Primer

X-chromosome inactivation

Mary F. Lyon

It is a well-known fact of popular science that nearly all tortoiseshell cats are female. Underlying this is X-chromosome inactivation, a phenomenon found in all mammals. In the sex-determining system of mammals — in which females carry two X chromosomes and males an X and a Y chromosome — the X chromosome is typically large and carries many genes unconnected with sex, whereas the Y chromosome is small and carries few genes, mainly concerned with spermatogenesis. Thus, females would seem to have two doses of the products of X-linked genes and males only one. X-chromosome inactivation is a dosage compensation mechanism that corrects this disparity.

One of the two X chromosomes in somatic cells of all female mammals becomes transcriptionally inactive early in development. Either the maternally derived or the paternally derived X chromosome can be inactivated at random, and once the choice has been made in any cell, the same X chromosome remains inactive throughout all further cell divisions in the life of that individual. Thus, in the adult there are large clumps of cells with the same active X chromosome.

All females that are heterozygous for an X-linked gene are mosaics of two types of cells, with one or other allele active, and if the gene concerned has a visible effect this can be seen as a variegated pattern. Tortoiseshell cats are heterozygous for an X-linked gene that gives a ginger coat and its normal homologue that gives a black or tabby coat. For X-linked genes that do not give a visible effect, the mosaicism in heterozygous females can be demonstrated by appropriate biochemical or histological tests.

X-chromosome inactivation (XCI) is found in all mammals, including marsupials, but in no other group. Dosage compensation is found in other animals with XX:XY sex-determining systems, including *Drosophila* and *Caenorhabditis elegans*, but its mechanism is different in each group.

Although, in general, XCI can affect either the matenally derived or the paternally derived X chromosome in different cells of the same animal, there are certain types of XCI in which the paternal X chromosome is the inactive one in all cells. This is seen in all cells of marsupials and in some cells of the mouse embryo, and is known as the 'imprinted' type of XCI. The name is derived from 'genetic imprinting', which is another phenomenon in mammals, in which only one homologue of certain genes is active in each cell. Activity is determined by the parental origin of the genes concerned. Imprinting affects individual genes — rather than a whole chromosome, as in XCI — and only a few genes, scattered throughout the genome. The silencing of one homologue is thought to result from an 'imprinting mark' inserted into the genes during gametogenesis, this mark being differential methylation of certain sites in the DNA. As discussed later, the imprinted type of XCI results from differential activity of a key X-linked gene.

XCI has been studied most in humans, mice and marsupials. In early mouse and human embryos both X chromosomes are active. In the mouse, XCI is seen first at the blastocyst stage (the first few days of development), in extraembryonic cells. At this stage XCI is of the imprinted type, with the paternal X chromosome inactive in all cells. Later, at the 6-day egg cylinder stage, XCI occurs in cells that give rise to the embryo proper, and here it affects the maternal X and paternal X randomly. The inactive state is highly stable and reversal occurs only in female germ cells, as they approach meiosis. In male germ cells the single X chromosome is inactivated during early spermatogenesis.

Properties of the inactive X

At the onset of XCI the inactive X chromosome takes on a set of distinctive properties. It replicates its DNA late during S phase of mitosis, it shows hypoacetylation of histone proteins and adopts a condensed state during interphase, when it forms the sex chromatin body. In the embryo proper of eutherian mammals, the CpG islands of genes on the inactive X are methylated — in contrast to the unmethylated CpG islands in the rest of the genome. (But such methylation is not seen in the inactive X of marsupials nor in eutherian germ cells, and probably also not in the extraembryonic lineages.) These properties of the inactive X resemble those of heterochromatin; thus, XCI converts typical euchromatin to the heterochromatic state.

Rare individuals are found with abnormal numbers of X chromosomes — XO, XXX, XXY, and so on — and in these people a single X chromosome remains active, no matter how many are present. By contrast, in individuals with triploid sets of chromosomes either one or two X chromosomes, and in tetraploids two X chromosomes, remain active. Thus, there must be a counting mechanism ensuring that one X chromosome remains active per two autosome sets.

Hence, the initiation of XCI in the embryo involves counting of X chromosomes, choice of which X chromosome remains active and initiation of the inactive state.

Initiation of XCI

The initiation of XCI in the early embryo originates from the X-inactivation centre on the X chromosome. Segments of X chromosome lacking an X-inactivation centre, through deletion or translocation, remain

active. From the X-inactivation centre, a gene termed *Xist* (X inactive specific transcript) has been cloned. *Xist* is transcribed from the inactive X but not from the active X chromosome, and is unique in this respect. Gene knockouts of *Xist* have shown that its transcription is essential for the initiation of XCI. Insertions of transgenes for *Xist* into autosomes have indicated that it is also sufficient for onset of inactivation, in autosomes as well as the X chromosome.

