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SOME MILESTONES IN THE HISTORY OF X-CHROMOSOME INACTIVATION

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INTRODUCTION

Although the discovery of X-chromosome inactivation is now over thirty years old, its mechanism is still largely an enigma. The original discovery came during the period of rapid burgeoning of knowledge in human and other mammalian genetics resulting from expansion of research due to concern over the hazards of atomic radiation after the Second World War. Over the years

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there have been various milestones when knowledge has taken a significant forward step. Now, with recent advances in genetics brought about by DNA technology, we may be reaching another stage when the time is ripe for a major increase in understanding.

THE SETTING FOR THE DISCOVERY

The story began in 1953 when the first mouse X-linked genes, tabby and mottled, were discovered, and the heterozygous females showed variegation. In 1959, chromosomally XO mice were shown to be viable fertile females, indicating that only a single X-chromosome was needed for normal female development in the mouse. At around the same time, the sex chromatin body, discovered ten years earlier by Barr & Bertram, was shown by Ohno to consist of one condensed X-chromosome (reviewed in 25; throughout this paper, wherever possible, reference is made to reviews rather than original articles). It was at this point that the discovery of another X-linked gene giving variegated heterozygotes triggered the formulation of the hypothesis that one X-chromosome was inactivated in the somatic cells of mammals (24). A very similar idea was put forward by Beutler et al (3) to account for the presence of two types of red blood cells in human females heterozygous for X-linked deficiency of glucose-6-phosphate dehydrogenase, and Russell (40) suggested a similar, but rather less complete, explanation for variegation in female mice carrying X-autosome translocations.

THE 1960s: DETAILS OF THE THEORY

After the discovery of the phenomenon, there then followed a rapid period of setting out the details. Within a short time the hypothesis was modified to say not that one X-chromosome was inactivated, but rather that a single X-chromosome remained active, in view of the number of sex chromatin bodies seen in individuals with abnormal numbers of sex chromosomes (e.g. XXX, XXY, XXXX). A problem for the theory was that these individuals showed phenotypic abnormalities, which were particularly severe in human XO females. To take account of this, it was suggested early on that there could be a noninactivated region on the X-chromosome that corresponded with a homologous region on the Y and so would not require dosage compensation (25). Another early development of the theory concerned germ cells. Ohno found that both X-chromosomes appeared noncondensed and presumably, therefore, active in female germ cells. A little later the single X-chromosome in male germ cells was shown to be inactive. Thus, single X-chromosome activity was a feature of somatic cells only (28). Germ cells were postulated to need differing X-chromosome dosage in the two sexes, with sterility resulting from germ cell death in sex chromosome aneuploids.

An early concept concerning the mechanism of X-inactivation was that of an X-inactivation center on the X-chromosome from which inactivation was postulated to spread (26, 41). This idea was suggested to take account of the effects seen in female mice with X-autosome translocations. Inactivation appeared to spread from the X into attached autosomal material, but only into one of the two segments of the translocation. Furthermore, the spread of inactivation into autosomal material appeared limited in comparison with that in the X itself. To understand the nature of the spreading signal it was appreciated that it would be necessary to find the causal relations among the properties of the inactive X: its condensation, late replication, and lack of transcription (27).

THE MILESTONE OF OHNO'S LAW

Thus, ideas on X-inactivation, broadly in line with those accepted today, were already in place in the 1960s, and it may be asked why progress was not more rapid. To answer this question, one must remember how little was known of mammalian genetics, whether of human, mouse, or any other species, at that stage. Suitable genetic variation for study was not available. A milestone came with the formulation of what is now known as Ohno's Law. Ohno (35) put forward the idea that, because of the different dosage relationships of autosomal and X-linked genes, translocations between the X and autosomes that occurred during evolution would be detrimental and would be eliminated. Hence, genes X-linked in one mammalian species would be X-linked in all. This hypothesis opened up a way to find X-linked genes for study of X-inactivation in any species and hence enabled advances in the field. Ohno's Law is now very well established with no exceptions so far known among eutherian mammals. However, there have been interesting findings recently concerning marsupials and monotremes. Genes on the long arm (Xq) of the present-day human X-chromosome are again X-linked in these groups, but genes on the human short arm (Xp) are autosomal both in marsupials and in the monotreme, the platypus, suggesting that these genes have been recruited from autosomes to the X-chromosome during the evolution of eutherian mammals (13). Genes on the human and mouse X-chromosomes have been rearranged relative to each other in evolution and genes from Xp are found in at least three separate segments of the mouse X-chromosome (31). Presumably, the arrangement on the human X is nearer to that on the primitive eutherian X-chromosome.

