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# The William Allan Memorial Award Address: X-Chromosome Inactivation and the Location and Expression of X-linked Genes

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## Introduction

X-chromosome inactivation has now been known for over 25 years, but it continues to provide new insights and ideas both on the nature and location of genes on the X and, particularly, on the control of gene expression, not only on the X but also in general.

## Identity and Location of X-linked Genes

Information on the identity of genes on the X comes from Ohno's (1967) Law, which states that the X chromosomes of all mammals carry homologous genes. The law, which is very well borne out, arose from the idea that the special method of control involved in X inactivation would make it difficult for genes to be exchanged between the X and autosomes during evolution. In man and the mouse knowledge has now reached the point where one can compare the distributions of homologous loci on the X chromosomes of the two species. This comparison shows that one need postulate only very few inversions or similar changes during evolution (Buckle et al. 1985). One group of loci extending from the centromere to the center of the long arm (Xq22) in man are similarly arranged together in the mouse (fig. 1) in bands D-F1. However, the *Hprt* and *G6pd* loci, which are distal to this group in man, in bands Xq26-Xq28, are on the opposite side of them in the mouse, suggesting the occurrence of one inversion (fig. 2). Furthermore, the mouse X has no short arm, and loci found in the human short arm are split into different regions of the mouse X. The *Oct* locus, which is fairly proximal in man, in band Xp21, is near the centromere in the mouse (Lyon et al. 1986*b*), whereas the *Sts* locus and the pairing segment are at the opposite, distal end of the mouse X (Keitges et al. 1985). This situation requires at least one further inversion to have occurred during evolution, altering the relative position of the centromere (fig. 2).

On the assumption that a minimum of two inversions have separated the mouse and human X chromosomes during evolution, it has been possible to postulate the positions of certain loci on the human X, and these predictions have been borne out. Examples include the locations of both the hypophosphatemia locus and the agammaglobulinemia locus. In the mouse, the homologue of agammaglobulinemia, *xid,* lies between *Pgk* and *Ags* (α-galactosidase) (Berning et al. 1980). Thus, it was expected to occupy a comparable position in the human X—and has indeed been found to do so (Mensink et al. 1986). The locus of hypophosphatemia in the mouse lies near the distal end of the X, in a region where the homology changes from one with the human long arm (*Ags*) to one with the distal human short arm (*Sts*). In man it might therefore be expected to lie in either of these regions. It has in fact been located in the distal short arm (Mächler et al. 1986; Read et al. 1986).

The accuracy of certain other predictions remains to be determined. For example, there are two closely linked genes for hypophosphatemia in the mouse, *Hyp* and gyro (*Gy*), the mutant gene *Gy* producing inner-ear defects as well as rickets (Lyon et al. 1986*a*). One might therefore expect that there are also two such loci in man. Thus, affected families may differ in which gene they have. Similarly, in the mouse there is an X-linked ichthyosis called scurfy (*sf*) that is not STS deficient, is accompanied by hypogonadism (Lyon 1986), and is located far from the

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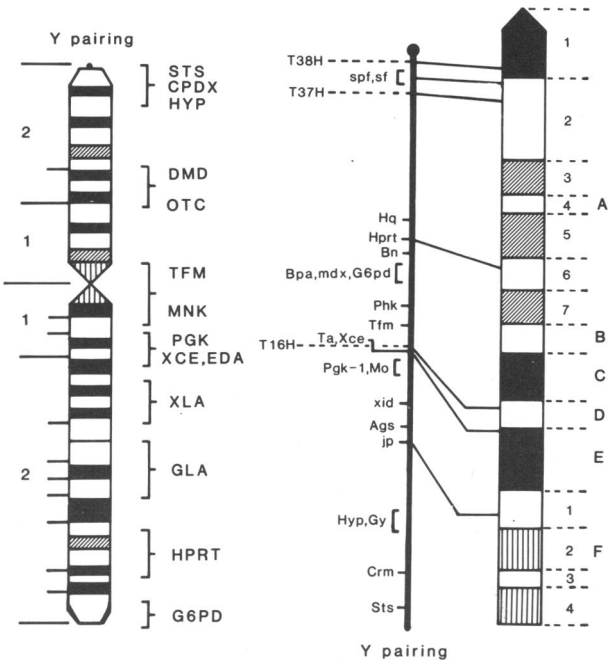


