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THE HISTORY OF X-CHROMOSOME INACTIVATION AND RELATION OF RECENT FINDINGS TO UNDERSTANDING OF HUMAN X-LINKED CONDITIONS*

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This paper represents the text of two lectures given on the occasion of a Workshop on the X-Chromosome held at Hacettepe University, Ankara, in September 1994. The history of the development of ideas concerning the mechanism of X-chromosome inactivation is traced from the finding of the sex chromatin body in 1949. Important recent findings concern the Xist gene, which is a candidate gene for the X-inactivation center, from which inactivation is initiated. These recent findings shed new light on the abnormalities seen in human patients with X-linked genetic diseases or with aneuploidy of the X-chromosome. *Key words: X-chromosome, X-inactivation, Xist gene.*

Preludes to the Theory:

The first clue that led to the discovery of X-chromosome inactivation came in 1949 when Barr and Bertram found that the cell nuclei of female mammals contained the sex chromatin or Barr body, lying against the nuclear membrane (1-3; throughout this paper wherever possible reference is made to reviews rather than original articles). Human females with Turner's syndrome were found to have no sex chromatin, and this led Paul Polani to the idea that they might have only a single X-chromosome. In the late 1950s good methods for studying human chromosomes became available, and in 1959 Polani with Charles Ford and other colleagues showed that Turner's females were indeed chromosomally XO. In the same year Welshons and Russell found that chromosomally XO mice were not only female, but unlike the human Turner's, morphologically normal. Thus, a female mouse needs only one X-chromosome for normal development. Another important clue in the same year was Ohno's finding that the sex chromation body was formed from a single condensed X-chromosome. Around the same time the first mouse X-linked genes were discovered, and it emerged that female mice heterozygous for X-linked genes affecting coat color or texture showed variegated coats with patches of mutant and normal fur. Putting these various facts together led to the suggestion that one X-chromosome of female mammals becomes

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inactive early in embryonic development[1,2]. Either the maternal or the paternally derived X may be inactivated in different cells. Once inactivation has occurred, the same X remains active throughout all cell divisions in the lifetime of the animal, with the result that in the adult there are large patches of cells with the same X active.

The function of X-chromosome inactivation was suggested to be dosage compensation, in that it resulted in males and females both having effectively a single dose of X-linked gene products. The inactive X-chromosome was not only transcriptionally inactive, but it was also condensed and heterochromatic and replicated its DNA late in the cell cycle.

Thus, the theory was first put forward in 1961, but still today the mechanism of X-inactivation is not understood. The present paper traces the milestones over the years in the development of ideas leading up to the present state of knowledge.

Development of Details of the Theory:

In the first period, in the 1960s, the theory was developed and extended. In the human, many X-chromosome aneuploids were found, such as XXY, XXX, or XXXXY, and in these cases the maximum number of sex chromatin bodies was always one less than the number of X-chromosomes present. Thus, the theory was modified to say not that one X was inactive but rather that a single X remained active. Later, triploids were found first in the human and then in the mouse, and these could have either one or two active X-chromosomes, whereas in tetraploid mouse embryos two X-chromosomes were active. Thus, it was considered that there was a counting mechanism, maintaining one X-chromosome active per two autosome sets[2,3].

Another point concerning X-chromosome aneuploids in the human was the need to explain their malformations, particularly those of XO females. If only one X-chromosome is active, one would expect XOs to be normal, as they are in the mouse, whereas in the human nearly all XOs die prenatally and the few survivors are malformed with Turner's syndrome. The suggestion was made that there might be a pairing segment between the X- and the Y- chromosomes, with homologous genes. For genes in the pairing segment, dosage compensation would not be needed and hence these genes might not be inactivated. The organism would thus need two doses of these genes, and the single dose present in XO individuals might lead to abnormality. It is now known that indeed in the human X, some genes do escape inactivation.

X-Chromosome Activity in Germ Cells:

In the germ cells a different picture was seen. Ohno showed that in female germ cells, at the oogonial or early stage, one X was condensed, just as in somatic

cells, but later, in oocytes, both X-chromosomes looked active. That is, there was reactivation of the X-chromosome. This was confirmed when Gartler showed that in human females heterozygous for the X-linked enzyme glucose-6-phosphate dehydrogenase (G6pd), both alleles were active in the same cell. In the mouse the reactivation was shown to take place just as the cells began to enter meiosis. In male germ cells, by contrast, the single X became condensed and inactive early in spermatogenesis. Thus single-X-chromosome activity is a feature of somatic cells only, and germ cells show a different pattern of activity in the two sexes[3].

