PERSPECTIVE

X-inactivation by chromosomal pairing events

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X-inactivation is the coordinated silencing of nearly all genes on one of the two X chromosomes in female mammals. X-inactivation requires the *cis*-acting *Xist* gene. The highly unusual properties of *Xist* and the extremely long distances over which *Xist* acts have made it difficult to reconcile X-inactivation with other examples of gene regulation. This paper presents new findings that suggest that X-inactivation involves transvection and harnesses heterochromatin association.

Role of chromatin in dosage compensation

Chromatin is the complex of DNA, histones, and other factors that compose chromosomes. Originally, eukaryotic chromosomes were believed to consist of euchromatin, regions that permit gene expression, and heterochromatin, chromosomal regions that are condensed and repressive to gene function (Heitz 1928; Karpen 1994). In general, the DNA of heterochromatin is more heavily methylated (in organisms that have DNA methylation) (Selker 1990; Klein and Costa 1997; Garrick et al. 1998), and replicates later in S phase (Taylor 1960) than the DNA of euchromatin. The histones of heterochromatin are also less acetylated (Turner 1998). Several lines of evidence indicate that the terms euchromatin and heterochromatin should both be considered umbrella terms for multiple chromatin structures.

There are two types of heterochromatin, constitutive heterochromatin and facultative heterochromatin. Constitutive heterochromatin forms over repetitive sequence and serves to maintain genome stability. Constitutive heterochromatin suppresses recombination between repetitive sequences and subdues the mutagenic potential of transposons by keeping them constitutively inactive (Yoder et al. 1997; Jensen et al. 1999). This is important, as ~35% of the human genome consists of repetitive transposon DNA sequence (Yoder et al. 1997). In contrast, facultative heterochromatin is formed and dismantled in a controlled manner by specific chromatin control sequences to regulate gene expression. The ability of specific chromatin structures to spread to nearby genes allows gene expression to be regulated.

Dosage compensation is the coordinate regulation of X-linked genes by chromatin remodeling to provide the two sexes, which have a twofold difference in X chromosome number, with equal levels of X chromosomal gene expression. In *Drosophila*, this is accomplished by large numbers of *cis*-acting elements, each of which controls a single gene or a small group of adjacent genes (Baker et al. 1994). These elements up-regulate gene expression twofold on the single male X chromosome by 'loosening' the chromatin of each gene to make it more euchromatic. In *Caenorhabditis elegans*, proteins associate with numerous sites along both of the X chromosomes of the XX individuals, down-regulating gene expression twofold by making each X chromosome slightly more heterochromatic (Nicoll et al. 1997; Dawes et al. 1999). The chromatin adjustments that produce twofold changes in gene expression in *Drosophila* and *C. elegans* underscore that a spectrum of chromatin structures are possible, spanning from very heterochromatic to very euchromatic.

Dosage compensation in mammals (X-inactivation) is fundamentally different from dosage compensation in *Drosophila* and *C. elegans*. The mammalian cell is thought to first 'count' the X chromosomes and, if two X chromosomes are present, activate the X-inactivation pathway. Rather than regulating all of the X-linked genes in a cell equally, the cell 'chooses' one X chromosome to be inactivated through the spread of heterochromatin across ~160 Mb. The *Xist* locus is involved in all three processes, namely counting, choosing, and long distance heterochromatin formation. *Xist*, therefore, is required for chromatin remodeling over a far greater distance than any other known *cis*-acting locus.

The *Xist* gene was first identified when a large (15 kb) untranslated RNA was cloned that is expressed exclusively from the inactive X chromosome (Borsani et al. 1991; Brown et al. 1991). Interestingly, the *Xist* RNA associates exclusively with the inactive X chromosome (Brown et al. 1992; Clemson et al. 1996; Panning and Jaenisch 1996). Targeted mutagenesis revealed that *Xist*-deficient X chromosomes are incapable of being inactivated in cultured cells (Penny et al. 1996) or during embryogenesis (Marahrens et al. 1997). A surprising discovery was the finding that a 450-kb transgene, that includes the *Xist* locus, could inactivate an autosome (Lee et al. 1996; Lee and Jaenisch 1997). The sequence

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required for *Xist* transgene-mediated inactivation was subsequently narrowed down to a 35 kb region that contains the *Xist* transcribed region and 9 kb of upstream sequence (Herzing et al. 1997).

