18

The X Chromosome, Dosage Compensation, and X Inactivation

The X chromosome is of medium size, making up 5.3% of the haploid karyotype. It is submetacentric, with a centromere index of 0.38 and a distinctive banding pattern (Fig. 17.1). In females, one X chromosome is condensed throughout interphase and is frequently visible in epithelial cells as a Barr body, or X heterochromatin. It is visible as a drumstick-shaped extrusion in 1–5% of polymorphonuclear white blood cells (Fig. 18.1a). The Barr body consists of a loop-shaped X chromosome in which the two telomeres lie close together at the nuclear membrane (Walker et al., 1991). Barr bodies can be scored in cells scraped from the mouth (buccal smears; Fig. 18.1b,c) or vagina, in cultured fibroblasts (Fig. 18.1d,e,f), or in follicle cells attached to a plucked hair. In normal females, a Barr body is visible in only 20–50% of buccal cells, in 30–80% of fibroblasts, and in over 90% of cells in amniotic membranes. In every individual (male or female) with two or more X chromosomes, the maximum

O. J. Miller et al., *Human Chromosomes*

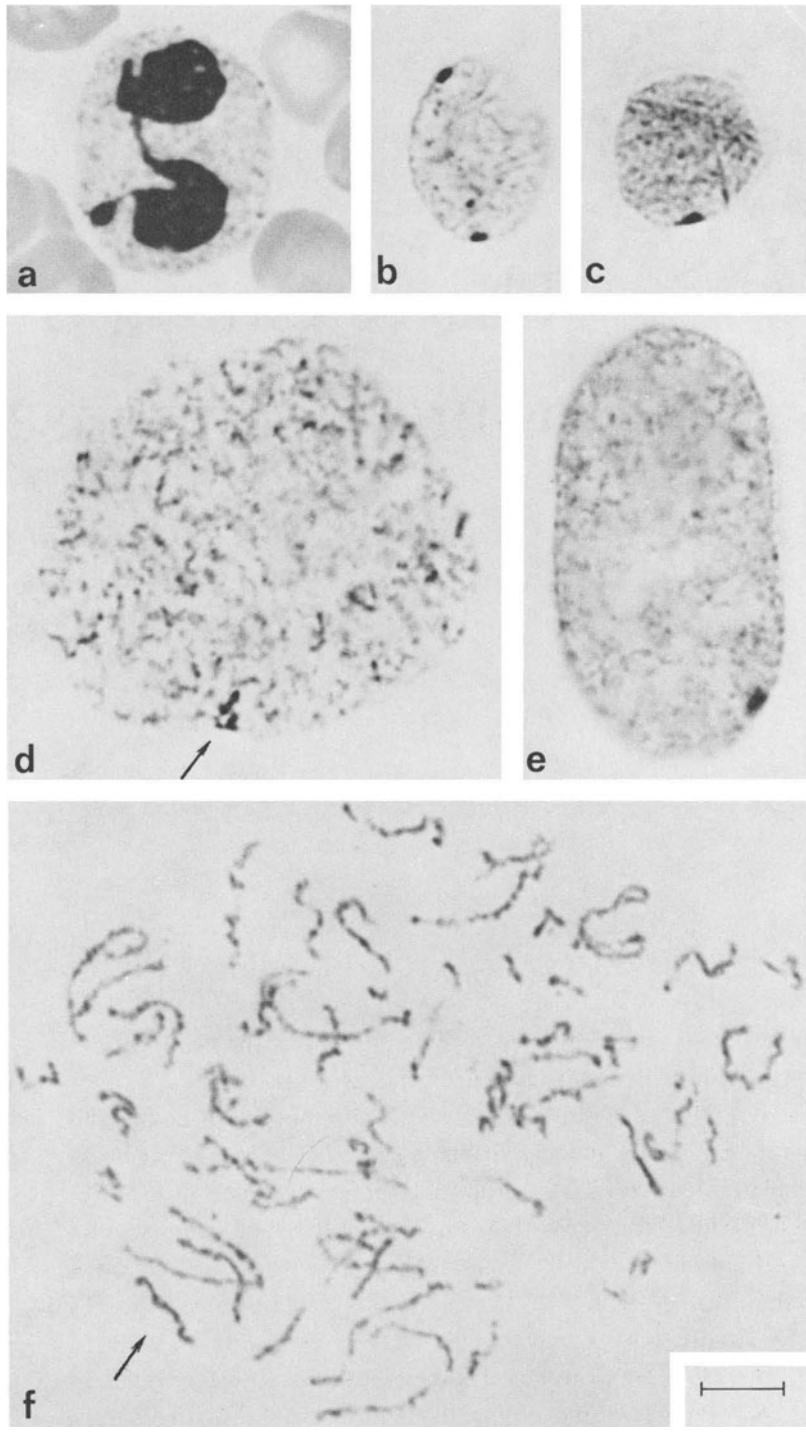

Figure 18.1. Inactive X chromosomes in interphase nuclei. (a) Drumstick in a neutrophil. (b) Two Barr bodies in a buccal nucleus from a 47,XXX woman. (c) Barr body **Figure 18.1.** (*cont.*) in a buccal nucleus from a 46,XX woman. (d) Heteropycnotic chromosome (arrow) in early prophase. (e) Barr body in a fibroblast nucleus. (f) Heteropycnotic chromosome (arrow) in late prophase (Feulgen staining; bar = 5 μm).

number of Barr bodies is one less than the number of X chromosomes. That is, one X remains euchromatic and the additional ones are heterochromatic. The heterochromatic X chromosomes replicate later in S than the euchromatic X, as demonstrated over 35 years ago by autoradiography (Chapter 3). The allocyclic, or out-of-step, behavior of the inactive X chromosome expresses itself in other ways, some described in Chapter 3. In both prophase and metaphase, the inactive X is often more condensed, and thus shorter, than the active X.