The gene knockouts of *Xist* showed that the counting and initiation of inactivation involve different regions of the *Xist* gene. The 5′ region of the gene is essential for initiation of inactivation, whereas deletion of the 3′ region prevents normal counting. Counting involves the selection of one X chromosome to remain active; if an X chromosome cannot be counted it is always inactivated. The gene knockouts also showed that *Xist* is needed for both the random and the imprinted type of XCI, although it might not be required for the type of XCI that occurs in male germ cells. Thus, the message from the knockouts is that a normal functioning *Xist* gene is needed for inactivation of the X chromosome, and for correct counting and for the choice of which X chromosome will remain active.

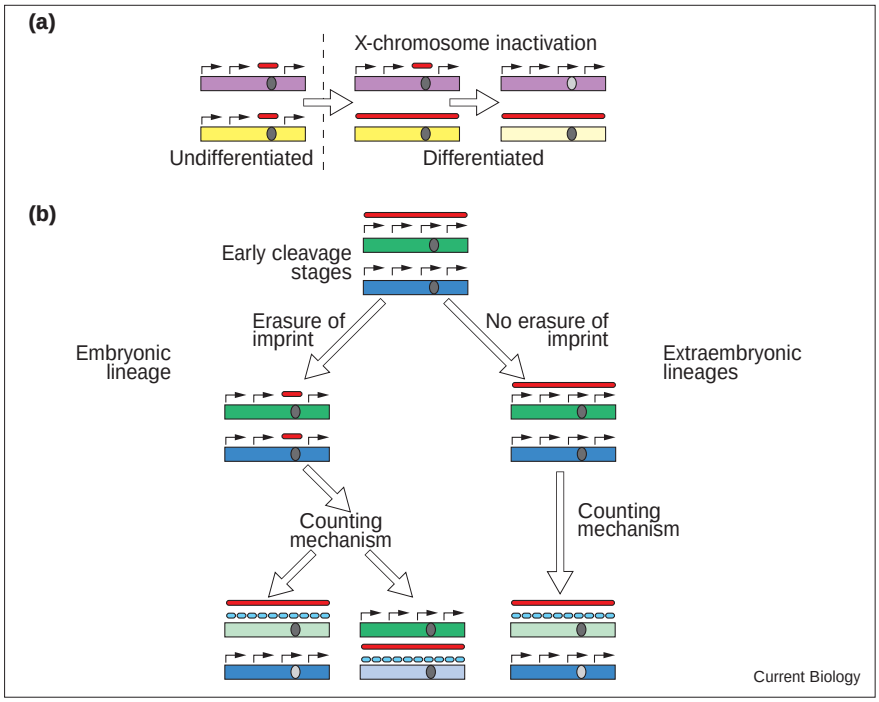
Methylation of *Xist* has been studied as a possible mechanism underlying the imprinted type of XCI but at present the results are controversial and this question remains open. After XCI has occurred, however, the *Xist* gene on the active X chromosome becomes methylated and there is evidence that this methylation is needed to maintain the repressed state of *Xist* and hence the activity of this chromosome.

Xist and the initiation of XCI

The *Xist* gene does not encode a protein but produces a large mRNA of 15–17 kb that is retained in the nucleus and, in cells that have already undergone XCI, seems to ‘coat’ the inactive X. In undifferentiated embryonic stem cells, where both X chromosomes are active, *Xist* expression is seen as a dot of RNA on each X chromosome, and the total level of *Xist* RNA is low. On differentiation, which leads to XCI, *Xist* RNA expands over the future inactive X to coat the chromosome, and the total level of RNA rises. For a short time the dot of *Xist* RNA on the future active X is also visible, but then expression from the active X is extinguished (Figure 1a).

The increased level of *Xist* RNA is due not to increased transcription, but to stabilization of the transcript, the stable and unstable forms being produced from different promoters. Stabilization and localization to the inactive X are separable effects; both are necessary for XCI but they are not sufficient. In early mouse preimplantation embryos there is a large clump of RNA over the paternal X, yet the paternal X remains active at this stage (Figure 1b). Hence, some unknown developmental factor is also needed for XCI. This developmental factor may be associated with counting, which apparently does not occur in very early embryos, where both X chromosomes are active. Possible developmental factors associated with *Xist* RNA include heteronuclear proteins that interact