The highly distinctive properties of *Xist* and the extremely long distance effects of the X-inactivation process have made it difficult to reconcile X-inactivation with other examples of gene regulation by chromatin. However, it is highly unlikely that a new and fundamentally different biological process has evolved to take care of the X chromosome gene dosage problem in mammals. The available evidence overwhelmingly indicates that basic biological processes are conserved among diverse organisms.

In this paper I consider the possibility that the processes responsible for X-inactivation are not unique to X-inactivation. I first highlight significant functional similarities between genomic imprinting and X-inactivation which culminate in recent findings by Rolf Ohlsson and colleagues. In considering these similarities, I propose that X-chromosome counting, choosing, and the initiation of heterochromatin formation are the genetic consequence of physical pairing interactions between the homologous X chromosomes (transvection). I next discuss recent evidence presented by Lyon (1998) that indicates the spread of heterochromatin over long distances is brought about by interactions involving repetitive elements distributed throughout the X chromosome. In light of recent findings by Gartler and colleagues (1999), Kominami and colleagues, and Henikoff and coworkers, I suggest that heterochromatin association is harnessed to control gene expression levels by heterochromatin spreading. Finally, I show how this new view of X-inactivation can be adapted in a straightforward manner to explain reports of skewed X-inactivation. The mechanisms discussed are useful in understanding a rapidly increasing number of characterized genetic diseases.

Similarities between autosomal imprinting and X-inactivation

Important clues explaining how X-inactivation might be initiated can be obtained from studies of autosomal imprinting. Imprinted gene expression is determined by the sex of the parent from which an allele is inherited. Imprinted genes are either exclusively silent on the maternal chromosome or are exclusively silent on the paternal chromosome (for review, see Bartolomei and Tilghman 1997). Like X-linked genes, imprinted genes are, therefore, silent on one of two homologous chromosomes and expressed from the other.

A direct relationship between autosomal imprinting and X-inactivation was established when it was demonstrated that X-inactivation is imprinted in the extraembryonic tissue of rodents (Takagi and Sasaki 1975) and in all tissues of marsupials (Cooper 1971; Sharman 1971). In these tissues, only the X chromosome from the father is inactivated. *Xist* has been shown to be required for both

random (Penny et al. 1996) and imprinted (Marahrens et al. 1997) X-inactivation, demonstrating that the two types of X-inactivation are mechanistically related.

Three different parameters indicate that, for both X-inactivation and autosomal imprinting, the allelic regions in question differ in their chromatin structures. First, extensive differences in methylation have been reported between the active and inactive X chromosomes (Mohandas et al. 1981; Migeon 1990) and between the two copies of autosomal imprinted regions (Bartolomei and Tilghman 1997). Demethylation results in loss of allelic differences in gene expression for both imprinted genes (Li et al. 1993) and for the X chromosome (Beard et al. 1995; Panning and Jaenisch 1996). Second, the histones of heterochromatin are less acetylated than in euchromatin (Grunstein 1997; Turner 1998). The inactive X chromosome is less acetylated than the active X chromosome (Jeppesen and Turner 1993). The treatment of cells undergoing X-inactivation with trichostatin, a histone deacetylase inhibitor presented normal inactivation (O'Neill et al. 1999). Trichostatin treatment of mouse conceptuses attempting to establish the monoallelic expression pattern of the imprinted *H19* gene similarly disrupts the silencing of one allele of the *H19* gene (Svensson et al. 1998). Finally, heterochromatin is later replicating than euchromatin and chromatin has been shown to be an important determinant of replication origin timing (Fangman and Brewer 1992). Both imprinted genes (Kitsberg et al. 1993) and X-chromosomal genes in females (Ohno and Hauschka 1960; Taylor 1960) are early replicating on one homologous chromosome and late replicating on the other.