X-inactivation in Marsupials

One line of work made possible by Ohno's Law was the study of X-inactivation in marsupials, in which the details of the phenomenon are rather different. The first surprise concerned the choice of X-chromosome for inactivation. In

contrast to the random inactivation of either the maternally or paternally derived X-chromosome in different cells of eutherian mammals, in marsupials the paternally derived X-chromosome is inactive in all cells (7, 43). This is an example of chromosome imprinting and was probably the first finding of this phenomenon in mammals. In view of the inactivity of the X-chromosome in male germ cells, the suggestion was made that paternal X-inactivation was the primitive form of the phenomenon, from which random inactivation had evolved (7). Evidence in support of this came when paternal X-inactivation was found in eutherian mammals in the trophectoderm and extraembryonic endoderm cells of rat and mouse embryos (44), and also—but rather less clearly—in human extraembryonic tissues.

X-chromosome Activity in Somatic and Germ Cells

The finding of paternal inactivation in marsupials also opened up the question of the relation of X-inactivation in somatic cells to that in germ cells. The speculation that variations in X-chromosome activity first arose through the need for differing dosages in germ cells, and that inactivation in somatic cells then followed, has attracted various authors. One view put forward by Miklos (34) and others and interestingly discussed by Jablonka & Lamb (17) is that the differing X-chromosome activity states in germ cells reflect a need for the euchromatic, active state for normal meiotic pairing. The presence of unpaired sites is postulated to be deleterious to a germ cell. Thus, where two X-chromosomes are present, in a female germ cell, both must be euchromatic for normal pairing, whereas in a spermatogenic cell, where the single X mainly has no homologues on the Y, inactivation of the X protects the pairing sites from deleterious effects. Such inactivation of sex chromosomes at male meiosis is found in other groups with an XX:XY sex-determining mechanism and thus could have been the primitive system from which somatic X-inactivation arose. Another possibility is that different levels of X-linked gene products are required for male and female germ cells, any level of such products being toxic to a male germ cell, but a two X-chromosome level being needed for female germ cells. As yet there is no evidence in favor of this idea, in that products of X-linked genes can be found in normal male germ cells. However, there remains a need to account for the death of germ cells in XX sex-reversed males. In mice of genotype XX *Sxr* all germ cells die at the spermatogonial stage, whereas in XO *Sxr* mice spermatogenesis proceeds to the late spermatid stage (reviewed in 30). Excess of X-linked gene products seems a possible explanation.

X-inactivation and Sex Determination

Another related question is whether X-inactivation could be in any way involved in sex-determination, by engendering a dosage difference at a critical locus with a homologue on the Y. Chandra suggested that the critical gene

was a regulatory one, with sequences on the Y as well as the X, and which thus, as a result of X-inactivation, achieved different levels of gene product in the two sexes. German and Page et al both favored structural genes with homologous or nearly homologous genes on X and Y (reviewed in 32). As it is now known that the critical Y-linked mammalian sex-determining gene, *Sry*, does not have a homologue on the X (14), these speculations are less interesting. However, the X-chromosome is clearly involved at some point in the sex-determination pathway, as XY sex reversal in wood lemmings depends on an abnormal X-chromosome (42).

THE 1970s: MILESTONES FROM EMBRYOLOGY

Another milestone reached in the early 1970s concerned the mechanism of inactivation, with the idea that the initiation and the subsequent maintenance of the inactive state should be considered separately (29). Initiation was an event that occurred at a specific time or stage in embryogeny and involved the choice of a single X-chromosome for activity and the onset of inactivation in the remainder. It apparently involved a counting mechanism, of the 2A:1X type where A is one autosome set, since there was evidence that two X-chromosomes could be active in triploids, (and now also in tetraploids (45)). Maintenance of X-inactivation might involve some form of feedback mechanism in which the active state of genetical material at replication led to a similar state in the daughter chromosomes. In eutherian mammals, the activity states are highly stable after initiation has occurred. In marsupials, by contrast, individual genes can become reactivated, either in cell culture, or even in some tissues in vivo (7). Thus, at a somewhat later stage, the idea of stabilization was added to initiation and maintenance as a further factor in the X-inactivation process, stabilization being weaker or absent in marsupials (11).