**Figure 1** Relative positions of some homologous loci on human and mouse X chromosomes. Postulated homologues with dissimilar symbols are: chondrodystrophia punctata (CPDX) and bare patches (*Bpa*), Duchenne muscular dystrophy (*DMD*) and muscular dystrophy (*mdx*), anhidrotic ectodermal dysplasia (*EDA*) and tabby (*Ta*), Menkes disease (*MNK*) and mottled (*Mo*), ornithine carbamoyl transferase (*OTC* and *spf*), X-chromosome controlling element (thought to be the inactivation center) (*XCE*), agammaglobulinemia (*XLA*), and X-linked immune defect (*xid*). The conserved central segment extends from *Tfm* to *GLA/Ags*.

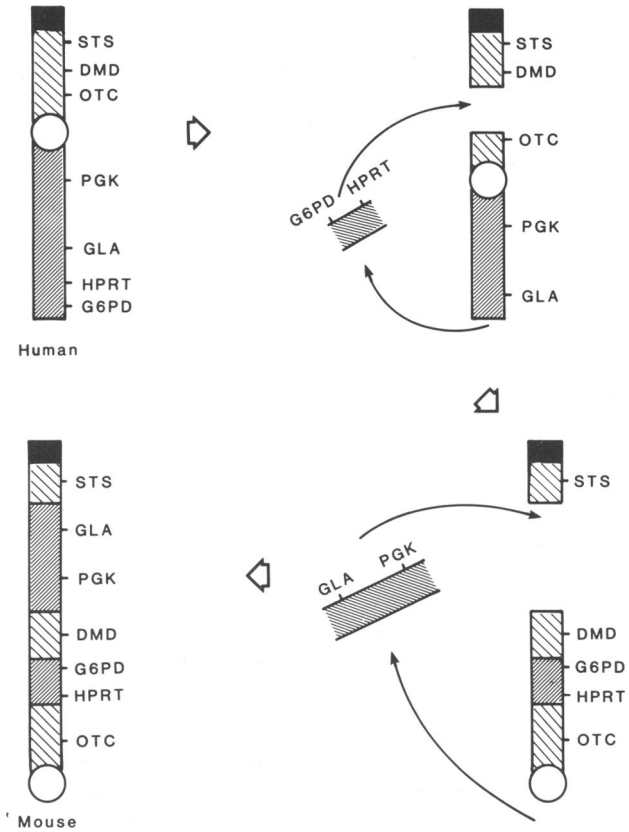


**Figure 2** Diagrammatic representation of change in gene order between human and mouse X chromosomes by means of two inversions. It is not suggested that these particular inversions actually occurred in evolution. The intention is rather to show that on the basis of present knowledge only a small number of rearrangements need to be postulated.

*Sts* locus. Therefore X-linked ichthyosis in man could again be heterogeneous. Indeed, there is already some evidence that this may be so. Some cases are accompanied by hypogonadism, as in the scurfy mouse (Ballabio et al. 1986), and among these some have STS deficiency (Ballabio et al. 1986) and others have not (Munke et al. 1983; Traupe et al. 1984). Perrin et al. (1976) described a large family having X-linked ichthyosis with hypogonadism and showed that the disease was not linked with Xg, suggesting that a locus distinct from the *STS* locus was involved; but no tests for STS activity were done.