For some examples of autosomal imprinting and X-inactivation, chromatin differences between the homologous chromosomes span large regions. Many, but not all imprinted genes are grouped into megabase-sized clusters. Some mutations within these gene clusters exhibit long distance effects on gene expression. One of the best studied imprinted clusters is on human chromosome 15 and is implicated in both Prader-Willi syndrome (PWS) and Angelman syndrome (AS) (Mutirangura et al. 1993; Driscoll 1994; DeLorey et al. 1998). Some PWS and AS patients carry mutations in this imprinted gene cluster that influence imprinted gene expression and/or DNA methylation patterns across hundreds of kilobases and, apparently, even across megabases (Glenn et al. 1993; Sutcliffe et al. 1994). Therefore, the imprinted autosomal regions share with the X chromosome, a capacity to influence gene expression over very long distances.

Both X-inactivation and at least one example of imprinted gene silencing are regulated by control loci that resemble each other. The autosomal *H19* gene (Bartolomei et al. 1991; Rachmilewitz et al. 1992; Zhang and Tycko 1992) resembles *Xist* in several ways (Pfeifer and Tilghman 1994). The phenotypes of *Xist* and *H19* knockout mice strongly indicate that the exclusive roles of both genes are to silence other genes on the same chromosome at one allele (Leighton et al. 1995; Marahrens et al. 1997). Both *Xist* and *H19* are genes with small introns that express untranslated RNAs from the allele that does

the silencing, whereas their promoters on the other allele are repressed and methylated (Ferguson-Smith et al. 1991; Bartolomei et al. 1993; Brandeis et al. 1993; Norris et al. 1994; Beard et al. 1995). The alleles of *H19* and *Xist* which do not silence other genes are transcriptionally silent yet reside in early replicating chromatin. The expressed allele which silences other genes resides in late replicating chromatin (Bartolomei and Tilghman 1997). Gene targeting revealed that the *H19* transcript is not required for the silencing function of *H19* (Ripoche et al. 1997; Jones et al. 1998). *H19*, therefore, may not really be a gene in this respect but rather a control locus for other genes that elicits a transcript. Gene targeting has also revealed that *Xist* functions as a *cis*-acting control locus. The similarity of *Xist* and *H19* suggests a common mechanism. However, unlike *H19*, *Xist* silences genes over extremely long distances and the RNA 'coats' the inactive X chromosome, suggesting it may play a functional role. Therefore, the similarity between the two genes may be the way they establish differences in the chromatin structures on their own homologous alleles.

Random vs. imprinted inactivation: A shared mechanism?

Two lines of evidence suggest that imprinted and random choosing of an X chromosome for inactivation proceeds by the same mechanism. First, X-inactivation can be made randomly in tissues where it is normally imprinted. In conceptuses where both X chromosomes are inherited from the mother, both X chromosomes are able to resist inactivation in the imprinted extraembryonic tissue where the paternal X chromosome is normally inactivated (Rastan et al. 1980; Endo and Takagi 1981; Shao and Takagi 1990; Tada and Takagi 1992). This imprinted resistance, however, is incomplete because a subset of the cells undergo random inactivation. Furthermore, inheritance of two paternal X chromosomes also results in random X-inactivation (Endo et al. 1982). This suggests that monoallelic *Xist* transcription is caused by a random inactivation mechanism which, in extraembryonic tissue, is rendered nonrandom by a discriminating imprint. The partial resistance, in parthenogenetic embryos, of both maternal X chromosomes to X-inactivation indicates that the imprint that skews X-inactivation is on the maternal X chromosome.

The second line of evidence that random and imprinted choosing are related was provided recently by analysis of imprinted autosomal genes. Imprints that restrict *H19* expression to the maternal chromosome and *Igf-2* expression to the paternal chromosome gene appear to be present on both maternal and paternal autosomes because both *H19* alleles are silent and both *Igf-2* alleles are expressed in mouse androgenetic (two paternal sets of chromosomes) embryos (Sasaki et al. 1995) while both *H19* alleles are expressed and both *Igf-2* alleles are silent in mouse (Walsh et al. 1994) and human (Mutter et al. 1993) parthenogenetic (two maternal sets of chromosomes) embryos. This is in contrast to X-inactivation in mouse extraembryonic tissue where there is evidence for

