transcriptional activation structures.

METHYLATION OF THE ACTIVE X-CHROMOSOME Specific methylation of sites on the active X chromosome has been observed. In certain cases this methylation is associated with nontranscribed sequences (22, 66, 80), and the significance of this methylation is unknown. In other cases, and more interestingly, it is sites in the body of X-linked genes, or 3′ to them, that are methylated on the active rather than the inactive X. This phenomenon has been observed for both eutherian and marsupial X-linked genes (15, 98, 104, 122, 124, 168, 238, 242). Identical methylation of the body of the human *MIC2* gene, which escapes X-inactivation, on both the active and inactive X chromosome may indicate that this methylation is specific to X-inactivated genes (145). The conservation and widespread nature of this type of methylation is suggestive of a function in X-inactivation.

STABILITY OF THE INACTIVE X CHROMOSOME

Species Differences in Stability

While our knowledge of marsupial X-inactivation is patchy, a major difference between eutherian and marsupial X-inactivation is in the relative instability of

X-inactivation in marsupials. The stability of the X-inactive state in marsupials differs both between species and between X-linked genes (51). Paternally inherited X-linked glucose-6-phosphate dehydrogenase (G6PD) activity is, for instance, found in all tissues of the Virginia opossum (*Didelphis virginiana*), whereas no such activity is found in either the euro (*Macropus robustus erubscens*) or the common wallaroo (*Macropus robustus robustus*) (228), although in vitro cultured fibroblasts of the latter show only partial inactivation of the paternal *G6pd* allele (124). The relation between this instability and both the likely absence of CpG island methylation on the marsupial inactive X chromosome and preferential inactivation of the paternally derived X chromosome in somatic tissues of marsupials remains, however, to be established.

While in eutherians the general rule appears to be that the inactive state once established remains immutable throughout the multiple cell divisions that characterize mammalian development, there are a number of specific situations in eutherians in which partial reactivation of the X chromosome may occur. These are detailed below.

X-Chromosome Reactivation and Aging

The first report of age-related X-chromosome reactivation concerned the ornithine transcarbamylase (*Otc*) locus. A large increase in cells staining for OTC activity in female animals as the animals aged was attributed to X-chromosome reactivation (231). Similar results obtained from studying coat pigmentation at the X-linked *Mottled (Atp7a*) locus (33) have been supported by preliminary analysis of transcript levels at this locus (PE Bennett-Baker & D Burke, personal communication). Age-linked reactivation was not, however, observed for either the *Tabby* locus in the mouse (40) or for the *Hprt* locus in man or mouse (135, 151). The observations on the *Tabby* and *Mottled* loci, both located close to the *Xic*, suggest that the differences between genes are unlikely to be related solely to the distance from the X-inactivation center (*Xic*). They are compatible with the idea of intrinsic differences in the temporal stability of mouse genes to X-inactivation (see also methylation section).

Such age-related reactivation is not restricted to endogenous X-linked genes, as similar findings have been made concerning autosomal genes such as the albino locus, when inserted into the X chromosome (39).

Age-related variation in the proportion of the human population showing skewed X-chromosome inactivation could be related to such age-related reactivation in the mouse (60).

Experimentally Induced X-Chromosome Reactivation

Most experiments to induce overall X-chromosome reactivation, using, for example, demethylating agents such as 5-azacytidine, have been unsuccessful. At

best, they have resulted in the local derepression of X-linked specific genes. In one of the more successful recent studies involving somatic cell hybrids retaining an inactive X chromosome, reactivation was obtained for the *PGK, HPRT, XPTC, IDS, FMR,* and *G6PD* genes (72). Overall reactivation of the X chromosome has, however, been suggested to occur when female mouse lymphocytes are fused with some, but not all, mouse EC cell lines. The resulting near-tetraploid hybrids, which have an EC cell morphology, in some cases reactivated the X-chromosome, as demonstrated by the absence of a late-replicating X chromosome (209) and the extinction of *Xist* expression (143). While the capacity for reactivation may be correlated to the methylation status of the *Xist* gene, this itself may be a reflection of the wider embryonic status of the reactivating cells.

Reactivation is not species specific since an inactive rat X chromosome (208) and a human X chromosome (243) could be reactivated in EC and microcell EC hybrids, respectively. However, several observations (243) suggest that this reactivation process does not fully constitute a true reversal of the X-inactivation process analogous to that occurring during meiosis. These include the continued transcription of human *XIST* from the reactivated human X chromosome and the failure to inactivate correctly the mouse or human X chromosome complements of such hybrid cells after in vitro differentiation.

GENES ESCAPING X-INACTIVATION

Some X-linked genes are expressed from both the active and the inactive X chromosome. For unknown reasons, this phenomenon seems far more frequent in man than in mouse. The smaller number of genes escaping inactivation in the mouse might explain the relatively normal phenotype of the XO mouse, characterized mainly by lower reproductive performance, compared to the severity of Turner's syndrome in man.

Genes Escaping Inactivation in Man

Assays for transcriptional activity of the inactive X in man have mainly been based on the analysis of somatic cell hybrids retaining the active or inactive X chromosomes. Nineteen human genes have been identified that are expressed from both the active and the inactive X chromosome [*GS1, KAL, ZFX, UBE1, ANT3, XE7, MIC2, SMCX* (*DXS1272E*), *XG, RPS4X, STS,* reviewed in (54); *ARSD* and *ARSE* (62); *SSX* (44); SB1.8 (*DXS423E*) (142); *PCTK1* (36); *DFFRX* the human homologue of the *Drosophila faf* gene (96); *IL9R* (229); and *WI-12682* (28)]. When present on the inactive X chromosome, genes escaping X-inactivation display features similar to those of genes present on the active X chromosome in term of replication timing (20), lack of CpG island methylation

as shown for *MIC2* (67), *ANT3* (201), *UBE1*, and *PCTK1* (36), and possibly hyperacetylation of histone H4 (93).

