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INVITED EDITORIAL
Genetic Control of X Inactivation and Processes Leading to X-Inactivation Skewing

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The chromosomal basis of sex determination (i.e., XX in females, XY in males) results in an inequality of gene copy number and content between males and females. In humans (and other mammals) the potential imbalance of gene expression from the two X chromosomes in females is resolved by inactivating one X in all the somatic tissues (Heard and Avner 1994; Willard 1995). Beginning in the late blastocyst stage of embryonic development, one of the two X chromosomes is globally down-regulated in each somatic cell, resulting in expression from only one allele at the vast majority of X-encoded loci. While the paternal X is selectively inactive in the extraembryonic tissues (*vide infra*), in the embryo proper the process of X inactivation is random between the maternal and paternal X chromosomes. The result is that most females have mosaic expression of maternal and paternal alleles of X chromosome loci. The mean contribution from each chromosome is 50%, but because the process is generally random, a normal female may vary considerably from the mean.

It is known, however, that many important disease processes affect the pattern of X inactivation. Extreme deviation away from the expected 50:50 contribution from each X chromosome, or skewing, results, in most cases, from selection against cells with either imbalanced gene expression or mutations that affect cell growth. Faster proliferation or unregulated proliferation (even giving rise to monoclonal outgrowth) due to mutation on the X chromosome may cause X-inactivation skewing. It would also be expected that polymorphisms or mutations that directly affect X inactivation could occur. Genetic analysis of X inactivation has already led to the identification of a unique gene, *XIST/Xist,* whose expression is intimately tied to the inactivation mechanism (Brown et al. 1991*a*). In addition, genetic polymorphism at the *Xce* locus in mice appears to directly affect X inactivation (Rastan 1982). The article by Naumova et al. (1996) in this issue of the *Journal* now reports a unique family in which there is heritable skewing of X inactivation. Several unique features of this family strongly suggest that X-inactivation skewing in the females is caused by mutation at a locus that directly affects one or more steps in the inactivation process. Families have previously been reported in which there was a rare conjunction of an X-linked disorder with nonrandom X inactivation (Ropers et al. 1977; Reddy et al. 1984; Ingerslev et al. 1989; Marcus et al. 1992). The family described by Naumova et al. is the first to be found by simply surveying patterns of X inactivation. Ascertainment of similar families may lead to identification of loci in addition to *XIST* that directly influence X inactivation.

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Molecular Biology of X Inactivation

X inactivation requires several coupled processes—initiation, spreading, and maintenance of inactivation (Willard 1995; Penny et al. 1996). Prior to establishment of X inactivation, an early embryonic cell senses how many X chromosomes are present. Only one may remain active, while the other (or others, as in cases of X chromosome aneuploidy, such as XXX, XXY, XXXXX, etc.) is subject to inactivation. X:autosome ratio is somehow assessed since in triploid cells (69,XXX) two X chromosomes may remain active. Once established, X inactivation is stable so that all the daughter cells of the cell originally undergoing X inactivation maintain the same active X throughout all future rounds of mitotic division. X chromosomal translocations have been used to infer the existence of an X inactivation center (XIC) in Xq13.3, which is responsible for X chromosome counting and initiation of inactivation (Rastan and Brown 1990; Brown et al. 1991*b;* Leppig et al. 1993). Chromosomes without XIC are not subject to inactivation. X:autosome translocations containing XIC may show partial spreading of X inactivation onto the autosomal segment. In interspecific chromosomal hybrids, XIC is not required for maintenance of X inactivation, however (Brown and Willard 1994).

Figure 1 Developmental regulation and imprinting of *Xist* expression. Two asterisks (**) above the X chromosome denotes *Xist* expression; shaded box denotes *Xist* methylation correlating with down regulation of *Xist* expression and imprinting. See text for discussion.