an imprint on the maternal but not the paternal X chromosome. However, in the extraembryonic tissue of human androgenetic (both sets of chromosomes inherited from the father) conceptuses, Ohlsson and coworkers showed recently that both *H19* and *Igf-2* are randomly inactivated (Ohlsson et al. 1999). Allele-specific in situ hybridization was used to show that, in the absence of the distinguishing imprints, the expression of both genes became variegated, suggesting random inactivation followed by clonal inheritance of the gene expression patterns. Therefore, the maternal, but not the paternal chromosome contains the imprint in human extraembryonic tissue. This implies that a random inactivation mechanism (as with *Xist* in somatic tissue) causes one allele of *H19* and *Igf-2* to be silenced and that this mechanism is rendered nonrandom by imprinting (as with *Xist* in mouse extraembryonic tissues).

If imprinted genes are subject to a random silencing mechanism that is skewed by a superimposed imprint, then it is reasonable to expect that autosomal genes, which are subject to the random inactivation mechanism but lack an imprint, also exist. This was shown to indeed be the case when it was demonstrated that the mouse autosomal *interleukin-2* (*IL-2*) gene is randomly inactivated (Hollander et al. 1998). The inactive allele of *IL-2* is late replicating while the expressed allele is early replicating, suggesting that chromatin similarly plays a role in *IL-2* silencing. The random formation of late replicating chromatin at one allele, therefore, is not unique to X-inactivation even under normal circumstances. This finding raises the very real possibility that many other genes are also randomly inactivated but that this has gone unnoticed. The striking difference between *IL-2* inactivation and X-chromosome inactivation is, of course, the distance the inactivation spreads; the random inactivation on the autosome appears to be confined to only the one gene (Hollander et al. 1998).

A transvection hypothesis for X-chromosome counting, choosing, and the initiation of heterochromatin formation

These results indicate that autosomal gene silencing and X-inactivation are related, and that imprinted and random inactivation share a common mechanism. How can one allele be randomly silenced while the corresponding region on the homologous chromosome remains expressed? A possible explanation is the discovery that two clusters of imprinted genes physically pair with their homologous counterparts during late S-phase (LaSalle and Lalande 1996). In contrast, none of several nonimprinted control regions tested showed homologous pairing (LaSalle and Lalande 1996). A large deletion in one copy of the imprinted region disrupted this homologous association (LaSalle and Lalande 1996). These findings raise the possibility that homologous association is required for the monoallelic expression of imprinted genes in the region. Changes in gene expression, caused by physical interactions between homologous chromo-

somes, are known to occur in nonmammalian organisms and are referred to as transvection.

Transvection was first discovered more than forty years ago in *Drosophila*, when a mutant phenotype for the *Ultrabithorax* (*Ubx*) gene was observed in flies heterozygous for a structural rearrangement that relocated *Ubx*, but not in flies lacking the rearrangement, or in flies homozygous for the rearrangement (Lewis 1954). It had previously been shown that homologous chromosomes physically pair during part of the mitotic cell cycle (Metz 1916) and the mutant phenotype was attributed to the rearrangement disrupting the pairing of the two alleles of *Ubx* (Lewis 1954). Several additional examples of transvection were described subsequently in *Drosophila* (Henikoff 1997) and related phenomena have been observed in plants (Matzke and Matzke 1995b; Meyer and Saedler 1996).

The observation that imprinted gene clusters in mammals associate homologously, raised questions whether this pairing results in transvection, as it does in *Drosophila*. Transvection was recently revealed at one of these regions using gene targeting (Duvillie et al. 1998). When the paternal allele of the imprinted *Ins-2* gene was disrupted in the gene body with a *neo* expression cassette, the *Ins-2* promoter on the homologous (maternal) chromosome was silenced (Duvillie et al. 1998). Physical interactions between the two alleles were somehow altered, resulting in the inactivation of the *Ins2* promoter on the maternal allele.