Further insights into X-inactivation came from advances in mammalian embryology. An important question had been whether the X-chromosome was inactive in very early development, so that initiation was in fact the activation of a single X, rather than inactivation. Results obtained by Epstein et al (9) showed that both X-chromosomes of female mice were active in the early mouse embryo and thus that inactivation was indeed a valid term for the phenomenon. An inactive X-chromosome was seen first in the trophoblast cells, which undergo paternal X-inactivation, at about 4-days gestation, and somewhat later in the primitive ectoderm that gives rise to the embryo proper (reviewed in 11). It is of interest in relation to imprinting that the paternally derived X-chromosome is apparently active early in embryogeny; the parental X-chromosome state does not simply persist in the embryo. Furthermore, imprinting in relation to X-inactivation is somewhat different from that in the

autosomes in that the state of the X-chromosome, whether active or inactive, is apparently not fixed by its parental origin. If the paternal X-chromosome, X^p, is the only one present, as in X^pO, it remains active, and in parthenogenetic X^mX^m embryos, the maternal X^m can undergo inactivation, although perhaps at a reduced frequency (11, 31, 32). Evidence from autosomal imprinting is that only specific genes are imprinted. Thus, by implication, in the X-chromosome the imprinted locus is likely to be the inactivation center, and the imprint affects the probability of its receiving the signal that blocks it.

Development of methods for culturing embryonic cells, either as embryocarcinoma (EC) cells or embryonic stem (ES) cells, led to further advances. Different lines of XX cultured EC cells have characteristic states of X-chromosome activity. Some show the typical embryonic state, with both X-chromosomes active, whereas in others, X-inactivation has already occurred, and is maintained in culture. Martin et al (33) found a cell line in which X-inactivation could be brought about by allowing the cells to differentiate. This provided a tool for the study of inactivation, as well as a clue to the mechanism, in that factors bringing about initiation of inactivation must be included among those involved in differentiation.

THE 1980s: MILESTONES FROM DNA TECHNOLOGY

Differential Methylation

Further milestones in the history of X-inactivation have come in the course of the general explosion of knowledge in genetics resulting from recombinant DNA technology. The first advance came with suggestions of the role of DNA methylation. Simultaneously, Riggs (37) and other authors suggested that methylation of DNA might be the mechanism of spreading of inactivation (11, 12). This idea was very tempting, since it appeared to fulfill all the necessary conditions. According to the suggestions, differential methylation was inserted at initiation of X-inactivation by a de novo methylase, which would run along the chromosome, thus providing a mechanism for the *cis*-limited travel of inactivation, affecting one homologue of the X but not the other. Subsequently, at DNA replication, a maintenance methylase would methylate already half-methylated sites. Thus, it appeared that known methylating enzymes could generate the necessary differences for both initiation and maintenance of inactivation. It is now clear that the active and inactive X-chromosomes indeed show characteristic differences in methylation patterns. In particular, the cytosines of CpG islands in 5′ promoter regions of genes are heavily methylated on the inactive X-chromosome (38, 39). Treatment of cultured cells with the demethylating agent 5-azacytidine leads to reactivation of some genes, and the reactivated genes have markedly reduced

methylation. Thus, there is evidence that the differential methylation has a functional effect. However, it is apparently not involved in the spreading process. The evidence for this is that differential methylation is not seen in marsupial X-linked genes. Further, it is apparently not present in the inactive X-chromosome of the extraembryonic endoderm of mice, since DNA from this source can transfect HPRT negative cell lines, and is thus evidently not modified (12, 38, 39). In both these cases, in marsupials and mouse extraembryonic endoderm, the X-chromosome undergoes the preferential paternal type of X-inactivation, and in addition the inactivation is less stable than in the random type of X-inactivation. Reversal of X-inactivation can occur in cultured cells of either type, and even in vivo in marsupials, as already mentioned. Thus, it is at present considered that methylation is part of the mechanism for stabilizing inactivation, after spreading has occurred. In line with this suggestion, Lock et al (22) found that differential methylation did not appear in mouse embryos until some time after the initiation of inactivation, and induction of inactivation in cultured EC cells is not immediately accompanied by methylation (2).