The function of changes in X-chromosome activity in germ cells is probably quite different from that in somatic cells and may be involved in meiotic pairing. Miklos[4] and also recently McKee and Handel[5] suggested that the presence of unpaired pairing sites at meiosis is in some way detrimental to a germ cell. The suggestion is that when chromatin is in an active state, it can take part in meiotic pairing, and if any pairing sites do not have a meiotic partner there is a harmful effect. When chromatin is in an inactive state, its pairing sites are in some way protected. Thus, in a female germ cell the reactivation of the inactive X-chromosome may be necessary for meiotic pairing and recombination, and saturation of the pairing sites. In the male germ cell, inactivation of the single X-chromosome is needed to protect the pairing sites which have no homologue on the Y. Such a system could explain the observed effects of X-autosome translocations on male fertility in the mouse. All reciprocal translocations known so far cause male sterility. This could be because inactivation spreads from the X into the attached autosome and inactivates pairing sites there, leaving unpaired and deleterious pairing sites on the homologous autosome. In Cattanach's translocation, on the other hand, there is an insertion of autosomal material into the X chromosome, and inactivation can pass through the insertion. There are two types of male with the Cattanach translocation: those with a corresponding deletion of the autosome, and those with two intact autosomes. Those with the deletion may be sterile and the others fertile, and this can be understood if the pairing sites of the inserted autosomal segment are inactivated. Animals with the deletion would then have unsaturated sites on the homologous autosome, whereas in those with two intact autosomes these sites would be fully paired[6].

There must in addition be other effects of differential X dosage in germ cells. In both the human and mouse, males with more than one X-chromosome, XXY or XX, are sterile due to death of all germ cells at the spermatogonial stage. In the mouse it is known that in such males any germ cells that by chance lose an X and become XO survive and undergo spermatogenesis[7]. The death of XX germ cells occurs too early to be concerned with pairing, and is in the wrong direction. Thus there must be some other effect of double X-chromosome dosage

which is lethal to male germ cells. Conversely, in XO females the rate of loss of oocytes from the ovary is abnormally high, leading to sterility in humans and curtailed reproductive life in mice. This could either be an effect of unsaturated pairing sites or of X gene dosage itself.

Ohno's Law:

Another important development in the 1960s was Ohno's Law[2]. This law states that a gene X-linked in one mammalian species is X-linked in all. Ohno's ationale in formulating this was that, because of single-X activity, if in evolution a gene was transferred by a translocation from the X-chromosome to an autosome or vice versa, there would be a wrong effective gene dosage and so such a translocation would be hamful and thus would be eliminated. There is now extensive evidence that Ohno's Law is true. Genes have been studied in many mammalian species, and the same genes are X-linked in all. This contrasts with the autosomes in which there is much exchange of genes. The two species in which knowledge of chromosome mapping is most detailed are man and mouse. Maps of where the human homologues of mapped mouse genes lie show that each mouse autosome carries genes with homologues on at least three different human autosomes. Thus, the X-chromosome, as Ohno postulated, indeed differs from the autosomes in having its genes conserved throughout mammalian evolution. However, the X-chromosome has clearly undergone rearrangements during evolution. The shapes of the mouse and human X-chromosomes are very different. On the basis of the specific genes carried, the chromosome can be divided into several segments. Within a segment the order of genes has been conserved in evolution, but the segments have been rearranged with respect to each other[8].

X-Chromosome Imprinting:

Ohno's Law has been very valuable, as it has enabled the study of X-linked genes in many species. In particular, it has enabled the study of X-inactivation in marsupials. A surprise, found in 1971, was that in marsupials, rather than random inactivation of one or other X-chromosome seen in eutherian mammals, the X from the father, the paternally derived X, was inactive in all cells[9]. This is an example of genetic imprinting and was probably the first example of this phenomenon found in mammals. Soon afterwards Takagi and Sasaki[10] showed that preferential inactivation of the paternal X-chromosome occurs also in the trophectoderm and primitive endoderm cell lineages of rat and mouse embryos. It may occur also in extraembryonic lineages of humans, but this is a controversial point. In parthenogenetic mouse embryos, in which all chromosomes are maternally derived, both in the embryo itself and in the extraembryonic membranes X-inactivation can still occur. In addition, in XO mice with only a

paternal X-chromosome, this remains active. Thus, the mechanism which maintains a single X active can override the imprint.

In view of the inactivation of the X in male germ cells, it was suggested that paternal X-inactivation might be the primitive type in evolution, and might have evolved from an earlier inactivation of the X in male germ cells[11, 12].

Ohno[13] pointed out that some fish and amphibia do not have distinguishable sex chromosomes and that the easily distinguishable X and Y of mammals must have arisen during the evolution of vertebrates. The suggestion was that in the early evolution of mammals, as the X and Y began to diverge in size there was a need for inactivation of the X-chromosome in male germ cells to protect the pairing sites which no longer had homologues on the Y. Later in evolution this inactivation of the X-chromosome in sperm was carried over into the new zygote at fertilization and formed a starting point for the evolution of somatic X-inactivation.