In view of the demonstrations of homologous pairing and transvection at imprinted regions in mammals, I propose that transvection is responsible for X-chromosome counting, the random choosing of one X chromosome, and the initiation of heterochromatin formation during X-inactivation. In this scenario, physical contact between the two *Xist* loci of female cells causes one *Xist* locus to be remodeled into heterochromatin while the other *Xist* locus remains euchromatic. Imprinted X-inactivation, like autosomal imprinting, proceeds by the same mechanism (Fig. 1A) with the additional presence of an imprint which causes the same allele to always form heterochromatin. In the absence of the imprint, pairing leads to random heterochromatin formation (Fig. 1B).

The pairing hypothesis for X-inactivation is supported by the recent finding that *Xist* transgenes will only inactivate autosomal sequences when they are in tandem multicopy arrays (Heard et al. 1999b). The ability of tandem multicopy, but not single copy, *Xist* transgenes to inactivate autosomal genes can be explained by pairing interactions between transgene copies that are facilitated by the immediate proximity of the repeats to each other. Homology-driven pairing interactions are thought to be responsible for the potent heterochromatin-forming ability of many tandem repeats (Selker 1999). Tandem inverted repeats, which simply need to fold over once to pair, exhibit the most potent heterochromatin-forming ability (Selker 1999).

Why do imprinted regions in mammals homologously associate while other, nonimprinted, regions do not?

Figure 1. Proposed role of transvection in X inactivation. In females, two homologous X chromosomes (blue lines) homologously pair at certain regions (jagged borders). This pairing may require stretches of heterochromatin (not shown). The homologous association juxtaposes the two *Xist* alleles causing one *Xist* locus to assume a heterochromatic structure (black box) and be transcribed (red arrow) while causing the other locus to assume a euchromatic structure (red box) and be transcriptionally silent. The *Xist* promoter, therefore, resembles the promoter of the *rolled* gene, which is expressed in heterochromatin and silent in euchromatin (Eberl et al. 1993). (A) Imprinting (triangles) in the extraembryonic tissue of rodents causes the paternal X chromosome to always be the one that is inactivated. Note that the imprint(s) may not be entirely on the maternal X chromosome as diagrammed. (B) In the absence of imprinting, homologous pairing leads to the random initiation of X-inactivation.

One possibility is that the chromosomes only pair at their heterochromatic repeats. The ability of heterochromatin to associate homologously is well documented (Lica et al. 1986; Cook and Karpen 1994; Dernburg et al. 1996b). The elements that *Xist* transgenes lack (Heard et al. 1999b) would, therefore, be heterochromatic repeats. The failure of autosomal homologs, homozygous for a single copy ectopic *Xist* locus, to initiate inactivation (Heard et al. 1999b) may be caused by insufficient patches of heterochromatin around the ectopic *Xist* loci to facilitate pairing. Similarly, a single copy *Xist* transgene would have difficulty pairing with the endogenous locus.

An important distinction between the transvection observed in *Drosophila* and the interactions proposed here is the outcome of the pairing event. In *Drosophila*, transvection between wild type alleles results in the up-regulation of both (Tartof and Henikoff 1991). The allelic interactions proposed here result in one allele forming heterochromatin.

A repeat hypothesis for the spread of X-inactivation

The transvection hypothesis fails to address how heterochromatin manages to spread throughout the entire X

chromosome once it has established itself at one of the two *Xist* loci. An important clue for solving this problem presents itself when we inspect X-autosome translocations. The heterochromatin generally fails to spread very far into autosomal sequence (Eicher 1970; White et al. 1998). However, in a small subset of chimeric chromosomes, inactivation readily spreads across large stretches of autosome (Eicher 1970; Lee and Jaenisch 1997; Lyon 1998). To account for the differing abilities of heterochromatin spread, Riggs proposed that 'way stations' or 'boosters,' positioned at intervals along the X chromosome, boost the X-inactivation signal (Riggs 1990).

Lyon reasoned that the boosters could be repetitive sequences (Lyon 1998). These studies compared the sequence content of the chromosomal regions that permit the spread of heterochromatin to the regions refractory to the spread of heterochromatin (Lyon 1998). This comparison uncovered a striking correlation: those chromosomal regions that promote the long distance spread of heterochromatin were also those regions that featured high concentrations of LINE transposable elements (Kazazian and Moran 1998; Lyon 1998). The X chromosome was the only chromosome with a high concentration of LINE elements throughout its entire length (Boyle et al. 1990). The autosomal regions refractory to the spread of heterochromatin were barren of LINE elements (Lyon 1998) as was the pseudoautosomal region which escapes X-inactivation (Boyle et al. 1990). This remarkable finding suggests that *Xist* cooperates with LINE elements to spread heterochromatin throughout the X chromosome.