Some genes (>12, so far) escape X inactivation, and, in a survey of cDNAs to identify such genes, a single unique transcript was identified whose expression is restricted to the inactive X chromosome (Brown et al. 1991*a*). Named "X inactivation–specific transcript," or *XIST* (*Xist* in the mouse; Brockdorff et al. 1991; Borsani et al. 1991), its expression with initiation of X chromosome inactivation has now been correlated in numerous studies. *XIST* maps within the XIC in both mouse and man. *XIST* encodes a 15–17 kb transcript that is conserved between mouse and humans but does not apparently code for a protein (Brown et al. 1992). *XIST/Xist* RNA is localized predominantly within the nucleus (Brockdorff et al. 1992) and is associated with the Barr body in human interphase nuclei.

A series of recent studies cement the relationship between initiation of X inactivation and *XIST/Xist* expression (see fig. 1). X inactivation and *XIST* expression are both imprinted by parental origin and developmentally regulated. Methylation of regulatory sites correlates with decreased or absent transcription in many X-linked genes. The *Xist* gene is hypermethylated at multiple CpG dinucleotide sites in both 5′ and 3′ elements as well as some sites within the gene in male somatic tissue (Kay et al. 1993). In females, methylation of *Xist* is incomplete but consistent with methylation of one allele (presumably the active X) in each cell. *Xist* becomes demethylated during spermatogenesis (again correlating temporally with onset of *Xist* expression) at the same time that the single X chromosome is thought to be silenced (Kay et al. 1994). In the mouse, *Xist* expression is first detected at the four-cell stage of embryogenesis, while completed X inactivation itself is not observed until differentiation of the extraembryonic trophectoderm (Norris et al. 1994). In this tissue, there is preferential inactivation of the paternally derived X chromosome, indicating that the inactivation process is imprinted. Detailed examination of the imprinting phenomenon has been highly informative, since it confines the X inactivation process to one chromosome and allows correlation to be made concerning the state of the antecedent chromosome prior to onset of *Xist* expression and X inactivation. In an elegant series of experiments, Kay et al. (1994) and Norris et al. (1994) demonstrated coupling of *Xist* expression and imprinting. They showed that *Xist* expression occurs from both alleles in X^PX^P androgenones (i.e., embryos derived from removal of the female pronucleus and replacement with a second male-derived pronucleus), indicating that the paternally derived X chromosomes are programmed to be permissive for *Xist* expression. The paternal X's seem to escape the X chromosome–counting process,

which allows one chromosome to remain active through repression of *Xist* expression. Zuccoti and Monk (1995) and Ariel et al. (1995) showed that *Xist* is hypermethylated and not expressed in ova. Maternal *Xist* remains methylated in gynogenones (embryos consisting of two maternally derived pronuclei) until it is expressed at blastocyst stage (Norris et al. 1994). These observations are all consistent with the idea that the imprinting signal is mediated by methylation at the *Xist* locus and that inhibition of *Xist* expression in the female germ line (or, alternatively, permissiveness in the male germ line) is crucial to selection of the active X in the extraembryonic tissues. Which chromosome is actually imprinted is a matter of semantics, since it appears that differential methylation plays a key role in producing differential gene expression from the two chromosomes. The methylation pattern is probably erased in the embryo proper, allowing for subsequent random initiation. The counting mechanism seems to allow one X to remain active, but all others are caused to express *Xist* and to be inactivated.