With syndromes in which the gene product is not known, it is more difficult to work out whether a particular mouse gene is homologous with a human one, and an example of this problem is muscular dystrophy. The single known X-linked muscular dystrophy locus in the mouse (*mdx*) is situated near the loci of *Hprt* and *G6pd,* and it is now known that the loci of factors VIII and IX are also in this general region (Avner et al. 1987; Mullins et al. 1987). If *mdx* were homologous with Duchenne, one would expect its locus to be near that of *Oct*. Its actual position raises the possibility that it is in fact a homologue of Emery-Dreifuss muscular dystrophy, which is located distally on human Xq (Hodgson et al. 1986). It is also possible, however, as shown in figure 1, that *mdx* is indeed homologous with Duchenne and that there is a third segment of the mouse X homologous with part of the human Xp. This segment would be located somewhere in the B-D region of the mouse X and would include the locus of *Bpa,* postulated to be homologous with chondrodysplasia punctata (Happle et al. 1983), as well as that of *mdx*. It will be interesting to know the results from use of the new probes of Kunkel et al. (Kunkel et al. 1986; Monaco and Kunkel 1987) in the mouse. There is already preliminary evidence that a cDNA

probe for the human Duchenne gene maps near the mouse *mdx* locus (Brockdorff et al. 1987; Heilig et al. 1987).

A difference between mouse and man concerns the *STS* locus. In man *STS* is on the X near, but not in, the pairing segment (reviewed by Shapiro 1985) and is only partially inactivated (Migeon et al. 1982). In the mouse, *Sts* apparently is in the pairing segment, since it has a homologue on the Y and, as expected for the pairing segment, is not inactivated (Keitges et al. 1985). This is interesting, since it suggests that mouse and man are at different stages in the evolution of the pairing segment (Craig and Tolley 1986). As pointed out by Ohno (1967), in the lower vertebrates the sex chromosomes are typically homomorphic. Thus, the heteromorphic X and Y of mammals have arisen by differentiation of the chromosomes during the evolution of the vertebrate phylum, presumably by a gradual reduction of the homologous (or pairing) region. In the mouse it appears that the *Sts* locus remains in the homologous region, but in man the locus of *Sts* is now in a nonhomologous segment. It was predicted very soon after the hypothesis of X inactivation was formulated that genes in the pairing segment would not be inactivated (Lyon 1962), and it is interesting to see this borne out.

## Expression of X-linked Genes

The control of X inactivation during development can be considered under four main aspects as follows: (1) the initiation of inactivation or differentiation of the X chromosomes in the early embryo, (2) the spreading of the inactivation process along the chromosome, (3) the maintenance of the active or inactive state throughout the further life of the animal, and (4) the reactivation of the inactive X in the oocyte or early embryo.

## Initiation of X Inactivation

Early in the study of X inactivation the concept was put forward that the process was initiated from an inactivation center on the X, from which it spread to the rest of the chromosome (Lyon 1963, pp. 67–68; Russell 1963, 1983). However, clear evidence for this has been obtained only recently, from study of X-autosome translocations, particularly in the mouse.

When an X is broken by an X-autosome translocation in the mouse, only one of the two segments shows the late replication (Russell and Cacheiro 1978; Takagi 1980) or dark Kanda staining (Rastan 1983) typical of inactivation. In addition, inactivation only spreads into the autosome from one of the two X-chromosome segments (Russell 1963; Russell and Montgomery 1970). This suggests that only one segment can undergo inactivation (Lyon 1963, pp. 67–68; 1966). This was tested using the translocations T(X;4)37H and T(X;11)38H and the gene sparse-fur (*spf*), which results in ornithine carbamoyl transferase (OCT) deficiency. The X-chromosome breakpoint is near the *Oct* (or *spf*) locus in both translocations. Animals heterozygous for T38H and having the *Oct*-deficient allele on the nontranslocated chromosome showed typical mosaic histochemical staining for OCT in their liver cells, with positive and negative patches (Lyon et al. 1986*b*). However, animals with T37H and the deficient allele on the nontranslocated chromosome were OCT positive in almost all cells. Chromosomal in situ hybridization showed that in T38H the *Oct* locus was distal to the translocation break, on the long marker chromosome, and that in T37H it was proximal to the break, on the short marker chromosome (Lyon et al. 1986*b*). Thus, the interpretation was that in T37H physical continuity between the *Oct* locus and the inactivation center was lost—and hence that the wild-type allele at the locus on the short translocation marker remained active in all cells.