Approximately 100,000 LINE elements are dispersed throughout mammalian chromosomes, constituting ~15% of the mammalian genome (Smit 1996) and posing a potential threat to genome stability. An elegant screening strategy for active elements revealed that numerous LINE elements in the human genome are potentially active (Sassaman et al. 1997) and could cause gene disruption if they transposed. LINE elements can also cause unwanted LINE-LINE recombination. LINE elements appear not to do more damage because they are kept inactive by heterochromatin formation. The highest concentrations of LINE elements are found in visible heterochromatin regions while more isolated elements, although not nested in cytologically visible heterochromatin, have been shown to be heavily methylated (Yoder et al. 1997) suggesting localized heterochromatin regions. Presumably this reflects the ability, which has been demonstrated in a variety of organisms (Pal-Bhadra et al. 1997; Matzke et al. 1994; Matzke and Matzke 1995a,b,c; Roche and Rio 1998; Jensen et al. 1999; Selker 1999), of dispersed transposons and dispersed repetitive transgenes, to induce heterochromatin.

An important clue as to how *Xist* might be interacting with LINE elements comes from recent studies into the chromatin structure of the *Xist* locus. It had always been assumed that the transcribed *Xist* allele on the inactive X chromosome is euchromatic. However, a closer examination of the replication timing of the active and inactive *Xist* alleles revealed that for *Xist*, the relationship between transcription and replication timing is reversed.

The silent *Xist* allele on the active X chromosome is early replicating while the transcribed *Xist* allele on the inactive X chromosome is late replicating (Hansen et al. 1995; Gartler et al. 1999). The implication that the transcribed gene has properties of heterochromatin was recently supported by the demonstration that the transcribed *Xist* allele fractionates with heterochromatin while the silent *Xist* allele fractionates with euchromatin (Endo et al. 1999).

Interactions between noncontiguous heterochromatic regions stimulate heterochromatin to spread

Analyses of the conditions that influence the spread of heterochromatin in *Drosophila* have uncovered properties of heterochromatin that, if applied to X-inactivation, provide a straightforward and logical explanation of how *Xist* can cause heterochromatin to spread throughout a 160-Mb chromosome. The studies revealed that two large but noncontiguous regions of heterochromatic repetitive sequence on a chromosome physically associated with each other (Csink and Henikoff 1996; Dernburg et al. 1996a). This association was shown to boost the distance that heterochromatin spreads from one repetitive region into the flanking nonrepetitive regions (Henikoff et al. 1995). For this boosting effect to occur, the two blocks of heterochromatin were required to be within a certain threshold distance of each other (Henikoff et al. 1995). Applying this property of heterochromatin to the X chromosome, if the heterochromatin that forms at and around one *Xist* allele physically associates with heterochromatin elsewhere on the X chromosome, it would stimulate the heterochromatin to spread. Because LINE elements are dispersed at a high density throughout the mammalian X chromosome, they provide sites from which heterochromatin can spread into the chromosomal regions between the elements until the entire X chromosome is heterochromatic.

The X-inactivation picture that emerges is as follows. In response to a developmental signal (Fig. 2A), physical interactions between the two *Xist* alleles cause heterochromatin to form at one allele, but not at the other allele (Fig. 2B). The heterochromatin that forms at and around the *Xist* allele physically interacts with other heterochromatin on the same chromosome (LINE elements shown) stimulating the heterochromatin to spread further into the adjoining euchromatic regions (Fig. 2C). Interactions with other heterochromatin regions induces spreading until X-inactivation is complete (Fig. 2D). The incomplete or variable spread of heterochromatin throughout a euchromatic region results in the genes in this region completely or partially escaping X-inactivation.