Recently, Penny et al. (1996) reported the creation of a 7-kb deletion in *Xist,* resulting in a null allele for expression. In female-derived embryonic stem cell clones, the X chromosome bearing this allele fails to inactivate but allows the X bearing the normal *Xist* allele to undergo inactivation. This result shows that an intact *Xist* gene is required in *cis* for inactivation to proceed. Although preferential inactivation of the normal X may result from disruption of the mechanism that chooses one X chromosome to remain active (primary nonrandom inactivation), Penny et al. favor the idea that cells that choose the mutant X for inactivation fail to inactivate either X and are then eliminated because of abnormal gene expression (secondary nonrandom inactivation). If the latter case were true, it would mean that *Xist* expression can be uncoupled from the counting mechanism. Nevertheless, the data directly implicate *Xist* in the spreading of X inactivation. Norris et al. (1994) and Lyon (1996) have proposed that *Xist* expression is regulated by *cis* sequences in XIC that are controlled by a trans-acting DNA-binding factor. Binding of the factor(s) would initiate *Xist* expression and begin the process of X inactivation. Methylation would inhibit the binding of the factor and prevent *Xist* expression, allowing that X to remain active. The mechanism by which a single X is selected for activation is a matter of conjecture but could result from either limiting amounts of the putative trans-acting factor or a specific chromosomal interaction that blocks *Xist* from transcriptional activation, for example.

The phenotype of rare individuals mosaic for small ring X chromosomes (usually 45,X/46,Xr(X)) is interesting and potentially informative about genetic control of the X-inactivation process (Migeon et al. 1993; Wolff et al. 1994). These patients typically have birth anomalies and mental retardation that is quite different from Turner syndrome. A unifying abnormality is the failure to express *XIST*, and so it is felt that the severe phenotype is due to abnormal overexpression of the remaining genes on the ring chromosome. *XIST* sequences are absent from some of r(X) chromosomes, but in others the *XIST* gene is present and fails to be expressed. Abnormal *XIST* expression from such chromosomes suggests the necessity of additional *cis* sequences for proper engagement of the X-inactivation process.

X-Inactivation Skewing

In a population of women, the mean contribution from each X chromosome to the active pool in somatic tissues is ~50%, but in individuals one may observe substantial deviations (Fialkow 1973; Puck et al. 1992; Allen et al. 1994; Fey et al. 1994). Most studies have been carried out on cells or DNA from the peripheral blood, but similar results have also been observed when other tissues have been sampled. Depending on the definition and quantitative accuracy of the measurement method, 5%–20% of apparently normal women have constitutional skewing of X inactivation. If, at the time of X inactivation, only one cell in the embryo was fated to be the precursor of the hematopoietic lineage, one would observe either one or the other X chromosomes active in all the cells. Under the assumption of a purely stochastic process, the variance of the distribution of X inactivation in humans suggests that 10–20 cells contribute to the pool of embryonic cells that go on to form the definitive hematopoietic system (Fialkow 1973; Puck et al. 1992; Allen et al. 1994). By other criteria, it is known that many more committed hematopoietic precursors are active simultaneously, so the most likely explanation is that the inferred small pool of cells represents the primitive mesodermal precursors that were fated to become the mature blood-producing system. No comparable data is available for other tissues, and there is currently no data concerning a systematic comparison of X-inactivation patterns in various tissues.

Females manifesting classical X-linked recessive traits are occasionally seen (reviewed in Willard 1995). This circumstance is most often attributed to "unfortunate Lyonization." Recently, this subject has been more formally investigated in manifesting carriers of Duchenne muscular dystrophy (DMD; Pegoraro et al. 1994; Azofeifa et al. 1995). Virtually all such individuals show skewing of X inactivation in peripheral blood. Whether such skewing is always purely stochastic is an unsettled question, but the paternal origin of all DMD mutations in the study by Pegoraro et al. (1994), the correlation of skewing with maternal patterns in the study of Azofeifa et al. (1995), and rare reports of familial clustering

of manifesting DMD carriers are all suggestive of additional modifying loci (Reddy et al. 1984).