Various conclusions follow from this result. First, it helps to explain some of the abnormalities seen in individuals with X-autosome translocations (Carpenter 1983; Zuffardi 1983). Such individuals will not only have inactivation of some autosomal loci attached to the X-chromosomal segment carrying the inactivation center but will also have excess activity of X-linked genes on the other segment. Obviously, this applies only to that minority of translocations in which a proportion of cells have the translocated X inactive (Carpenter 1983). Second, the result shows that inactivation is a positive process and that the X does not simply spontaneously lose activity. Rather, loss of activity requires some signal, which is lacking when X-chromosomal material is not in continuity with the inactivation center. Furthermore, evidence is provided that spreading of the inactivation signal along the X does indeed occur. Previously the spreading of inactivation to autosomal genes attached to the X was interpretable as being really a position effect that was due to proximity to the heterochromatin of

the inactive X (Cattanach 1974), but here we are dealing with a true X-linked gene.

Initiation, and also reactivation, obviously require specific developmental signals. Experimentally, X inactivation has been initiated in embryonic cell lines, either by allowing them to differentiate (as first shown by Martin et al. [1978]) or by cell fusion with a differentiated cell (shown by Takagi [1983]). Until recently, true reactivation of the whole X (as opposed to one or a few loci) had only been achieved by Takagi et al. (1983), by fusing an adult somatic cell with an early embryonic cell line. Recently, Migeon et al. (1986) have also obtained complete reactivation by fusing a human chorionic villus cell line with mouse cells. The mechanism both for inactivation in the embryo and for reactivation in the oocyte remains unknown. Clearly, something developmentally specific is required, and probably—but not certainly—it involves the activation center.

### Spreading

The nature of the spreading signal also remains unknown. It was suggested in 1975 by Holliday and Pugh (1975), Riggs (1975), and Sager and Kitchin (1975) that spreading might be achieved by a methylating enzyme that ran along the chromosome; and this may indeed be so. Further possible evidence concerning methylation comes from the phenomenon of imprinting.

### Imprinting of the X

Initiation of X inactivation in the mouse embryo occurs at different times in different cell lineages. The earliest lineages are the trophectoderm and primitive endoderm (Takagi 1978; Monk and Harper 1979; Sugawara et al. 1985), and in both of these, as shown by Takagi (Takagi and Sasaki 1975; Takagi 1978), there is nonrandom inactivation, with the paternal X inactive in all cells. It is only in the primitive ectoderm lineage, which gives rise to the future embryo and undergoes X inactivation last (Rastan 1982; Gardner et al. 1985), that there is random inactivation.

The nonrandomness in the trophectoderm and endoderm is said to be due to the maternal or paternal chromosomes or both bearing some imprint that they presumably acquire during gametogenesis (Lyon and Rastan 1984). Imprinting is not confined to the X but is also seen in autosomes (Cattanach 1986), and it is regarded as providing the explanation for inviability of parthenogenetic embryos in mammals. Embryos must have one maternally and one paternally imprinted chromosome set for normal development (McGrath and Solter 1984, 1986; Surani et al. 1986). Study of diploid parthenogenetic embryos that have two maternally derived X chromosomes has shown that X inactivation does in fact occur normally in them, both in the embryo itself (Kaufman et al. 1978) and in the primitive endoderm (Rastan et al. 1980; Endo and Takagi 1981). Similarly, in androgenetic human diploid moles that have two paternally derived X chromosomes, one X remains active (Tsukahara and Kajii 1985); and in $X^PO$ mouse embryos the single paternal X remains active in the yolk-sac endoderm (Papaioannou and West 1981). Thus, the correct imprint is not an essential part of the mechanism of X inactivation. Rather, the effect of imprinting is merely to alter the probability of an X being inactivated. Either a maternal X bears an imprint, increasing its probability of being singled out to remain active, or the reverse occurs in the paternal X (Lyon and Rastan 1984).