Genes That Escape Inactivation

More recent developments have addressed some of the questions that arose when X-inactivation was first discovered. Studies of gene expression have identified several genes on the human X-chromosome that are not inactivated. These include *STS, ZFX, UBE1* (formerly *A1S9*), *RPS4X,* and *MIC2*. Homologues of some of these genes, *Zfx, Ube1,* and *Rps4x,* have been studied on the mouse X-chromosome, and undergo inactivation normally (1, 20). Thus, this difference in inactivation could account for the difference between human and mouse in the phenotype of XO individuals. In the human, nearly all XO females die prenatally and the few survivors have Turner's syndrome, whereas in the mouse, XO females are normal and fertile. It was suggested early on that this difference could be due to the presence of noninactivated genes on the human X-chromosome, which were required in double dose. A surprise is that some of the human noninactivated genes are not located in the segment of pairing with the Y, where it was originally supposed that genes needed in double dose would be found. Nevertheless, some do have near homologues on the Y. Evidently this work has uncovered differences in the evolution of the X-chromosome in human and mouse. Viable XO individuals have been reported in various species of rodents, in some cases as the normal type (e.g. *Microtus oregoni*; reviewed in 17), suggesting that the situation seen in the mouse may be typical of myomorph rodents. Little is known of X-inactivation in other orders of mammals. There is information so far from man, the mouse, marsupials, and monotremes, and all are different. Presumably, there are further differences to be uncovered among other orders, since at the cytogenetic level

there is much variation (10), potentially providing a rich source of material for study of the details of X-inactivation.

Study of the known differences between human and mouse may provide insight into the phenomena of spreading of X-inactivation. There is the question whether genes respond individually to the spreading signal or in blocks. As found early in the history of X-inactivation, results from X-autosome translocations in the mouse suggest that X-inactivation spreads less readily in autosomal material. This finding led Riggs (37) to postulate "way stations," later termed booster elements (38, 39), along the X-chromosome that promoted the spread. In addition, there may be individual responses of genes to the signal as it arrives. It is of interest that in *Drosophila* the product of a gene concerned in dosage compensation binds at hundreds of sites along the X-chromosome (21). However, the mechanism of dosage compensation in *Drosophila* differs from that in mammals in that there is no inactivation center and X-chromosome segments behave autonomously when translocated to autosomes (23). Detailed study of genes whose inactivation status differs in mouse and man may reveal sites concerned in response to the spreading or the stabilizing signals.

THE 1990s: CLONING THE INACTIVATION CENTER

A further major milestone has been the cloning, in both human and mouse, of the *XIST* gene, a candidate for a role in the X-inactivation center. Over the years, detailed studies of the properties of numerous translocations and deletions had led to precise mapping of the center, to band Xq13 in human and band D in mouse. In the course of a search for genes that failed to undergo inactivation, a human gene *XIST* was found that mapped to the region of the X-inactivation center, and that was expressed from the inactive X-chromosome only, and not from the active X-chromosome (6). Soon after, a homologous gene, similarly mapping to the inactivation center and expressed only from the inactive X, was cloned in the mouse (4, 5). From the combination of its location and its expression, this gene is a strong candidate for a role in the X-inactivation center. A surprise is that *Xist* is expressed in all tissues in the adult. The inactivation center is postulated to be involved in the initiation of X-inactivation, or change of activity state of the X. Such changes take place only at a specific time in embryogeny, associated with differentiation, and at specific stages in development of the germ cells. This suggests that the gene or genes that trigger changes of state at these times remain to be discovered. In the male, *Xist* is expressed only in the germ cells (36), where the X-chromosome is inactive, suggesting that inactivation in the female soma and male germ cell may involve similar mechanisms.