Chromatin effects and nonrandom X-inactivation

The model for X-inactivation helps explain how different *XIST* mutations might have opposite effects on X-chromosome choosing. One 15-kb deletion in the *Xist* locus

Figure 2. Model for X inactivation. (A) A developmental signal, in conjunction with homologous pairing of the two *Xist* alleles, is responsible for the chromatin remodeling that follows. (B) One *Xist* allele assumes a heterochromatic structure and the other allele a euchromatic structure. (C) Heterochromatin encompassing the *Xist* locus physically associates with other heterochromatic regions, stimulating the inactive chromatin structures to spread into adjacent regions. (D) Heterochromatin association induces spreading from multiple heterochromatin sites until inactivation is complete. (Yellow lightning) Developmental signal; (brown jagged regions) heterochromatin; (black arrows) the direction of heterochromatin spreading; (red box) transcriptionally silent *Xist* allele; (black box) actively transcribed *Xist* allele; (gray boxes) individual heterochromatic LINE elements or clusters of heterochromatic LINE elements.

causes the wild type X chromosome to be inactivated preferentially in female embryos (Marahrens et al. 1998). I infer that this deletion does not prevent homologous pairing because X-inactivation does occur. However, the mutation modifies transvection causing the wild type allele to be epigenetically altered. Since homologous pairing should cause the intact *Xist* gene to loop out, the potential of the intact *Xist* allele to interact with heterochromatin on the wild type X chromosome might be increased. Another targeted deletion removes ≥ 3.1 kb from the 3' portion of the *Xist* gene (Hong et al. 1999) and an additional 62 kb 3' of the gene (Clerc and Avner 1998). This second deletion causes the X chromosome bearing the deletion to always be inactivated, even when it is the only X chromosome in the cell (Clerc and Avner 1998). The second deletion clearly does not abolish the ability of the mutant *Xist* locus to induce X-inactivation. Perhaps this 65-kb deletion causes a nearby region of heterochromatin to be closely juxtaposed with *Xist*, making the *Xist* locus more heterochromatic. A more heterochromatic state of *Xist* prior to X-inactivation may fa-

cilitate heterochromatinization when X-inactivation occurs.

If flanking regions influence the chromatin state of *Xist* prior to X-inactivation, then allelic differences in the effect on the chromatin structure of *Xist* may result in the preferential inactivation of the X chromosome of one allele over the X chromosome of the other allele. A difference in the size or proximity of nearby heterochromatin might explain the influence of the X-controlling element (*Xce*), which maps just 3' to *Xist* (Heard et al. 1999a), on X-chromosome choosing. Heterozygosity at the *Xce* locus results in the X chromosome bearing one *Xce* allele that is preferentially inactivated over the X chromosome carrying the other allele (Cattanach and Williams 1972).

Medical implications

The view of X-inactivation presented here highlights interactions that may also play a role in patients with mutations that are hundreds of kilobases away from an intact, but misexpressed, disease gene (for review see, Kleinjan and van Heyningen 1998). Among the most extensively studied are long-distance mutations involving the imprinted gene cluster implicated in PWS and AS. In some patients with PWS the causative mutations disturb gene expression over megabases, while in other patients the causative mutations may be even tens of megabases away from disease genes (Glenn et al. 1993). In several unrelated patients with X-linked deafness, misexpression of the intact *POU34* gene is caused by a minideletion >400 kb upstream of the gene (de Kok et al. 1995a,b) while five additional patients with the same disease have microdeletions 900 kb upstream of the disease-causing gene (de Kok et al. 1996). Mouse mutations that influence gene expression over long distances have also been identified (Cordes and Barsh 1994; Miller et al. 1994; Bedell et al. 1995). Some of the mutations that act over long distances are known to remove DNA repeats (Kleinjan and van Heyningen 1998) suggesting that the pairing of repetitive sequences may bring distant sites together. Progress in understanding how pairing and other long distance interactions influence gene expression may prove useful for the development of therapies for patients suffering from long distance mutations and for the development of gene therapy in general.

Note added in proof

Lossi et al. (*Am. J. Hum. Genet.* 1999. **65**: 558–562) have recently reported an X-inactivation defect in patients with a mutation in the *ATR-X* gene. The *ATR-X* gene product interacts with the homolog of a *Drosophila* protein known to be important for transvection.

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