### Stability of Maintenance of the Inactive State

X inactivation in the trophectoderm and endoderm lineages also differs in other ways from that in the embryo proper. Liskay and Evans (1980) showed that DNA from the inactive X of adults is modified in such a way that it cannot transfect HPRT-negative cells. However, Kratzer et al. (1983) showed that mouse yolk-sac endoderm DNA can so transfect. Moreover, in both mouse and man there is now evidence of incompleteness or instability of X inactivation in these lineages. In the mouse Krumlauf et al. (1986) have shown that an α-fetoprotein gene introduced into the X of transgenic mice does not appear inactivated in the endoderm lineage, although it is inactivated in adult somatic cells. In the human, Harrison and Warburton (1986) found apparently normal inactivation in chorionic villus cells not cultured in vitro, with the paternal X inactive in most but not all cells. However, Migeon et al. (1985) found that in cultured chorionic villus cells both alleles of G6PD may be active in the same cell—and furthermore, as mentioned above, that fusion of chorionic villus cells with adult somatic cells in culture would cause complete reactivation of the inactive X in the chorionic villus cell (Migeon et al. 1986). Thus, the general picture is that X inactivation in trophec-

toderm or endoderm lineages is rather unstable. A similar association of preferential paternal X inactivation and apparent instability of inactivation is seen in marsupials. Partial activity of the paternally derived G6PD and PGK alleles in cultured fibroblasts is probably due to reactivation (Cooper et al. 1977; Johnston et al. 1978).

### Possible Role of Methylation

A possible explanation of the association of imprinting and relative instability of inactivation in these cases is that methylation of DNA is involved in both phenomena. The exact role of methylation in all the various aspects of X inactivation—such as initiation, spreading, imprinting, stabilization, and reactivation—remains to be elucidated. The evidence that it has some role is clear. The patterns of methylation of specific gene sequences on the active and inactive X chromosomes are different (Lock et al. 1986; Monk 1986), and treatment of cells with the demethylating agent 5-azacytidine can lead to partial derepression of the inactive X (reviewed in Monk 1986). However, the effects of methylation are clearly complex, in that (1) 5-azacytidine does not lead to complete derepression of the inactive X and (2) the specific pattern of methylation of the inactive X includes undermethylation of some sites as well as methylation of others. The possibility of a role of methylation in imprinting is suggested by the work of Chapman and colleagues (Chapman et al. 1984; Sanford et al. 1984, 1985; also see Monk et al. 1987), who showed that the DNA of the trophectoderm and endoderm lineages of the mouse embryo is undermethylated and that marked de novo methylation of DNA occurs in the ectoderm—i.e., embryonic—lineage of the early embryo. It is possible that the imprint is a result of differential methylation of DNA in the male and female gametes. This imprint is then erased by the de novo methylation in the embryonic lineage, and simultaneously the presence of the additional methylated sites in this lineage helps to stabilize the inactivated state.