Although genes known to escape X-inactivation are scattered along the entire X chromosome, many are clustered around the pseudoautosomal region and Xp11. This clustering may indicate that X-inactivation is a regional phenomenon, with entire blocks of genes being coordinately regulated. For instance, *PCTK1* and *UBE1*, whose respective 5′ and 3′ ends map to within 5 kb of each other, both escape inactivation (36). An approximately 1-Mb domain in Xp11.23 includes the *SSX*, *SMCX*, and *DXS423E* genes, all of which escape inactivation (30, 142). This domain, however, also contains genes such as *DXS6672E* and *DXS1008E* that are subject to inactivation (142). More detailed analysis of the clustering along the chromosome of genes refractory to inactivation should allow clarification of whether escape is determined by elements operating at the level of either single genes or whole chromosomal regions.

Several genes that escape from inactivation in man have a functional Y-linked homologue. In such cases, escape from inactivation of the X homologue is thought to assure an equal level of gene expression in males and females. Genes located in the pseudoautosomal region (XpPAR) of the short arm of the human X chromosome, such as *ANT3, XE7, MIC2, XG* (reviewed in 54), and *Il9R* (229), fall into this category. Certain genes located outside the PARs also have functional homologues on the Y and escape X-inactivation [*ZFX*, *RPS4X*, *SMCX*, *AMELX*, reviewed in (54)]. The *SYBL1* gene located in the Xq PAR on the long arm of the X chromosome is, however, subject to X-inactivation (53). Surprisingly, *SYBL1* dosage compensation seems to be maintained through inactivation of the Y-linked copy. Finally, certain genes escape from X-inactivation, but have no functional Y homologue (*KAL1* and *STS*) or no Y homologue at all (*UBE1, SB1.8*). The biological significance of the potentially higher level of expression of these human genes in females is still unresolved.

Genes Escaping Inactivation in Mouse

Only two genes that escape X-inactivation in man, *Sts* and *Smcx*, also escape inactivation in the mouse (24, 54, 188, 202).

Unlike its human homologue, *Sts* maps to the murine PAR and was therefore expected to escape X-inactivation (187). Murine *Smcx* is not a PAR gene (3, 241) but has, like its human homologue, a widely expressed Y-linked homologue. In both cases, expression from the inactive mouse X chromosome suggests only partial escape from inactivation. Precise quantification of the level of transcription of *Smcx* from the inactive X in the adult revealed that transcript levels of the *Smcx* allele carried by the inactive X chromosome are 30%–70% of those for the allele carried by the active X chromosome (194, 37). Moreover, levels of *Smcx* expression from the inactive X homologue varied

from tissue to tissue, and for a given tissue, from one individual to another. The intermediate levels of expression observed are not due to cellular mosaicism (37, 194). The lower levels of enzymatic activity from the inactive X reported for the mouse *Sts* gene in at least certain strains of mouse also suggest that activity levels of less than 50% may be occurring (95, 103). The *STS* locus on the inactive X chromosome in man has similarly been reported to show lower activity than that on the active X chromosome (140). The biological significance of such partial escape from inactivation, and the associated issue of male tissues expressing more *Smcx* or *Sts* than female tissues remains to be addressed.

Observations made on both early mouse embryos and differentiating ES cells have led to the suggestion that *Smcx* may be inactivated at first, and then undergo partial reactivation (37, 194; CM Disteche, personal communication). Analysis of *Smcx* expression in individual cells of the 6.5-day mouse embryos showed that *Smcx* is totally inactivated in some cells, whereas other cells already show escape from inactivation; cells with complete *SmcX*-inactivation persisted in the embryo at least until day 13.5 (CM Disteche, personal communication). It is unclear whether this is something specific to the mouse or indeed to the *Smcx* gene itself.

DEFINITION AND CHARACTERIZATION OF THE X-INACTIVATION CENTER (*Xic*)

General Characteristics

The existence of the *Xic* locus and its localization have been deduced from studies of the X-inactivation status of cells carrying structurally altered X chromosomes (185, 216). In man, the candidate region has been refined to a region lying within band Xq13 on the proximal long arm of the human X chromosome (237). The size of this region is between 660 and 1200 kb (31, 110, 112, 118). In the mouse, the candidate region is defined proximally by Searle's translocation to band XD (174) and distally by the deletion breakpoint HD3 carried by one of a series of ES cell lines analyzed by Rastan & Robertson (177). The murine candidate region so defined is considerably larger than that in man. The overall linkage conservation between the Xq11-Xq23 region of the human X chromosome and part of the mouse X chromosome has led to the assumption that the region as defined in man can be directly extrapolated to the mouse. The mouse *Xic* candidate region so defined contains at least six genes: *XIST/Xist* (see below) (21, 25, 27); *XPCT/Xpct* (E Debrand, E Heard & P Avner, submitted), a widely expressed gene coding for a novel transmembrane transporter protein (111); *BPX/Bpx*, a gene specifically expressed in the brain that contains a domain showing strong homology to genes encoding nucleosome assembly

proteins (184); *CDX4/Cdx4*, a homologue of the Drosophila caudal 4 gene (85); *BRX/Brx*, a gene of unknown function that is preferentially expressed in the brain (196a); and *Tsx*, a gene of unknown function whose expression in the adult is specific to the testis (196; D Cunningham, D Arnaud, D Segretain & P Avner, in preparation).