Measurement of X Inactivation in Humans

To measure X inactivation one must distinguish between the two chromosomes and determine whether the products of those chromosomes are equally active. Evaluation of replication timing is an excellent quantitative procedure but is only applicable when there is a structural difference between the two chromosomes that allows them to be distinguished, for example, an X:autosome translocation (Boggs and Chinault 1994). Expression of glucose 6-phosphate dehydrogenase (G6PD) has been used (Gartler et al. 1969; Fialkow 1973) but is limited by the fact that the G6PD polymorphism is rather rare in the Caucasian population (~1% heterozygosity). The production of rodent/human hybrids is a very reliable and quantitative technique for studying X inactivation. In this assay human blood cells are fused to mouse or hamster fibroblasts that are deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT) (Gorski 1991; Puck et al. 1992).

The most widely applicable approach to analysis of X inactivation is to detect differences in methylation on the active and inactive X. Methylation at the HPRT (Lock et al. 1986, 1987) and phosphoglycerate kinase (PGK) loci (Singer-Sam et al. 1990) has been found to correlate with X inactivation. The RFLPs adjacent to these CpG islands are heterozygous in ~35%–40% of women. Testing of X inactivation can be conducted by Southern analysis (HPRT or PGK) or by PCR (PGK). Another X chromosome marker used for X-inactivation analysis, DXS255 or M27β, is much more polymorphic than either HPRT or PGK. This marker defines a VNTR polymorphism with 90% heterozygosity. At DXS255 the active X chromosome is hypermethylated (Boyd and Fraser 1990). In a small percentage of women the methylation pattern does not seem to be consistent with X inactivation (Cachia et al. 1992; Fey et al. 1994). The human androgen receptor (AR) locus contains a highly polymorphic (90% heterozygosity) trinucleotide repeat in the first exon. This repeat is within ~100 bp of two *Hpa*II and HhaI restriction enzyme sites that are methylated on the inactive X chromosome but unmethylated on the active X (Allen et al. 1992). As exploited by Naumova et al. and others, this marker lends itself to large-scale studies because of its high informative heterozygosity and because it is easily quantitated.

Negative Selection

Balanced X:autosome translocations are associated with skewing of X inactivation (Zabel et al. 1978). In general, there is preference for inactivation of the normal chromosome, since spreading of inactivation into the autosomal segment would give rise to a functional monosomy. Presumably, cells with a severe degree of gene imbalance are at a selective disadvantage and cells with balanced expression either outgrow them in the early embryo or they die outright. Recent reconsideration of X:autosome translocations associated with hypomelanosis of Ito (probably includes all cases of incontinentia pigmenti type I) leads to the conclusion that functional disomy for the involved X is also deleterious (Hatchwell 1996; Hatchwell et al. 1996). The finding of the normal X active within affected areas of the skin suggests that selection against the functionally disomic cells occurs relatively late in embryonic development. Such patients typically have severe skewing of X inactivation in the blood because of continuing intense selective pressure. In individuals with unbalanced X:autosome translocations, one observes skewed X inactivation and attenuation of the severe phenotype potentially associated with the partial autosomal trisomy (Kulharya et al. 1995). The translocated X is selectively inactivated with spreading of inactivation to the autosomal segment.

Extreme skewing of X inactivation can also result from single gene mutations that affect cell survival or growth. It is worth noting that even relatively mild selective advantage can lead to severe skewing in blood cells, because hematopoiesis is continuous throughout life; because many blood cells have intrinsically short life spans and high turnover; and because a large number of mitoses occur between the active precursor pool and the terminally differentiated cell. Examples of recessive X-linked gene defects that cause complex somatic phenotypes in affected males and also give rise to skewed X inactivation in the blood of asymptomatic carrier females are HPRT deficiency, X-linked α-thalassemia with mental retardation syndrome (ATRX) (Gibbons et al. 1992), incontinentia pigmenti type 2 (Harris et al. 1992), focal dermal hypoplasia (Gorski 1991), and X-linked dyskeratosis congenita (Langlois et al. 1993).