### Evolutionary Relationship of Paternal and Random X Inactivation

It is interesting to consider the possible functional significance of imprinting. In X inactivation there is the rather unusual situation that two homologues of a chromosome must behave differently in the same cell. Such a situation also occurs in other animal groups, including coccid insects (in which one whole chromosome set becomes inactive in embryos destined to become male) and flies of the genus *Sciara* (in which one or two of three X chromosomes is eliminated from somatic or germ-line cells). In these cases, too, imprinting is seen, again with the paternal homologue inactive or eliminated. In coccids the paternal set becomes heterochromatic or is eliminated in males, and in *Sciara* the paternal X chromosomes are preferentially eliminated (reviewed in Chandra and Brown 1975; Lyon and Rastan 1984). It is possible that imprinting has a selective advantage in these cases. When one homologue of a pair is required to behave differently, there must be the risk of error that both homologues will behave the same, both becoming either active or inactive. If the homologues bear imprints giving them different probabilities of remaining active, the risk of error may be reduced. However, this imprint may, in the mammalian X, also have the concomitant disadvantage of reduced stability of inactivation. In the trophectoderm and primitive endoderm, which persist only until birth in the extraembryonic membranes, this may be a relatively unimportant consequence. However, in the embryo proper, which gives rise to tissues persisting throughout life, the reduction of error through imprinting may have been traded off against a greater selective advantage conferred by the more stable dosage compensation obtained in the random type of X inactivation that is initiated later in embryogeny.

It is also possible that random inactivation has an advantage over preferential paternal inactivation in causing heterozygous females to be mosaics. One advantage could be that heterozygous females would not express the full harmful phenotype of deleterious genes. Those cells with the mutant deleterious gene active are in some cases eliminated during development, e.g., in the case of the blood cells of human heterozygotes for HPRT deficiency (Seegmiller 1976). Another possibility is that the heterozygote might actually have an advantage over either homozygote or hemizygote. This is seen in the resistance to malaria of heterozygotes for G6PD deficiency (Luzzatto and Battistuzzi 1985). Another very recent example of this was the finding of Neitz and Jacobs (1986) on color vision. Human females heterozygous for polymorphic genes for different long-wave photopigments had more accurate color-matching powers than did either type of hemizygous male. Mollon and colleagues (Mollon et al. 1984; Mollon 1986) had

previously found a similar type of effect in new-world monkeys, in which females heterozygous for genes for retinal photopigments had trichromatic color vision whereas males and homozygous females had only dichromatic vision. Thus, both increased stability of dosage compensation and an advantage of a mosaic phenotype in heterozygotes may have been factors in the evolution of random X inactivation.

### Reactivation of X-linked Genes with Aging

However, recent evidence indicates that the stability of inactivation in somatic cells may not in fact be as complete as previously thought. This comes from work on the mouse X-autosome translocation T16H, in which there is nonrandom inactivation with the translocated X active in all cells. Thus, animals with a gene for OCT deficiency on the translocated X and the wild-type allele on the normal X would be expected to show uniformly negative staining for OCT in the liver. In fact, in young animals a few positive cells were seen. The patches were small, as if formed later in development than usual, and this led us to suspect reactivation of the X. We therefore looked at older animals and found that the proportion of positive cells increased with age (Wareham et al. 1987). The patches remained the same size and were formed of a similar small number of cells, but they became more numerous. We concluded that this was due to some form of reactivation. Reactivation had been found before, by Cattanach (1974), again using translocation T16H to give nonrandomness but this time having Cattanach's translocation on the inactive X. He saw apparent reactivation of the autosomal coat color genes involved in Cattanach's translocation. However, attached autosomal genes may behave differently from true X-linked genes, and this may be the first report of reactivation of a true X-linked gene in a eutherian mammal in vivo.

This finding of reactivation opens up many questions that can only be answered in the future. Is this effect peculiar to OCT, or is it found with other genes? If so, what factors are important? OCT is a long distance from the inactivation center. Is the signal for stable inactivation attenuated with distance? Alternatively, is the gene structure important? We know that inactive X-linked genes have specific clusters of methylated sequences (reviewed in Monk 1986). Is the number or location of these important? And, of greater interest, is this reactivation with age a phenomenon peculiar to the X chromosome, or is it, like imprinting, an example of an effect that also occurs with autosomal genes? In other words, is this reactivation an example of a general breakdown of gene control with age, a breakdown that could contribute to carcinogenesis and other problems of aging? In the past X inactivation has provided information about a variety of topics important in medicine, such as the monoclonal origin of various tumors. It is to be hoped that with the further investigation of both reactivation and imprinting—and of their relation to stability of gene control—the X will continue to give us important answers.

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