X-Chromosome Activity in Early Embryos:

Other major advances in understanding of X-inactivation in the 1970s were brought about by increased knowledge of early mammalian embryos. An early question was whether in the embryo the X-chromosomes were inactive and a single one was activated, or the reverse, with both Xes at first active and one being inactivated. Epstein, studying activity of the X-linked enzyme HPRT in early embryos, showed that the latter was correct[3]. In the early female embryo both X-chromosomes are active, and it is indeed correct to speak of inactivation. Nobuo Takagi and Sohaila Rastan and others showed that an inactive X appeared first in the trophectoderm at the late blastocyst stage at about four days and somewhat later, at about five days, in the primitive ectoderm which gives rise to the embryo proper.

Another development in the 1970s was the study of cultured embryonic cells, first embryonal carcinoma or EC cells, and later embryonic stem cells, ES cells. In some lines of EC cells and in most ES cells, both X chromosomes in female cell lines are active. Martin et al.[14] showed that when such cell lines were allowed to differentiate, X-inactivation took place. This was an important step forward because it meant that ES cell lines constituted a suitable system in which X-inactivation could be experimentally studied.

Initiation of X-Inactivation and the X-Inactivation Center:

Another important step was the concept that X-inactivation could be considered as having two main components: initiation and maintenance[3] initiation is the onset of X-inactivation in the early embryo. Once initiation has occurred, each X-chromosome faithfully replicates its active or inactive state at each cell division throughout the life of that animal, and this is the maintenance or stabilization of inactivation. Initiation of X-inactivation is thought to take place through the

X-inactivation center on the X-chromosome. Evidence for this comes from the study of mouse X-autosome translocations.

When autosomal coat color genes are translocated to the X-chromosome, inactivation spreads into the attached autosomal material and causes variegation for these color genes, but it only spreads into one of the two autosomal segments. In addition, only one of the two segments into which the X is broken shows late replication and dark Kanda staining. An example is T37H in which the large translocation product shows dark Kanda staining but the small one does not. This suggests that only one of the X-chromosomal segments is undergoing inactivation. This was studied in the translocations T37H and T38H, both of which have a breakpoint near the sparse-fur or ornithine transcarbamylase (Otc) locus, sparse-fur being a deficient allele of Otc. Animals heterozygous for the translocation and for sparse-fur were bred, and OTC activity was studied by histochemical staining of the liver. Translocation T38H showed the expected mosaic pattern of dark positive cells and light OTC negative cells. With T37H, however, all cells stained positive for OTC, indicating that the normal allele on the translocated X-chromosome was active in all cells[3]. By in situ hybridization it was shown that in the two translocations, the locus of Otc was on opposite sides of the breakpoint. The interpretation was that in T37H the Otc locus is separated from the inactivation center and so is remaining active in all cells.

As already mentioned, there is thought to be a counting mechanism which maintains one X-chromosome active per two autosome sets, and it is suggested that this acts by blocking one inactivation centre. The chromosome with the blocked center remains active. All other centers in the cell (normally one) then initiate inactivation which spreads in a cis-limited manner along the chromosome in both directions. Segments without a center remain active.

By studying different translocations and deletions, the position of the center has been found to be in chromosome band D in the mouse and band Xq13 in the human. From this region a human gene was cloned, termed XIST, or X-inactive specific transcript, with a unique pattern of expression. It is expressed only from the inactive and not the active X-chromosome[15]. The corresponding gene maps to the region of the inactivation center in the mouse also, and shows a similar expression pattern[16, 17]. From both its position and its unique expression from the inactive X-chromosome only, it is thought that Xist must have some important significance in inactivation.

Sohaila Rastan and colleagues studied the expression of Xist in the mouse embryo[18, 19]. It was not expressed at the 2-cell stage, but was expressed at both the 4-cell and 8-cell stages. At first only the paternal allele was expressed, and expression of the maternal allele was not seen until the late blastocyst stage.

In the extraembryonic tissues, where paternal X-inactivation occurs, only the paternal allele was expressed. Others showed that in male mice Xist was expressed only in the testes, and specifically in the germ cells, where the X is inactive[20-22].

In female germ cells, where reactivation of the X occurs, expression of Xist disappears around the time of reactivation[22]. In XX embryonic stem cells Xist was not expressed while the cells were undifferentiated and both X-chromosomes were active, but Xist expression appeared when the cells were allowed to differentiate and X-inactivation occurred[18]. Thus, in various respects the expression of Xist is appropriate for it to have a causal role in X-inactivation. Expression occurs before inactivation, and is imprinted when it first appears and so on. However, in other respects the expression is an enigma. In the embryo it occurs too early, when both X-chromosomes are active, and so some other factor must be needed to actually bring about inactivation. In addition, Xist is expressed in all cells of the adult female, and at present the significance of this is not known.

The sequence of Xist has been studied in humans and mouse by Hunt Willard[23] and Sohaila Rastan[24], respectively. The structure of the gene is well conserved among mammals. In both human and mouse there is no substantial open reading frame. The transcript remained in the nucleus and appeared associated with the inactive X. Thus it appears that Xist does not code for a protein and may act through RNA.