Another fascinating category of X-linked disorders that cause skewing of inactivation in some or all blood cell are those involved with primary immune deficiencies (reviewed by Belmont [1995]). In females carrying mutations at the X-linked severe combined immunodeficiency (XLSCID), agammaglobulinemia (XLA), or Wiskott-Aldrich loci, cell competition and compensation mechanisms lead to decreased contribution of cells expressing the mutant allele among affected cell types. In XLA, the B cell lineage shows selective use of the nonmutant active X (Conley et al. 1986). In XSCID, skewing of X inactivation is observed in B cells, T cells, and NK cells (Puck et al. 1987). In Wiskott-Aldrich syndrome some degree of skewing of X inactivation can be observed in all hematopoietic lineages but is most prominent and consistent in the T cells (Goodship et al. 1991). The lineage-specific pattern of X-inactivation skewing in both

XSCID and XLA are essentially pathognomonic for the carrier state.

Positive Selection and Monoclonality

Selection for cells bearing an active mutant X chromosome could theoretically take place. An example is provided by the carriers of X-linked adrenoleukodystrophy (in which there is skewing in favor of the mutant X), although the mechanism is unknown (Migeon et al. 1981). More commonly, skewed X inactivation caused by positive selection reflects monoclonality. Following bone marrow transplantation, rare individuals may show only a single X active, presumably reflecting monoclonal reconstitution (Turhan et al. 1989). In those cases a single hematopoietic stem cell has undergone extensive self-renewal replication in vivo and has contributed all the cells of the active precursor pools for both myeloerythroid and lymphoid lineages (a remarkable 10^{15} daughter cells in an adult). Virtually any preneoplastic process affecting the blood may lead to monoclonal proliferation, as is observed in myelodysplasia (Tsukamoto et al. 1993) or histiocytosis X (Willman et al. 1994). Paroxysmal nocturnal hemoglobinuria (PNH) provides an interesting example of a hematopoietic disease in which there is a somatically acquired X-linked mutation leading to monoclonal proliferation. Mutations in the PIG-A gene lead to defective expression of a variety of glycosylphospho-inositol (GPI)–anchored cell-surface proteins (Josten et al. 1991; Rosse and Ware 1995). Absent expression of one or more of these cell-surface proteins (several of which are known to regulate cell growth) confers a proliferation advantage in blood precursors so that all the blood cells derive from the single stem cell in which the mutation occurred. A variety of other benign or premalignant lesions in nonblood tissues have been shown to have completely skewed pattern of X inactivation. These include uterine leiomyomata, intestinal polyps, and premalignant cervical adenomas. Skewed X inactivation has been advanced as a potential method for helping to judge the malignant potential of certain pathologically uncertain lesions (Mutter et al. 1995).

Twinning

There are now numerous published examples of female MZ twins discordant for typical X-linked recessive disorders (Richards et al. 1990; Lupski et al. 1991; Jørgensen et al. 1992; Winchester et al. 1992). It seems that there is an excess of such occurrences, which reflects an increased rate of X-inactivation skewing in MZ twins. Several explanations have been suggested: the twinning process itself might directly affect X inactivation; defects in X inactivation might increase the frequency of MZ twinning; or the twinning process might act as a bottleneck reducing the number of cells contributing to the embryo and thus increasing the chance that the X-inactivation pattern is skewed. Because of the high incidence of vascular anastomoses between monochorionic/monoamniotic twins (resulting in sharing of hematopoietic stem cells), studies of X-inactivation patterns in the blood of MZ twins are potentially misleading. Recent data using prospectively sampled umbilical cord or placental tissue are consistent with an increased rate of extreme skewing (18.6% vs. 6%, $P < .001$) of X inactivation in MZ twins (Bamforth et al. 1996; Goodship et al. 1996). No consistent "mirror image" inactivation pattern was seen, arguing against a primary role for asymmetric splitting of the embryo on the basis of X chromosome activity.