That portion of the *Xic* region immediately surrounding *Xist* seems to be undergoing particularly rapid evolutionary change. This change affects both *Xist* itself, which has yet to be identified in marsupials, and the nearby *Tsx* gene, which is found in rodents but has yet to be identified in man (196). This genetic plasticity cannot be restricted to coding sequences alone, since the amount of genomic DNA separating both *Xist* from the neighboring *Brx* and *Cdx4* genes and *Xist* from *Xpct* is considerably reduced in size in mouse as compared to man (184, 196, 196a; E Debrand, E Heard & P Avner, submitted)

Of the known genes in the *Xic*, only *Xist* is expressed from the inactive X chromosome. Interestingly, *Tsx*, which lies some 40 kb 3′ to *Xist*, has been shown by an RT-PCR approach to be subject to X-inactivation in the female (D Cunningham, D Arnaud, D Segretain & P Avner, in preparation). The sensitivity of the RT-PCR approach allows detection of low levels of "illegitimate transcription" (47), which appear subject to X-inactivation (64). Elements separating the expression domain of *Xist* that is not subject to inactivation from one that is subject to X-inactivation must lie, therefore, within the 40-kb region separating *Xist* from *Tsx*, a region that is, as already discussed, subject to major evolutionary change.

Defining the Xic *by Transgenesis*

Several laboratories have adopted a transgenesis approach to refine the minimal *Xic* region necessary and sufficient for *Xic* function. Jaenisch and colleagues (115, 116) showed that a YAC covering a 450-kb region, including *Xist*, transfected into male ES cells, upon differentiation not only synthesized *Xist* but also induced inactivation of genes carried by the YAC. Colocalization of the *Xist* transcript and the autosome carrying the YAC transgenes has been observed, by fluorescent DNA/RNA in situ hybridization, to accompany this inactivation and has been termed decoration (116) (see below). These data are compatible with the idea that this YAC was recognized and eventually counted as an intact *Xic*. These results contrast with those obtained by direct microinjection of YACs containing a very similar region around *Xist* into fertilized oocytes to generate transgenic mice. *Xist* expression and X-inactivation were not observed using this approach (75, 130), except for a transgene on the Y chromosome where although *Xist* expression was observed, inactivation could not be assessed (130). One possibility in such in vivo experiments is that embryonic lethality associated with the insertion of a YAC-"*Xic*" transgene into an autosome might have caused the loss of precisely those animals in which the YAC was capable of

inducing inactivation. Partial monosomy might result from the inactivation of the neighboring autosomal sequences.

More recently, a cosmid containing the *Xist* gene itself with only 9 kb of upstream 5′ sequence and 6 kb of 3′ sequence has been transfected into male ES cells (83). *Xist* was not expressed in the undifferentiated transfected ES cell lines. Differentiation was associated with *Xist* expression, which suggests that the genetic elements necessary for regulated expression of *Xist* were present and could be activated on differentiation. Whether all the genetic elements necessary for other aspects of the initiation of X-inactivation, such as counting and choice of chromosome, are present is unclear. The multicopy nature of the ES cell line transgenic insertions leaves some uncertainty as to what is happening, since the interpretation of these results assumes that counting of *Xic* copies on the same chromosome always occurs.

XIC Duplication in Man

XIC duplications in man are an alternative source of information concerning the recognition of multiple *Xic*/XIC copies in *cis*. Several convincing cases of duplications of part of the long arm of the X chromosome that should include the *XIC*, e.g. Xq12-Xq13 (153), Xq12-Xq22 (190), suggest that counting of the two Xics in *cis* does not necessarily occur. The male patient reported by Schmidt is particularly interesting, since the same duplicated X chromosome was present in the patient's mother and underwent inactivation. Thus non-inactivation could not have been due to chromosome duplication occurring after the X-inactivation counting step had been completed. Duplication after the critical period of counting has been suggested to account for cases of functional maternal X disomy (136) and may account for some of the observations made concerning supernumerary X chromosome-derived, tiny ring chromosomes. The presence of such X ring chromosomes is often associated with an absence of X-inactivation, leading to abnormal dosage of X-linked genes on the ring, and abnormal phenotypes and/or mental retardation. Most X ring chromosomes that do not inactivate contain neither the *XIC* region nor *XIST* (92, 137, 240). In a minority of cases, however, the *XIST* gene is present within the ring chromosome but not expressed. Thus either the *XIST* gene alone may be insufficient for X-inactivation and other X chromosome elements not contained in the ring chromosome are necessary, or the rings arose after the onset of inactivation, or were otherwise refractory to X-inactivation (137, 92).

XIST AND X-INACTIVATION

Characteristics of XIST Transcription

Both the human and mouse *Xist* transcripts are large molecules (around 17 kb), ubiquitously expressed in adult female somatic tissues, which are characterized

by a low level of sequence homology (26, 29, 196). The degree of conservation of the murine and human *Xist* sequences is similar to that observed for the human and murine *H19* sequences (77). This rapid sequence divergence could possibly explain the difficulty in identifying a marsupial *Xist* homologue. Both the human and murine *Xist* lack open reading frames of significant length (26, 29).

Many of the genetic elements necessary for regulated expression of *Xist* are present in the cosmid exploited by Herzing et al (83; see above) containing 9 kb of sequence upstream of the *Xist* gene itself and 6 kb of 3′ sequence. Analysis of a 1.2-kb region upstream of the *Xist* gene has defined a minimal constitutional promoter region functioning in mouse fibroblasts, lying between −81 and +1 relative to the transcription start site. A positive regulatory element lying between −41 and −15 was also described, as well as a possible negative regulatory element lying between −441 and −231 (167), and an alternating purine-pyrimidine repeat located 25 kb upstream of the *XIST* promoter showing negative suppressor effects (78). A number of conserved repeat sequences within the 5′ end of the *Xist* gene that have a positive stimulatory effect on reporter gene activity may also play a role in controlling *Xist* expression (78). Four subregions within the minimal promoter region of the transcribed *Xist* allele, including two weak Sp1 sites, exhibit in vivo footprints suggestive of binding of transcription factors (108). The silent *Xist* allele is devoid of such binding.