The Role of DNA Methylation in X-Inactivation:

The question is what the inactivation center is actually doing. It is presumed to be initiating the spread of inactivation along the chromosome, and thus one needs to know the mechanism of spreading. In 1975 Art Riggs, Holliday and Pugh suggested that DNA methylation might be the basis of spreading. A methylase might be an enzyme that could run along the chromosome in an appropriate way. Since then evidence has been obtained that the inactive X-chromosome indeed shows differential methylation[3, 25-27].

The CpG islands in the 5' promoter regions of genes on the inactive X are heavily methylated. Treatment with demethylating agents, such as 5-azacytidine, will cause partial reactivation, and the reactivated genes can be shown to have lost their methylation. Thus, the methylation does have a functional effect. However, this methylation is not found in marsupials, nor is it in mouse germ cells, and probably not in mouse extraembryonic lineages, which show paternal X-inactivation[3].

Thus, X-inactivation can occur without methylation of CpG islands. In addition, methylation appears later in embryonic development than the initiation of X-inactivation. Thus, it is thought that methylation of CpG islands is not the spreading mechanism. The various types of X-inactivation in which methylation is not seen are all less stable than in typical random inactivation in the embryo

proper of eutherians. In marsupials, reversal of X-inactivation can occur either in cultured cells or in some tissues in vivo[9]. In female germ cells of eutherians, reactivation occurs as the cells enter meiosis, and in extraembryonic cells reactivation has been reported. Thus, the suggestion is that methylation is involved in the stabilization and maintenance of inactivation, and exactly what is involved in the spreading of inactivation along the chromosome still remains unknown. Recently a new property of the inactive X-chromosome lack of acetylated histone 4, has been discovered[28]. The set of properties of the inactive X, including condensation, late replication and lack of acetylated histone 4, are those of heterochromatin. Thus, the spreading mechanism is causing normally euchromatic material to behave as heterochromatin, but it remains to be discovered how this is brought about.

Relation of Recent Findings to X-Inactivation in Humans:

The recent advances in knowledge of X-inactivation are not only of interest because of the potential insight they may give into the mechanism of inactivation. In addition they help to give better understanding of the findings in humans with X-chromosome aneuploidies or X-linked genetic diseases.

A first point to consider concerns the shapes, sizes and numbers of patches seen in females heterozygous for X-linked genes. If mouse coat color is taken as an example, the pattern in heterozygotes is complicated with some large patches of solid color and other areas where small patches of one or another color intermingle. A similar pattern is seen in chimaeras produced by experimentally aggregating two embryos of different color. Thus, the actual pattern is not peculiar to X-inactivation but is due to the way cells have grown and mingled in development. Certain features of the pattern are that the patches tend to run transversely and not to cross the midline. This can be understood by remembering that the pigment is formed by melanoblasts that migrate out from the neural crest. Each spot is formed by clonal growth of one or more precursor cells, and the different sizes of spots are due to coalescing of various spots of the same color. Different regions of the body may be populated by the precursors of one or a few pigment-forming cells. One gets the best match to the observed pattern by imagining that each region of the body is populated by two precursor cells. If they are both pigmented or both white, a large patch of solid color results. However, if a region is populated by one white and one pigmented precursor cell, the two types of cell mingle as they spread across the body and give a region with many small spots of color[29]. Pigment cells have been used merely as an example. Using other means of marking cells, such as by histochemical staining, one can look at various tissues, and similarly work out whether particular structures are derived from a single precursor cell or many. Dillwyn Williams[30] has used staining for ornithine transcarbamylase and for glucose 6 phosphate

dehydrogenase to study the patches in various mouse tissues. He has found that intestinal crypts are formed from a single cell, but the villi from many precursor cells. Thyroid acini are of multicellular origin, but thyroid nodules are of single cell origin. X-inactivation has been used to study the single or multicellular origin of tumors, but obviously it is important to know that the tissue of origin of the tumor has a multicellular origin before studying the tumor. Thus, in every human X-linked disease, the mosaic pattern of patches varies with the particular cell type involved. One particular pattern of interest is Blaschko's lines, which are lines on the skin in a pattern somewhat resembling that in mouse coat color, and which are seen in females with the X-linked disease incontinentia pigmenti.

X-Choromosome Aneuploidies and Escape of Genes From Inactivation:

In considering the various chromosome abnormalities affecting the X-chromosome, as mentioned earlier one would expect XO females to be normal, as they are in the mouse, but in humans most die prenatally and the few survivors have Turner's syndrome. It was postulated early on that there might be a pairing segment of the X-chromosome carrying genes with homologues on the Y, and that genes in such a pairing segment might escape inactivation, as they would not need dosage compensation. It is now known that there are various genes on the human X that escape inactivation[3, 31, 32], and thus the abnormalities of human XOs may indeed be due to wrong dosage of some of these genes. Some of the genes concerned, such as MIC2, are in the pairing segment, and others such as ZFX and STS are near it. However, others, such as UBE1 and RPS4X, are some distance away. This is of interest in relation to the mechanism of X-inactivation because these genes have normally inactivated genes on both sides of them. This means that the signal for spreading of inactivation must have run past these genes, and either the genes resisted the inactivation or the stabilizing mechanism must have failed so that reactivation promptly occurred. In any case, study of these genes may provide valuable information on spreading.