Primary Skewing X Inactivation

The X chromosome controlling element (*Xce*) locus is the best example and the most extensively characterized of any potential direct regulator of X-inactivation initiation. *Xce* has three well-characterized alleles Xce^{a}, Xce^{b}, and Xce^{c}, with a > b > c with respect to likelihood of inactivation (Rastan 1982). The *Xce* effect is present from the earliest time at which X inactivation can be detected, strongly suggesting that *Xce* biases the choice of inactive X rather than contributing to some kind of selective phenomenon. *Xce* may be involved in the sensing mechanism that determines which X chromosome remains active. *Xce* maps to the same critical interval as the XIC but can be distinguished by recombination from *Xist* (Simmler et al. 1993). Methylation at sites ~15 kb downstream from *Xist* correlate with the intensity of *Xce* allele such that hypermethylated alleles are co-inherited with the active X. (Courtier et al. 1995). Given that methylation may also play a role in maintaining X inactivation, the methylation pattern of this region 3′ distal to *Xist* may reflect establishment of the propensity of the allele for X inactivation. This segment of methylated CpG islands is not conserved between mouse and man, however.

Finally we come to the very unusual family described by Naumova et al. They report, in part, the results of the largest survey to date of quantitative analysis and family analysis of patterns of human X inactivation. An important finding is that 22% of women have skewing of X inactivation >80%, and 10% have skewing >90%. This distribution of X inactivation is essentially identical to that observed in smaller previous studies and, as noted above, is consistent with a small pool of embryonic cells undergoing stochastic X chromosome inactivation. But Naumova et al. also detected a very unique family, in which extreme skewing appears to be inherited. In this family seven daughters of a single individual all have extreme skewing of X inactivation

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such that in each individual the paternally derived X chromosome is predominantly active. This fact effectively rules out the possibility that the observed skewing is the result of a direct negative-selection process. While positive selection is a possibility, the single known trait associated with positive selection in vivo is X-linked adrenoleukodystrophy. It is interesting that the paternal grandmother of these sisters also shows extreme skewing of X inactivation. As noted in their discussion, Naumova et al. feel that the most likely pattern of inheritance is X linked because it is unlikely that all seven daughters would inherit the same autosomal allele, while, by necessity, they must inherit the same paternal X chromosome. The father of these women has apparently inherited a putative X-inactivation modifier locus from his mother, since she also shows skewed X inactivation. The paternal grandmother's preferentially active X bears the other allele at the AR locus used for quantification of X inactivation, however. Evaluation of the X chromosome haplotypes between AR and an *XIST* polymorphism indicated that there was no recombination between *XIST* and AR. This evidence leads to the suggestion that there has been a recombination between *XIST* and the putative skewing locus. Could this be a human equivalent of *Xce*? Unfortunately, further haplotyping would not be very informative in this family, since there is only a single typeable meiosis. However, the ascertainment of this family suggests that an X-inactivation modifier locus may be mapped by finding other families in which extreme skewing occurs as a heritable trait. Key questions to be answered are whether such a modifier, itself, shows parental imprinting effects and which step(s) in the X-inactivation process (initiation, *XIST* expression, spreading, or maintenance) is affected by it.

Appendix

Causes of Skewed X Inactivation

- Primary
  - Imprinting
  - *Xce*
  - *Xist* deficiency
- Selection
  - Positive
    - X-linked adrenoleukodystrophy
  - Negative
    - Constitutional
      - Balanced X:autosome translocation
      - Unbalanced X:autosome translocation
      - HPRT deficiency
      - Incontinentia pigmenti
      - Focal dermal hypoplasia
      - α-Thalassemia with mental retardation (ATRX)
      - X-linked dyskeratosis congenita
    - Blood cell lineage-specific
      - Agammaglobulinemia (XLA)
      - X-linked SCID
      - Wiskott-Aldrich syndrome
- Monoclonality
  - Aplastic anemia
  - Myelodysplasia
  - Histiocytosis X
  - Paroxysmal nocturnal hemoglobinuria (PNH)
  - Bone marrow transplantation
- MZ twinning

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