Xist *Developmental Regulation*

During mouse development *Xist* expression is first detected at the 4-cell stage, at least one day before the first signs of X-inactivation, and continues through the blastocyst and egg cylinder stages and in the adult soma (101, 102), i.e. before, during, and after the initiation of X-inactivation. Expression of *Xist* prior to the egg cylinder stage is imprinted as is X-inactivation itself (see below), with random *Xist* expression and random X-inactivation occurring later (102). Since the low levels of *Xist* transcript seen in undifferentiated ES cells when both X chromosomes are active (16, 206) is restricted to the vicinity of the *Xist* gene itself (160), one would expect, by analogy, the *Xist* transcripts present at the 4-cell stage of female embryogenesis, prior to X-inactivation, to be similarly localized. Specific association of *Xist* with the X chromosome (see below), on the other hand, probably only occurs after X-inactivation (115, 116). Such results imply either that additional factors or alterations in the nature of the *Xist* transcript itself, through, for example, alternative splicing, must be involved in the onset of the inactivation process during embryogenesis.

Evidence for a Role of Xist *in X-Inactivation*

Direct evidence for a role for *Xist* in the inactivation process was first obtained by Penny et al (163), who deleted 7 kb of exon 1 and part of the minimal promoter

sequence of one of the two *Xist* alleles in a female ES cell line. X-inactivation of the targeted X chromosome was disrupted, which demonstrated that the *Xist* gene is required in *cis* for inactivation to occur. The authors suggested that the phenotype of the mutated ES cell line results from secondary nonrandom X-inactivation of the chromosome bearing the nondeleted *Xist* allele. Thus cells that choose the mutant X chromosome for inactivation fail to inactive either X chromosome and are eliminated. Such an interpretation implies that both the counting of X chromosome copy number in the cell and the choice of the X chromosome to inactivate may not involve the *Xist* gene itself, and that *Xist* may only be involved in the propagation step. The phenotype of the *Xist* knockout mice recently described by Marahrens et al (128) has confirmed these results.

It has not been possible to address directly the role of *Xist* in the maintenance of X-inactivation using the mouse *Xist* mutations currently available. To address this question in man, Brown & Willard (32) exploited a series of somatic cell hybrids deleted for various parts of the inactive X chromosome including the *XIC*. It was demonstrated that the inactivated state is apparently maintained in the absence of the *XIC*, which suggests that neither *XIST* nor indeed the *XIC* are necessary to maintain X-inactivation in adult somatic cells (32). Studies of tumor material from two female patients with myelodysplasia, concomitant with the presence of an isodicentric X chromosome with a breakpoint in Xq13 and the loss of the *XIST* gene, have similarly indicated that *XIST* is not necessary to maintain X-inactivation (172).

Mechanisms of Xist *Action*

The lack of any open reading frames of significant length in the *Xist* transcript led to suggestions that it was either *XIST* transcription per se that was important or that *XIST* action was mediated directly through its RNA product (26, 29). The mutated phenotype of a *Xist* knockout in which the functional promoter and overall *Xist* transcription were left intact while several exons were deleted suggests, however, that it is not transcription of the *Xist* gene per se that is important in the inactivation process (128). A role for *XIST* as a functional RNA is, on the other hand, supported by observations that *XIST* RNA not only remains in the nucleus but also often encircles the heterochromatic Barr body (49). An association between *Xist* RNA and the chromosome from which it is transcribed has been observed in transgenes carrying active copies of the *Xist* gene (83, 116), while *Xist* RNA seems not to be directly associated with the chromosomal DNA itself (49). The close association of *XIST* RNA with the nuclear matrix fraction led Clemson and colleagues (49) to suggest that *XIST* may constitute an architectural element, possibly a component of non-chromatin nuclear structure that specifically associates with the territory of the

inactive X chromosome. Transgenesis experiments, however, indicate that *Xist* RNA does not specifically recognize the X chromosome per se, but seems to coat in *cis* the chromosome containing its site of synthesis. This coating in *cis* by the transgenic *Xist* RNA appears to be extensive and continuous, and skipped regions without *Xist* RNA decoration were not observed. Such regions might, perhaps naively, have been predicted on the basis of the only partial spread of inactivation into autosomal genes seen in X-autosome translocations (185) or of the only partial inactivation of the *Yy1* locus, the most distal gene to the site of the YAC transgenic insertion examined (116).

If *Xist* were to bind in a regularly spaced manner in *cis* along the length of the inactive X, i.e. in a pattern consistent with that observed in cells carrying the *Xist* transgene in multiple copies (83, 116), at least 7000 molecules of the transcript would be required (35). Fewer than 2000 copies of *Xist* RNA have, however, been suggested to exist in the female adult kidney cell and cells of the 7.5 d.p.c. embryo (35). Such comparisons, though subject to error, illustrate the need for caution in extrapolating directly the results obtained with the cell lines transgenic for *Xist* to the endogenous *Xist* gene.