In the mouse, from the work of Sohaila Rastan and colleagues, the homologous genes, including Zfx and Rps4, undergo inactivation normally[3]. Up to now only one mouse gene is known which unequivocally escapes inactivation[33]. Thus, this provides a plausible explanation for the fact that XO mice are normal, in contrast to human XOs.

Another type of X-chromosome anomaly for which an explanation has recently been found is the small ring-X chromosome. Several females with one normal X and one small ring-X have been found, and these females have shown severe phenotypes with various congenital malformations and with mental retardation[34]. It was not clear why such a small chromosome should produce a severe effect when females with larger deleted X-chromosomes were less severely affected.

Barbara Migeon showed that in these small ring X-chromosomes, in some cases the Xist gene was deleted, and in other cases although it was present it was not expressed[34]. Thus, the interpretation was that the ring X lacked a functional X-inactivation center and was remaining active. Studies of the expression of some X-linked genes showed this to be so[35]. The abnormal phenotype of these females was thus thought to be due to excess dosage of cretain X-linked genes.

Some Causes of Non-Random Inactivation:

1. Imprinting:

In the mouse, an abnormal phenotype thought to be due to excess X-chromosome dosage is seen in animals with additional X-chromosomes, if these are derived from the mother, In the human, XXY or XXX females in which two X-chromosomes are of maternal origin are normally viable, but in the mouse such females die early in gestation. Nobuo Takagi[36] showed that such embryos tended to have two X-chromosomes active in the extraembryonic cell lineages, where imprinting was occurring, and the development of these extraembryonic tissues was abnormal. Thus, this work suggests that the maternal X in the mouse bears an imprint tending to prevent its inactivation in the relevant tissues. In the human, Barbara Migeon[37] has found that either X-chromosome can be expressed in the extraembryonic tissues, and this, together with the viability of X^mX^mY and $X^mX^mX^p$ humans, is part of the evidence that imprinting may not occur in human X-inactivation.

Sohaila Rastan and her colleagues have carried out two pieces of work on the imprinting of the Xist gene in the mouse which are relevant to the question of imprinting of the X-chromosome in humans.

In the first[38] they studied the methylation of sites in the 5' region of the gene. As already mentioned, the inactive X shows differential methylation; in addition, methylation has been suggested as a basis for imprinting, and thus methylation is a reasonable feature to study. In the male, where the Xist gene is inactive, its 5' region was heavily methylated, whereas in the female the allele on the active X was methylated, and that on the inactive X unmethylated. In the male germ cell, the Xist gene showed demethylation in the course of spermatogenesis. Thus, it is possible that differential methylation of Xist in male and female germ cells may constitute the imprint. The allele in the female germ cell, where both X-chromosomes are active, may well be methylated, but this is not known. Thus, there is no conclusive evidence at present that differential methylation does indeed constitute the imprint, but it is a possibility.

In the second piece of work, Kay et al.[19] studied the expression of Xist in parthenogenotes, gynogenotes and androgenotes. In androgenotes, which are

embryos with all their chromosomes derived from the father, and therefore with demethylated Xist, the gene was expressed at the four-cell stage as in normal embryos, whereas in parthenogenotes and gynogenotes, both of which have only maternally derived chromosomes, Xist expression appeared only later at the morula to blastocyst stage. Thus, this provides evidence that the expression of Xist does indeed show imprinting and that the imprint apparently disappears with time. There were also other findings. When expression did appear in gynogenotes, either allele could be expressed, which is as one might expect since both are maternally either allele could be expressed, which is as one might expect since both are maternally derived. In contrast to gynogenotes, androgenotes can be chromosomally of three types: X^pX^p, X^pY, or YY. The YY embryos would be expected to die early because of their lack of X-linked gene products. The evidence indicated that Xist was expressed in all the surviving embryos, both XX and XY. Thus, the counting mechanism which maintains one X active was apparently not functional at this early stage, but must develop later. Thus, what we see is that an imprint is present early, preventing expression of Xist in maternally derived X-chromosomes of gynogenotes and parthenogenotes, and this disappears as development proceeds. Conversely, the counting mechanism is not present at first and appears as development continues. A further finding in androgenotes was that Xist expression was not maintained as development progressed, as it is in normal embryos, but was down-regulated at the eight-cell stage and disappeared by the morula to blastocyst stage. The authors interpret this to mean that there must be a maternally expressed gene involved in maintaining Xist expression in the early embryo[19].