Figure 1



Changes in *Xist* RNA production at initiation of X inactivation. **(a)** In embryonic stem cells imprinting is absent. In undifferentiated cells both X chromosomes are transcribed (small arrows) and there is a dot of *Xist* RNA (red) over each X-inactivation centre (grey). At differentiation XCI is initiated. *Xist* RNA (red) over the future inactive X (yellow) expands to coat the chromosome, while a dot persists on the active X (purple). Then *Xist* RNA production on the active X ceases and the inactive X takes on its distinctive heterochromatic state (light yellow). **(b)** In embryos, imprinting is present and the effects show that XCI requires a developmental factor (bright blue dashed line) as well as *Xist* RNA (red). In early cleavage stages the paternal X (green) has stable *Xist* RNA but is transcribed. In extraembryonic lineages this proceeds via counting and a developmental factor to inactivation of the paternal X (light green) in all cells. The maternal X is shown in blue. In the embryonic lineage the imprint is lost, and a dot of *Xist* RNA is present on both X chromosomes, followed by random XCI.

with the 5′ region of the gene and a type of histone, macro H2A1, which is concentrated in the inactive X.

Properties of the inactive X

How does *Xist* RNA bring about the heterochromatic properties of the inactive X? Late replication of DNA and hypoacetylation of histones, which are seen in all forms of XCI, are associated in other systems with lack of transcription. Condensation indicates some altered chromatin state, and the presence of histone macro H2A1 implies a different nucleosome structure. But the relation of these properties to each other, and the role of *Xist* RNA in establishing them, remain unknown.

The mechanism of travel of XCI along the X chromosome is also not understood. In X–autosome translocations (where an X chromosome and an autosome exchange segments) XCI spreads into the autosomal segment attached to the part of the X chromosome carrying the X-inactivation centre, and can travel many megabases. Similarly, when an *Xist* transgene is inserted into an autosome its effects spread for a considerable distance. Travel of XCI in autosomes, however, is less efficient and is variable; the altered properties do not always extend to the end of the autosomal segment, and some autosomal genes escape inactivation. That XCI can travel in autosomes shows that X-specific sequences are not required. But the restriction of travel implies that during evolution the X chromosome has become enriched in sequences that facilitate the spread of XCI. The identity of these remains unknown.

The escape of some autosomal genes from XCI suggests that responses of individual genes or groups of genes to the inactivating signal may be important. A small proportion of genes on the X chromosome itself also escape XCI. Some of these have homologues on the Y chromosome and so do not require dosage compensation. In a few cases escape has been shown to be due to reversal of inactivation after it had occurred, but in most cases the possibility of resistance to the original inactivating signal remains open.

Maintenance of the inactive state

The *Xist* gene is transcribed in all cells of adult females, suggesting that it has a role in maintaining the inactive state but this role, if any, is not clear. There is good evidence that methylation of CpG islands on the inactive X of eutherians is important in maintaining XCI. Demethylating agents can partially reverse XCI in cultured cells. Late replication of the inactive X chromosome is also thought to be a maintenance mechanism, with both methylation and late replication being thought to establish self-sustaining feedback loops. In marsupials, which have late replication but no differential methylation, the inactive state is less stable than in eutherians. In most eutherian somatic cells loss of *Xist* does not lead to reactivation of the inactive X. But in some types of eutherian cells that lack differential methylation, loss of *Xist* activity is associated with reactivation. Thus, the maintenance of XCI seems to be complex, with various levels of control, of which *Xist* activity might be one.

Evolution of XCI

XCI apparently arose early in the evolution of mammals. Monotremes show late replication of a part of the X chromosome, which may indicate a rudimentary form of XCI. Prevailing opinion is that the imprinted form of XCI, with the paternal X inactive in all cells, is the primitive form. Both the random XCI and the differential methylation seen in eutherians are regarded as later developments, giving a more complete and stable dosage compensation. Interestingly, the *Xist* gene has not yet been found in marsupials, although it might be present but poorly conserved.

Because of the dosage compensation of X-linked genes, transfer of genes between X chromosome and autosomes in evolution would have harmful results. This led Ohno to suggest Ohno's Law: that a gene X-linked in one mammalian species is X-linked in all. Early in eutherian evolution, however, blocks of genes are thought to have been added to the X and Y chromosomes, followed by spreading of XCI into the newly acquired X-chromosome segments. In the human X chromosome most of the genes that escape XCI are in these 'recently' acquired segments, suggesting that the susceptibility of these regions to XCI is less well evolved.

Unanswered questions

Recent discoveries of the key role of *Xist* in XCI have led to major advances in understanding. But the mechanism by which *Xist* RNA brings about the change of the inactive X to the silent heterochromatic state is still unknown. The identity of the developmental factor required in addition to *Xist* RNA to bring about this change is a key question to be answered. The interaction of the various properties of the inactive X, the role of histone macro H2A1, and the nature of the counting signal, are also major points to be addressed. Thus, despite recent advances the mechanism of X-chromosome inactivation promises to be a fascinating subject for some years yet.

Key references

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Address: MRC Mammalian Genetics Unit, Harwell, Didcot, Oxon OX11 ORD, UK.