Xist *and Gametogenesis*

Based on the concept that X-inactivation in the female might be mechanistically similar to X-inactivation during male meiosis, *Xist* expression was looked for during male gametogenesis (131, 180, 189). The levels of *Xist* transcript detected in the testis were 1000-fold lower than in female somatic tissues (102). Expression of *Xist* during male gametogenesis occurs around day 14 postpartum shortly after the onset of meiosis (D Cunningham, D Arnaud, D Segretain & P Avner, in preparation), although other reports suggest its presence earlier (180). Such *Xist* transcripts may play a role in X-inactivation in the testis, or represent illegitimate transcription, the consequence of methylation erasure from the *Xist* gene prior to imprinted paternal *Xist* expression during early female development. Spermatogenesis appears to be normal in mice carrying a deleted *Xist* gene, as the males were fully fertile (128). Thus either *Xist* is not required for X-inactivation associated with male spermatogenesis, which differs mechanistically from inactivation in the female, or X-inactivation itself is not essential for spermatogenesis.

Although the inactive X in male spermatogenesis is late replicating like its counterpart in female somatic cells (107), further differences between the two X-inactivation processes are suggested by the absence in the male germline of methylation of X-linked CpG islands that are methylated in the female soma. Hyperacetylation of histone H4 on the inactive X chromosome observed in male germ cells undergoing meiosis similarly contrasts strongly with the hypoacetylation of the inactive X in female somatic cells (7).

SKEWING OF X-INACTIVATION

Nonrandom X-inactivation can result from several distinct causes. Primary nonrandom inactivation implies distortion of the randomness of the X-inactivation process itself, whereas secondary nonrandom X-chromosome inactivation implies selection for or against cells carrying the active or the inactive X chromosomes. Nonrandom inactivation in the extraembryonic tissues due to imprinting (see below) and X-controlling element (*Xce*) effects in the mouse fall in the former category.

Skewing in the Mouse: The Xce *Locus*

The X-controlling element (*Xce*) influences in an as yet undefined manner the randomness of the X-inactivation process in the mouse (43). Three alleles at the *Xce* locus have been identified: Xce^a, Xce^b, and Xce^c (195). In Xce^a/Xce^b heterozygotes, the X carrying the Xce^a allele is more likely to be inactivated than is the X carrying the Xce^b allele; similarly in Xce^b/Xce^c heterozygotes, the X carrying the Xce^b allele is more likely to be inactivated in most cells. Most extreme nonrandomness is seen in Xce^a/Xce^c heterozygotes. The two feral mouse species *Mus castaneus* and *Mus spretus* that have been characterized both carry an Xce^c type allele. The possibility that one or other may be carrying a more extreme allele cannot be completely ruled out with the *Tabby*/vibrissae test system routinely used for *Xce* typing (42).

The *Xce* effect is not specific for endogenous X chromosome genes, as inactivation of autosomal genes inserted into the X chromosome is influenced by *Xce* or *Xce*-like effects (38).

A possible interaction between imprinting effects and skewing in adult tissues due to *Xce* or *Xce*-like effects has been noted in several studies (41, 59). The ability of the *Xce* locus to influence imprinted inactivation in extraembryonic tissues remains controversial (175, 233). It may indeed be logical to imagine that *Xce* would not influence X-inactivation when the choice of the chromosome to inactivate has already been predetermined, as in the case of an imprinted chromosome.

Clear evidence that the *Xce* locus exerts its effect at the level of primary nonrandom X-inactivation or chromosome choice, rather than through a secondary cell selection effect was provided by Rastan (173), using differential Kanda staining of the X chromosomes of embryos as early as 6.5 d.p.c.

If *Xce* acts to modify the choice of chromosome to be inactivated, all X-linked markers subject to inactivation would be expected to reflect its action and direct analysis at the transcriptional level should demonstrate the effect of *Xce*. The skewed presence of allelic forms of transcripts at the *Smcx* locus in differentiated cells derived from a female ES cell line heterozygous for *Xce* has been ascribed

to just such an effect (163). A correlation between the levels of skewing seen for the *Pgk1* locus in Xce^c/Xce^a heterozygotes and skewed expression of *Xist* transcripts has similarly been reported by Buzin et al (35). The absolute levels of *Xist* seen in mouse strains carrying different *Xce* alleles may be more difficult to interpret. Reduced levels of *Xist* RNA in *Mus spretus* females compared to C57BL/6 females were ascribed to an *Xce* effect (25), but other groups have failed to see consistent and marked differences in *Xist* RNA levels between strains carrying the Xce^a, Xce^b, or Xce^c alleles (35).

The original mapping data suggested that *Xce* localized to the *Ta-Mo* (*Atp7a*) region, coincident with, but considerably more extensive than, the *Xic* candidate region itself (43). Using three polymorphic microsatellite markers lying on either side of *Xist*, Simmler et al (195) showed that the *Xce* locus cosegregated with these markers, which confirmed the localization of *Xce* within the central part of the *Ta-Mo* region. Analysis of the standard *Xce* typing strains Ju/H and more recently TfH/H have suggested that *Xce* lies distal to *Xist* itself (195; MC Simmler, BM Cattanach & P Avner, unpublished data).

Hypermethylation of a region lying close to the 3′ end of *Xist* varies in different *Xce* strains, suggesting that *Xce* may directly or indirectly affect the methylation status of this region (52). This hypermethylation, like that seen in the 5′ region of *Xist* (see below), is associated with the active X chromosome. It has been suggested that such *Xce*-sensitive methylation may precede X-inactivation itself and could thus be associated with the choice of chromosome to be inactivated (52).