This work raises the question whether the counting mechanism is a relatively late evolutionary development in X-inactivation. In normal embryos such a mechanism is not needed if there is imprinting. Males are always X^mY and would have an active X-chromosome, and females are X^mX^p and would have one active and one inactive X. In eutherian mammals we know of the counting mechanism through many aneuploids and polyploids. In marsupials, far fewer aneuploids are known, and it remains possible that marsupials do not have a counting mechanism but rely on imprinting. It may be that imprinting is the ancestral form of X-inactivation. In eutherian mammals X-inactivation has been delayed until a stage when the imprint has worn off, and a counting mechanism developed in its place, and this is apparently associated with a more stable type of X-inactivation. It may be that in the human embryo X-inactivation occurs relatively later than in the mouse, so that even in the extraembryonic membranes the imprint has been lost.

2. Chromosome Anomalies:

An important feature seen in humans with X-chromosome anomalies such as translocations and deletions is non-random inactivation. Evidence concerning the explanation for this comes from mice. In mice most translocations show

inactivation of one or another X-chromosome, but one, called T16H or Searle's translocation, shows non-random inactivation with the translocated X active in all cells. The result is that mice heterozygous for this translocation, and for a mutant gene, express the allele of the gene on the translocated X only, and show a phenotype like that of a male. The question is whether the actual initiation of inactivation is abnormal, so that only one inactivation center responds, or whether inactivation first occurs at random and then cell selection eliminates cells with the translocated X inactive. Takagi[39] showed that cell selection was the explanation. He studied embryos at the time of inactivation and found some cells with one segment of the translocated X-inactive but the other segment remaining active, presumably through lack of the inactivation center. Thus, such cells have a double abnormality. They have excess X-chromosome activity, and also a deficiency of autosomal gene activity through the spread of inactivation into the autosomal segment attached to the segment of X-chromosome undergoing inactivation. When Takagi looked at somewhat older embryos, cells with the translocated X inactive had disappeared and only cells with the normal X inactive and the translocated X active remained. Thus, by analogy with the mouse situation, it is thought that the non-random inactivation seen in humans heterozygous for X-autosome translocations is again due to cell selection. Similarly, in humans heterozygous for deleted X-chromosomes, the normal X is active in all cells, and again this is thought due to cell selection. In such females any mutant genes on the active X are expressed fully as in a male. This is particularly important in translocations where the translocation break itself may interrupt genes and so cause a mutation. For example, some females affected with Duchenne muscular dystrophy have been found to carry translocations disrupting the gene.

3. Cell Selection in Certain Tissues:

In some cases non-random inactivation is seen only in certain tissues. This is thought to be due to cell selection against cells with the mutant allele active, in those cell lineages in which the gene acts. This is seen for example in females heterozygous for X-linked immunodeficiencies, such as Wiskott-Aldrich syndrome. Here, in heterozygotes, B and T lymphocytes, granulocytes and platelets all have the normal X active in all cells, but erythrocytes and fibroblasts have a mixture of cells with the normal or the mutant X active[40-42].

In diseases where the gene product is not known, as in Wiskott-Aldrich syndrome until very recently, this non-random inactivation is useful in detecting heterozygotes. One looks in the fibroblasts for X-linked markers for which the female might be heterozygous. If in the lymphocytes there is non-random inactivation, detected either by gene expression or by differential methylation, then the female is a heterozygote for the disease.

Another possible cause of apparent non-random inactivation is passage of the gene product from one cell to another. An example is Duchenne's muscular dystrophy. Each fiber in skeletal muscle is multinucleate and may include cells of both types with the normal or the abnormal X-chromosome active. If any cells with the normal X active are present in a particular fiber, dystrophin will be formed and the fiber will look normal. Thus, the proportion of normal-looking fibers is well above 50 percent.

4. Reactivation of X-linked genes:

In the mouse another type of departure from typical inactivation that has been seen is due to reactivation of specific genes occurring in some cells. Typically X-inactivation is very stable in eutherian mammals and it used to be thought that reactivation did not occur in somatic cells, but it is now known that it can occur at a low level. This was seen using the translocation T16H, which as discussed above causes non-random inactivation with the translocated X active. Females were bred heterozygous for the translocation and for sparse-fur, the deficient allele of ornithine transcarbamylase (OTC), with the deficient allele on the active X. On histochemical staining for OTC in the liver one would expect the mutant deficient allele to be active in all cells, and hence the sections should look uniformly negative for OTC. In fact a few positive cells were seen, and these became more numerous with age. This was attributed to reactivation of the Otc gene in some cells[8]. A similar phenomenon had been seen earlier by Cattanach in animals carrying his translocation opposite T16H. Cattanach's translocation carries the normal allele of albino translocated to the X-chromosome, and the animals were bred so that with the Cattanach translocation inactive in all cells, they should have been albino. In fact they had small patches of pigment, and became darker with age. Thus, this phenomenon is not confined to the Otc locus. However, it does not occur with all genes and has not yet been found in man, although the possibility of it occuring with particular X-linked diseases cannot be excluded.