The Blocking Signal

The exact function of the center remains an enigma. One center in each cell receives some signal, which blocks it, resulting in that X-chromosome remaining active. The nature of this blocking signal remains unknown. Over the years suggestions have ranged widely, including an episome, attachment to a specific site in the cell, and a "single informational entity" (11), with recent models involving cooperative binding of activator molecules (11, 12). As X-inactivation is widely thought to involve a 2A:1X balance, the action of an autosomal gene on the X-inactivation center is postulated in various theories. In *Drosophila melanogaster* and *Caenorhabditis elegans,* where autosome:X ratios are again involved in sex determination and dosage compensation, specific autosomal genes involved have been identified (16, 23), and it is tempting to speculate that similar mechanisms may underlie sex determination throughout the animal kingdom. However, in mammals no such genes have yet been found. Jacobs & Migeon (18) studied X-chromosome replication in human fetuses with 18 of the possible 22 autosomal trisomies, and found no anomalies. Light on the role of the center in choice of X for inactivation may come from study of the mouse X-controlling element, *Xce*. This was a discovery of the 1960s, now coming into its own. The *Xce* locus maps to the region of the inactivation center, and there are various alleles that affect the choice of X for inactivation (19). Detailed study of the *Xist* gene in strains with different *Xce* alleles should prove very interesting.

The Spreading Signal

When the blocking of one center has occurred, the next step is the initiation of the spreading signal, presumably by the inactivation center. In considering the possible nature of this signal, one must return to the question posed when X-inactivation was first discovered, of the causal connections among its various properties. The originally known properties included condensation to form the sex-chromatin body, and late replication. To these must now be added differential methylation and differential DNAse sensitivity. As already discussed, methylation is unlikely to be the primary spreading signal, but instead a later factor involved in stabilization. The state of the chromatin fibre and the time of replication have both had their proponents for the primary effect. Condensation to form the sex-chromatin body appears later in embryogeny than late replication (15), and hence it too seems unlikely to be the primary effect. Grant & Chapman (12) and Riggs (38, 39) have pointed out that late replication could provide a self-maintaining feedback system for the maintenance of X-inactivation, if delayed replication prevents transcription and lack of transcription leads to delayed replication. Riggs (38, 39) has proposed that late replication and differential methylation are both self-maintaining mechanisms for stabilizing inactivation. Marsupials have late replication only,

accounting for their less stable X-inactivation, and this would be the more primitive form, with the eutherian mammals having a more advanced form of X-inactivation with both late replication and methylation as stabilizing mechanisms. For the spreading signal itself, Riggs suggests a role for a type of restriction enzyme involving type I DNA reeling. The suggestion is that, beginning from the inactivation center, the enzyme would reel in DNA until it reached a neighboring element, when further activity would be triggered. The travel could occur in both directions and would be *cis*-limited, in accord with the known properties of spreading of X-inactivation. The reeling would bring together scaffold attachment regions (SAR) for chromosome loops. Such a system could produce different conformations of the active and inactive X-chromosomes, and the conformation could be reproduced at replication.

This is a very interesting model, which accounts for most of the properties of spreading. A problem concerns the results from mouse X-autosome translocations, in which inactivation spreads into attached autosomal material for distances of up to several G-bands or many megabases (32). Thus, the signal must be able to travel large distances without a need for X-chromosome specific sequences. The spread into attached autosomal material has often been compared with position effect variegation in *Drosophila,* where genes in euchromatin undergo inactivation when translocated near to heterochromatin, and where again the effect can cover considerable distances. In this instance a protein, heterochromatin protein 1, HP-1, has been found to be associated with position effect variegation (8). It would be interesting to know the role of this or similar proteins in mammals. The model of activation or inactivation of genes by DNA looping has also another relevance. Boundary elements, or scaffold attachment elements, on either side of genes insulate them from the suppressive effect of surrounding chromatin (8). Clearly, this is of relevance to genes that escape or resist inactivation, and to the evidence that the spreading signal can pass through genes but leave them still active. Could these genes have similar insulating boundary elements?

CONCLUSION

Thus, after the passing of several milestones, the state of knowledge of X-inactivation appears, as at the time of its discovery thirty years ago, to have reached a stage set for further rapid developments. On the one hand, there is the cloning of the *Xist* gene, providing a candidate for the X-inactivation center; there are models for the spreading signal, and for the maintenance and stabilization of inactivation; and there are suitable materials for testing models and candidates in the form of embryo cell lines of suitable inactivation status, genes which escape inactivation, and variations among species.

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