Skewing in Man

Studies of skewing of X-inactivation in the normal human population rely mainly on methylation patterns of polymorphic markers to distinguish the active from the inactive X chromosome. Of the normal female population without known X-linked disease, 5–20% appear to be subject to important skewing effects (17, 60). Indeed, approximately 10% of the population shows skewing as extreme as 90/10 (155). Interestingly, evidence from studies of X-inactivation in manifesting and healthy carriers of dystrophinopathies suggests some degree of concordance in skewing behavior between first-degree relatives. Carrier daughters with markedly asymmetric X-inactivation patterns often had mothers with skewed inactivation, although the active/inactive X chromosome in question was not necessarily the same (10). Naumova et al (155) likewise reported on a three-generation family in which the direction of skewing varied in successive generations. Skewing was associated with the inactive X in the paternal grandmother but with the active X chromosome in all seven granddaughters. Although the data are highly suggestive of an X-linked gene effect, no evidence could be deduced from haplotype analysis for the involvement in

the skewing of a human homologue of *Xce*. Such studies underline the need for caution in attributing to *Xce* cases of skewing in both man and mouse that have not been appropriately mapped to the *XIC/Xic*. Any genetic factor that alters the size of the pool of cells destined to form the embryo proper at the moment of X-inactivation [estimated at between 44 and 47 cells (11)], either directly, or indirectly by changing the time at which inactivation occurs, could induce skewing, just as could genetic determinants that directly influence the randomness of the allocation of cells to particular lineages. The family showing exceptionally high skewing ratios of 99:1, reported in preliminary form by Plenge and colleagues (169), could fall into this category, even though it has been suggested that *XIST* itself is involved.

Intriguing observations have been made about skewing in monozygotic (MZ) twins with discordant expression of sex-linked disorders. Examples of such discordancy in female MZ twins for the expression of X-linked clinical syndromes include red-green color blindness (97), Duchenne muscular dystrophy (179), and fragile X syndrome (221). In such cases, reciprocal skewing of X-inactivation has been observed in the affected and the unaffected twin, with no reported examples of concordant twins (237). Differential uneven X-inactivation probably occurs early in the development of MZ twins since several different tissues often show such skewing effects. One possible explanation is a special relationship between monozygosity and lyonization that could be interrelated in either a temporal or mechanistic manner during embryonic development.

Secondary Skewing Effects Due to Selection

Skewing is often not due to an effect occurring during the primary inactivation process itself but rather to a positive or negative selection for cells expressing the allele carried by one of the X chromosomes (reviewed in 17, 46, 237). In all cases except that of adrenoleucodystrophy (138), the chromosome carrying the mutated allele is selected against. In mouse, similar skewing has been reported in B cells for X-linked immune deficiency (*Xid*). Extreme skewing can result from even a relatively mild selective advantage if the tissue in which the mutated gene is expressed is one in which cell division and turnover are important and lifelong.

An additional important class of skewing by cell selection is provided by X chromosome rearrangements. Nonrandom X-inactivation is usually observed in individuals with structurally abnormal karyotypes involving interstitial or terminal deletions, isochromosomes, ring chromosomes, or translocations. Nonrandom inactivation minimizes the potential genetic imbalance associated with the chromosomal abnormality. In mouse embryos, cell selection normally leads to complete elimination of cells in which the translocation chromosome

is inactivated, because the partial autosomal monosomy induced is only rarely tolerated. This feature has been widely exploited in the mouse with the T16H or Searle's translocation to assess the inactivation status of various X-linked genes (3, 184).

IMPRINTING AND X-INACTIVATION

Preferential inactivation of the paternal X chromosome is found in the extraembryonic tissues of eutherian mammals (212; see above) and in most if not all tissues of marsupials (50). Studies in the mouse suggest that resistance of the maternal X and a predisposition of the paternal X chromosome to inactivation in early development both contribute to this phenomenon. The epigenetic modifications resulting in the parent-of-origin specific differences underlying imprinted X-inactivation probably occur during gametogenesis and may involve modifications to the X chromosome as a whole. An alternative hypothesis, given the importance of *Xist* expression in the initiation of X-inactivation, is that the imprinting seen in X-inactivation is mediated through the *Xist* gene.

Imprinted Xist *Expression*

The expression profile of *Xist* during early mouse embryogenesis is imprinted. *Xist* transcripts detected prior to X-inactivation are of paternal origin and expression of the paternal allele continues in extraembryonic tissues of the female embryo (101, 102). Subsequent random expression of either the maternal or paternal *Xist* allele at 5.5–6.5 d.p.c. coincides with the onset of random X-inactivation and implies erasure of the paternal and maternal imprints by this stage of development. Studies with parthenogenetic and gynogenetic embryos (two maternally derived pronuclei), which carry two maternally derived X chromosomes (XmXm), show that *Xist* is not expressed until the morula or blastocyst stages. The maternal *Xist* allele may therefore carry a silencing imprint prior to this transition (101). In androgenetic embryos (two male-derived pronuclei) with either one or two X chromosomes (XpY or XpXp), paternal *Xist* expression is detected by the 4-cell stage. Since in such XpXp androgenotes, both *Xist* alleles are expressed at this stage, the counting mechanism may not yet be functional. Alternatively, counting may not occur while the paternal imprint is maintained.

Direct evidence for a role for *Xist* in imprinted X-inactivation was recently obtained by Marahrens et al (128) in mice carrying a *Xist* deletion. Female embryos that inherit the deleted *Xist* allele on the maternal X (Xm) develop normally into fertile adults, whereas embryos that inherit the deleted *Xist* allele on the paternal X die soon after implantation. This suggests that the maternally

inherited *Xist* gene is incapable of initiating X-inactivation in the extraembryonic lineage, unlike the paternally inherited *Xist* gene. The defects exhibited by the embryos carrying a mutated paternal *Xist* allele, namely poorly developed extraembryonic tissues, are similar to those found in parthenogenones and embryos with supernumerary maternal X chromosomes. These results are consistent with earlier studies of embryos with maternal X disomy (XmXm) that led to the notion of imprinted resistance of the maternal X chromosome to inactivation early in development (193, 205).