A question that has arisen lately is whether loss of the Xist gene from a chromosome that had already been inactivated would cause its reactivation. Two pieces of work have been done on this. In one case, Brown and Willard[43] took cultured human cells and induced deletions of the Xist gene in them. In the other case, Tony Monaco and Veronica Buckle and colleagues[44] studied two isodicentric X chromosomes found in patients with leukemia, and found these two chromosomes had lost the Xist gene. In both cases these X-chromosomes without the Xist gene remained inactivated. Thus, there is no evidence that the presence of the Xist gene is needed to maintain inactivation once it has occurred.

5. Non-Random Initiation of X-Inactivation:

Another question is whether there can be true departure from random inactivation at the time of the actual initiation. In the mouse this has been found. Cattanach

found a genetic factor termed Xce. Various strains of mice had different alleles of Xce which affected the probability of the X carrying them being the active one. The allele Xce^c was most likely to remain active, with Xce^b next, and Xce^a weakest. The result was that in females that were genetically heterozygous for Xce^c and Xce^a, instead of a 50 percent ratio, 70 percent of cells had the X-chromosome with Xce^c active, and only 30 percent had Xce^a active. This was thought not to be due to cell selection but to be a true effect on the choice of X for inactivation at the time of initiation. The Xce factor maps to the same point as the inactivation center and is thought probably to be part of the center[3]. There is some evidence that the Xce effect might be produced by different alleles of the Xist gene. Kay et al.[18] found that expression of Xist varied in different Xce alleles, with the strongest Xce allele having the weakest Xist expression. This is interesting but not conclusive evidence that Xce and Xist may be different aspects of the same thing. However, Simmler et al.[45] found apparent crossing-over between Xce and Xist, suggesting that they involve distinct genetic loci. In humans, it is possible that some cases of heterozygotes showing non-random inactivation may be due to similar Xce-like effects, but this is very difficult to investigate in humans.

Conclusions:

Thus, consideration of the history of X-inactivation shows that the present time is an exciting one. The situation is poised for possible further important developments, particularly concerning the role of the Xist gene and its relation to the X-inactivation center. Furthermore, recent developments have provided new insights into the abnormalities seen in human X-linked diseases and X-chromosome aneuploidies. It is likely that further understanding will emerge in the near future.

REFERENCES

1. Lyon MF. Sex chromatin and gene action in the mammalian X-chromosome. Amer J Hum Genet 1962; 14: 135-148.
2. Lyon MF. Some milestones in the history of X-chromosome inactivation. Annu Rev Genet 1992; 26: 16-28.
3. Lyon MF. X-chromosome inactivation. In: Wachtel SS (ed). Molecular Genetics of Sex Determination. San Diego: Academic Press; 1994: 123-142.
4. Miklos GL. Sex-chromosome pairing and male fertility. Cytogenet Cell Genet 1974; 13: 558-577.
5. McKee BD, Handel MA. Sex chromosomes, recombination, and chromatin conformation. Cahromosoma 1993; 102: 71-80.
6. Lyon MF. X-chromosome inactivation as a system of gene dosage compensation to regulate gene expression. Prog Nucleic Acid Res Mol Biol 1989; 36: 119-130.
7. Lyon MF. Sex chromosome activity in germ cells. In: Coutinho E, Fuchs F (eds). Physiology and Genetics of Reproduction, Vol 4 A. New York: Plenum Press; 1974: 63-71.

8. Lyon MF. The William Allan memorial award address: X-chromosome inactivation and the location and expression of X-linked genes. Am J Hum Genet 1988; 42: 8-16.
9. Cooper DW, Johnston PG, Watson JM, Graves JA. X-inactivation in marsupials and monotremes. Sem Dev Biol 1993; 4: 117-128.
10. Takagi N, Sasaki M. Preferential inactivation of the paternally derived X-chromosome in the extraembryonic membranes of the mouse. Nature 1975; 256: 640-642.
11. Cooper DW. A directed genetic change model for X-chromosome inactivation in eutherian mammals. Nature 1971; 230: 292-294.
12. Lifschytz E, Lindsley DL. The role of X-chromosome inactivation during spermatogenesis. Proc Natl Acad Sci USA 1972; 69: 182-186.
13. Ohno S. Sex Chromosomes and Sex-linked Genes. Berlin: Springer-Verlag; 1967.
14. Martin GR, Epstein CJ, Travis B, et al. X-chromosome inactivation during differentiation of female teratocarcinoma stem cells in vitro. Nature 1978; 271: 329-333.
15. Brown CJ, Ballabio A, Rupert JL, et al. A gene from the region of the human X-inactivation centre is expressed exclusively from the inactive X-chromosome. Nature 1991; 349: 38-44.
16. Brockdorff N, Ashworth A, Kay GF, et al. Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. Nature 1991; 351: 329-331.
17. Borsani G, Tonlorenzi R, Simmler MC, et al. Characterization of a murine gene expressed from the inactive X chromosome. Nature 1991; 351: 325-329.
18. Kay GF, Penny GD, Patel D, Ashworth A, Brockdorff N, Rastan S. Expression of Xist during mouse development suggests a role in the initiation of X-chromosome inactivation. Cell 1993; 72: 171-182.
19. Kay GF, Barton SC, Surani MA, Rastan S. Imprinting and X chromosome counting mechanisms determine Xist expression in early mouse development. Cell 1994; 77: 639-650.
20. Richler C, Soreq H, Wahrman J. Inactivation in mammalian testis is correlated with inactive X-specific transcription. Nature Genetics 1992; 2: 192-195.
21. Salido EC, Yen PH, Mohandas TK, Shapiro LJ. Expression of the X-inactivation-associated gene Xist during spermatogenesis. Nature Genetics 1991; 2: 196-199.
22. McCarrey JR, Dilworth DD. Expression of Xist in mouse germ cells correlates with X-chromosome inactivation. Nature Genetics 1992; 2: 200-203.
23. Brown CJ. Hendrich BD, Rupert JL, et al. The human XIST gene; analysis of a 17kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell 1992; 71: 527-542.
24. Brockdorff N, Ashworth A, Kay GF, et al. The product of the mouse Xist gene is a 15kb inactive X-sepicific transcript containing no conserved ORF and located in the nucleus. Cell 1992; 71: 515-526.
25. Riggs AD. DNa methylation and late replication probably aid cell memory, and type I DNA reeling could aid chromosome folding and enhanced function. Phil Trans Roy Soc B 1990; 326: 285-297.
26. Riggs AD, Pfeifer GP. X-chromosome inactivation and cell memory. Trends in Genetics 1992; 8: 169-174.
27. Bird AP. The essentials of DNA methylation. Cell (Cambridge, Mass.) 1992; 70: 5-8.
28. Jeppesen P, Turner BM. The inactive X-chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell 1993; 74: 281-289.
29. Lyon MF. Genetic activity of sex chromosomes in somatic cells of mammals. Phil Trans Roy Soc B 1970; 259: 41-52.