The counting mechanism that maintains one active X per two autosome sets is thus apparently unable to override this maternal imprint. Such data conflict with those from some studies of parthenogenones. Although an inactive X is rarely seen in parthenogenones at the blastocyst stage (204), extraembryonic cells of parthenogenetic 9.5–10.5 d.p.c. embryos contain an inactive X (99, 176, 177). The unresolved question is whether these 9.5–10.5 d.p.c. embryos are exceptional survivors that happen to develop to midgestation precisely because the maternal X can occasionally escape from, or lose its imprint.

Methylation as a Possible Imprint for Xist

If *Xist* expression is involved in imprinted X-inactivation, what might be the imprinting signal(s) involved? Methylation, evoked as a likely mechanism for genomic imprinting in general, is an attractive candidate (178), and there is strong evidence supporting a role for methylation in regulating parent-specific *Xist* expression. The methylation status of *Xist* correlates well with its transcriptional status as manifested by the full methylation of *Xist* found on the active X chromosome in both somatic and extraembryonic tissues (157). In the male germline, sites in the promoter and first exon of *Xist* are similarly fully methylated during early spermatogenesis until 18.5–21.5 d.p.c. (i.e. in perinatal spermatogonia) when they become demethylated, as in mature sperm (6, 245). In the female germline, reactivation of the inactive X chromosome also coincides with the cessation of *Xist* expression (131). Sites in the promoter and first exon of both *Xist* alleles become fully methylated at this stage (6). The maternal *Xist* allele is thus fully methylated in its 5′ region in the oocyte while the sperm *Xist* allele enters the zygote hypomethylated and poised for expression.

In undifferentiated female ES cells, pre-emptive methylation of one of the two *Xist* alleles was thought to mark the X chromosome for inactivation (157), as in the early embryo. This partial methylation is, however, both mosaic and clonally unstable, suggesting that the *Xist* imprint is not fully retained in such cells (143, 186).

Perhaps the strongest evidence that methylation is crucial to the control of *Xist* expression comes from studies of mouse embryos carrying a homozygous

deficiency for the DNA methyltransferase gene. Here, *Xist* is hypomethylated and expressed even in males (16) with evidence for associated X-inactivation (160). Whether it is the global absence of methylation in such embryos or just that of *Xist* that is involved in the control of X-inactivation remains, however, to be determined.

The maternal methylation imprint on *Xist* escapes the global demethylation that occurs between the 8-cell and blastocyst stages (148, 178). Specific erasure, however, may allow for the random *Xist* expression and X-inactivation that occurs in the embryo proper. Interestingly, two other genes that are imprinted, *H19* and *Igf2r*, also retain methyl groups preferentially when DNA methyltransferase is limiting (16, 119).

Predisposition of the Paternal X Chromosome to X-Inactivation

The overall chromatin configuration of the X chromosome derived from the paternal gamete may contribute to imprinted X-inactivation. One early hypothesis was that the inactive state of the X chromosome during spermatogenesis was carried over into the early embryo, predisposing the paternal X to X-inactivation in the earliest differentiating lineages (149). Testis-specific and spermatogenesis-specific proteins appear to associate with the XY body, and histones such as TH2A, TH2B, and H1t are present in spermatocytes but not oocytes (133). Furthermore, in the late stages of spermatogenesis, most histones appear to be replaced by protamines (12). The male-derived X chromosome that enters the zygote may retain such chromatin-specific modifications that could influence imprinted *Xist* expression in the early embryo and also cause the partial suppression of paternal X-linked gene expression that is observed in early embryogenesis.

Paternal alleles of a number of X-linked genes exhibit lower transcriptional activity than do their maternal counterparts during the cleavage stages of embryogenesis, prior to the onset of X-inactivation (reviewed in 90). This partial repression may anticipate the occurrence of paternal X-inactivation in extraembryonic tissues. Clearly, this modification does not definitively mark the paternal X for inactivation, as in XpO females the single X can remain active in extraembryonic tissues (161). This partial repression of the paternal X may have a retarding effect on early mouse development. While adult XO female mice seem to be phenotypically normal and fertile irrespective of the parental origin of the single X chromosome, on closer examination XpO embryos are slightly growth retarded during pre-implantation and immediate post-implantation stages, compared to their XmO and XmXp counterparts, and only catch up later (13, 34, 205).

Species-Specific Differences in Imprinted X-Inactivation

The developmental importance of imprinted X-inactivation seems to vary between different mammalian species. The presence of an extra copy of a maternally inherited X chromosome (Xm) is highly detrimental to early mouse development, probably as a result of the resistance of the mouse X to inactivation in the extraembryonic lineage. In contrast, humans with maternal X chromosome disomy develop normally (9, 171) and in trisomy X and Klinefelter's individuals (XXX and XXY, respectively), the extra X can be of either maternal or paternal origin (reviewed in 237). The imprint on the human maternal X is thus either weak, unstable, or erased before initiation of X-inactivation in progenitors of extraembryonic tissues. Alternatively, complete X-inactivation may not be essential in human extraembryonic tissues.

Imprinted paternal X-inactivation in marsupials also appears to be both incomplete and less stable (reviewed in 70). Such imprinted, but less stable X-inactivation may be more highly conserved than the random, stable X-inactivation observed in the embryonic cells of eutherians. It may therefore represent the primitive mechanism of X-inactivation. The significance of *Xist* in this evolutionary perspective is intriguing, since its apparent absence in marsupials conflicts with its seemingly fundamental role in imprinted X-inactivation, as indicated by the data of Marahrens et al (128).