30. Thomas GE, Williams ED. The demonstration of tissue clonality by X-linked enzyme histochemistry. J Pathol 1988; 155: 101-108.

31. Migeon BR. X-chromosome inactivation: molecular mechanisms and genetic consequences. Trends in Genetics 1994; 10: 230-235.

32. Ellison J, Passage M, Yu L-C, Yen P, Mohandas TK, Shapiro L. Directed isolation of human genes that escape X-inactivation. Somat Cell Mol Genet 1992; 18: 259-268.

33. Agulnik AI, Mitchell MJ, Mattei M-G, et al. A novel X gene with a widely transcribed Y-linked homologue escapes X-inactivation in mouse and human. Hum Molec Genet 1994; 879-984.

34. Migeon BR, Luo S, Stasiowski BA, et al. Deficient transcription of XIST in females with tiny ring X chromosomes and severe phenotypes. Proc Natl Acad Sci 1993; 90: 12025-12029.

35. Migeon BR, Luo S, Jani M, et al. The severe phenotype of females with tiny ring X chromosomes is associated with inability of these chromosomes to undergo X inactivation. 1994; 55: 497-504.

36. Shaof C, Takagi N, An extra maternally derived X chromosome is deleterious to early mouse development. Development 1990; 110: 969-975.

37. Migeon BR, Wolf SF, Axelman J, Kaslow DC, Schmidt M. Incomplete X chromosome dosage compensation in chorionic villi of human placenta. Proc Natl Acad Sci USA 1985; 82: 3390-3394.

38. Norris DP, Patel D, Kay GF, et al. Evidence that random and imprinted Xist expression is controlled by preemptive methylation. Cell 1994; 77: 41-51.

39. Takagi N. Primary and secondary nonrandom X chromosome inactivation in early female mouse embryos carrying Searle's translocation T (X;16)16H. Chromosoma 1980; 81: 439-459.

40. Gealy WJ, Dwyer JM, Harley JB. Allelic exclusion of glucose-6-phosphate dehydrogenase in platelets and T lymphocytes from a Wiskott-Aldrich syndrome carrier. Lancet 1980; 1: 63-65.

41. Greer WL, Kwong PC, Peacocke M, Ip P, Rubin LA, Siminovitch KA. X-chromosome inactivation in the Wiskott-Aldrich syndrome: a marker for detection of the carrier state and identification of cell lineages expressing the gene defect. Genomics 1989; 4: 60-67.

42. Prchal JT, Caroll AJ, Prchal JF, et al. Wiskott-Aldrich syndrome: cellular impairments and their implication for carrier detection. Blood 1980; 56: 1048-1054.

43. Brown CJ, Willard HF. The human X-inactivation center is not required for maintenance of X-chromosome inactivation. Nature 1994; 368: 154-156.

44. Rack KA, Chelly J, Gibbons RJ, et al. Absence of the XIST gene from late-replicating isodicentric X chromosomes in leukaemia. Hum Molec Genet 1994; 3: 1053-1059.

45. Simmler M-C, Cattanach BM, Rasberry C, Rougeulle C, Avner P. Mapping the murine Xce locus with $(CA)_n$ repeats. Mamm Genome 1993; 4: 523-530.