PERSPECTIVES

Many of the characteristics of the inactive X chromosome described in this review, such as transcriptional inactivation, hypoacetylation of histones, replication delay, and DNA methylation, are physical and biological characteristics associated with the formation of conditional heterochromatin and occur in a number of epigenetically controlled phenomena, including dosage compensation, imprinting, and position effect variegation (reviewed in 79). Transcriptional silencing, for example, is almost universally associated with replication allocycly in epigenetic control phenomena, suggesting that gene expression and replication are tightly linked.

Mammalian X-inactivation in the female, imprinting, and allelic exclusion, however, differ from other epigenetic phenomena in that genes or chromosomal homologues within the same cell are identified and treated differently. In this respect, mammalian X-inactivation is clearly different from the dosage-compensation strategies of the nematode and the fruit fly. Specific mechanisms for establishing monoallelic gene expression, not necessarily shared by other epigenetic systems, would therefore be expected to exist. Methylation is one such characteristic of X-inactivation. A high mutation rate (conversion of 5-methylcytosines to thymines) could, in an evolutionary sense, be the price

that has to be paid, when methylation modification of the DNA is adopted, to stabilize the inactive state. Methylation may therefore be adopted only where both discrimination between alleles within a single cell is necessary and the length of the life cycle or the multicellular nature of the organism confers an overriding premium on the stability of inactivation.

Nuclear compartmentalization, like methylation, provides another possible mechanism by which the discrimination between alleles implicit in different forms of monoallelic expression could be established or maintained. Nuclear compartmentalization (48, 144) could influence the access of the inactive and active X chromosomes to transcription factors and chromosomal proteins implicated in the maintenance of the inactive state.

More recently, a potential role has been suggested for noncoding RNAs in the regulation of chromatin activity associated with several different epigenetic phenomena. Noncoding RNAs in mammals have been implicated not only in X-inactivation, as for *Xist*, but also, as for *H19* (117, 159) and *IPW* (235, 236), in the selective silencing of neighboring genes that exhibit parental imprinting. The role of other recently described mammalian noncoding RNAs, the product of the *His-1* gene (8), *NTT* (121), *gadd7* (84), the DGCR5 RNA associated with the DiGeorge syndrome (203), and the RNA product of the UHG gene (226) all remain to be defined. Interestingly, two noncoding RNAs, *Rox1* and *Rox2* (4, 134), are associated with the chromatin changes underlying dosage compensation in Drosophila. *Rox1* and *Rox2* RNAs seem to associate with the protein products of the four *msl* genes to "paint" the hypertranscribed male X chromosome. Unlike *Xist* transcripts that associate with an inactive X chromosome and only in *cis*, the *Rox-1* and *Rox-2* RNAs associate with portions of the hypertranscribed male X chromosome in both *cis* and *trans*. The nature of the component of the male X chromosome conferring this specificity remains to be identified. Taken overall, noncoding RNAs are likely implicated in the selective regulation of gene expression in a variety of ways. Intriguingly, a family of *Xenopus* RNAs carrying small interspersed repeats, homologous to those present in *Xist*, have been implicated in ensuring the cellular localization of other unique heterologous RNAs. The repeat sequences appear to play a critical role in this process (106). As yet undefined RNAs also seem to be involved in the demethylation occurring during early mouse development that may be involved in establishing site-specific methylation of the type implicated in X-inactivation and imprinting (232).

Underlying similarities between the dosage compensation strategies used by different species may extend further than the epigenetic modifications already discussed. It is likely, for example, that genes on the active X chromosome of mammals are transcriptionally uprated compared to their autosomal

homologues. An example concerns the X-linked *Clc4* gene in *M. spretus* that shows a level of transcriptional activity double of that shown by this same *Clc4* gene when carried on an autosome as in laboratory mouse strains (CM Disteche, personal communication). Hypertranscription may not therefore be the appanage of the *Drosophila* X chromosome alone.

The nonrandom distribution of genes escaping X-inactivation in man and the presence of cytologically identifiable regions of the inactive X chromosome in which histones remain hyperacetylated suggest that the X chromosome may be organized into domains with distinct properties. If genes like *SSX* and *SMCX*, which escape inactivation, were to belong to the same domain as the *DXS1008E* and *DXS6672E* genes, which are inactivated (142), then clearly additional gene-specific characteristics would have to be invoked. Characterization of the inactivation status of additional genes, facilitated by the availability of transcription maps of the human X chromosome, and better biochemical characterization of, for example, histone acetylation of individual genes lying within such postulated domains should allow this issue to be clarified.

Differences between mammals in the X-inactivation process raise interesting questions from an evolutionary standpoint. The recent identification of an X-linked gene involved in mouse placental growth (244), for instance, supports the idea that paternally imprinted X-inactivation might have evolved to avoid sex ratio distortion due to unequal nutrient supply in XX and XY individuals (150). This could explain the seemingly crucial importance of imprinted X-inactivation for extraembryonic tissue development in mice. In marsupials, on the other hand, imprinted X-inactivation may have been maintained as part of a mechanism regulating the dosage of X-linked genes critical for sexual determination (51).

As we understand more about X-inactivation at the molecular level, unforeseen layers of complexity common to imprinting and X-inactivation will likely be discovered and improve our understanding of such questions. The oft quoted schematic classification of X-inactivation into three phases is, for example, likely to prove no more than that—a schema—with molecular mechanisms such as methylation intervening at several distinct levels. Major progress will almost certainly be conditioned by, and will in turn influence, our general understanding of chromatin structure and chromosome function.

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