The Single Active X (Lyon) Hypothesis

Gene dosage effects can have a profound effect on the phenotype, as shown by autosomal deletions, duplications, and trisomies. However, the presence of a single X chromosome in males, in contrast to the two X chromosomes in females, has no apparent deleterious effect. Lyon (1961) proposed the single active X hypothesis, which provides a mechanism for preventing gene dosage differences between males and females (Fig. 18.2). The hypothesis, based on observations of X-linked genes in mice and Barr bodies in humans, has inspired an enormous amount of research. It has several key features. Each somatic cell has one active X chromosome, and any additional X chromosomes are inactivated early in embryonic development, at an estimated 1000- to 2000-cell stage, if not earlier (Lyon, 1974). The inactivation of the paternal or maternal X chromosome occurs at random. The same X remains active in all the descendants of any cell that underwent X inactivation, thus constituting a clone of cells with the same X active. Most genes on an inactive X are not transcribed; the chromosome is facultatively heterochromatic and may or may not be visible as a Barr body. The low percentage of cells with Barr bodies is not due to reactivation of the inactive X in the other cells, because only one allele at various X-linked loci is expressed in clonal lines despite the abundance of cells without a Barr body.

The single active X hypothesis implies that a female heterozygous for a gene on the X chromosome is a *genetic mosaic* of two different cell populations, one with the maternal X active, the other with the paternal X active. Like XY males, each of the two populations is functionally hemizygous for all the genes subject to X inactivation. The patchy appearance of the skin or eye defect in females heterozygous for such X-linked disorders as anhydrotic ectodermal dysplasia or ocular albinism is easily explained by the Lyon hypothesis. This hypothesis has

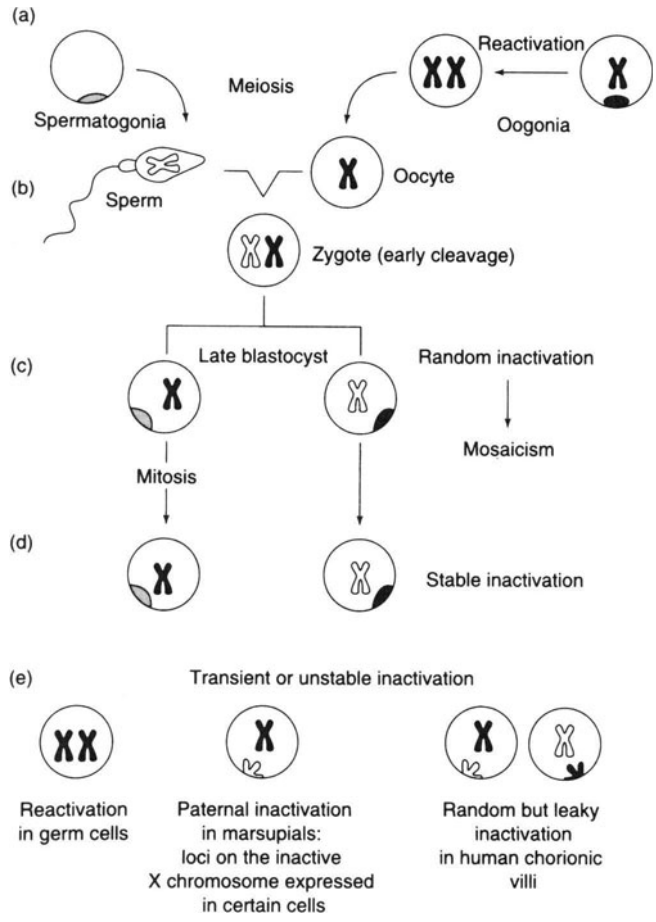

Figure 18.2. Diagram illustrating the single active X hypothesis. Illustration of the single active X hypothesis. (a) The clumped mass at periphery indicates the inactive X in spermatogonia and early oogonia. Note the reactivation of the inactive X in late oogonia. (b) Fusion of sperm and oocyte produces a zygote in which both the paternal (light) and maternal (dark) X chromosomes are active. (c and d) Random inactivation of either the paternal or maternal X occurs, and the same X remains inactive through most subsequent mitotic division differentration. (e) Cell types in which X inactivation is transient or unstable (reprinted from Trends Genet, v10, Migeon, X-chromosome inactivation: molecular mechanisms and genetic consequences, pp 230–235, copyright 1994, with permission of Elsevier Science).

withstood so many critical tests at the cellular level that the analysis of clonal populations is now the standard test of whether an X-linked gene is subject to or escapes X inactivation (facultative heterochromatinization). Davidson et al. (1963) cloned fibroblasts from a woman who was heterozygous at the glucose-6-phosphate dehydrogenase (*G6PD*) locus on the X chromosome. On starch-gel electrophoresis, the initial mass culture contained the enzyme products of both alleles, whereas each clone of cells contained the product of only one or the other allele. Cancer cells are usually clonal, as shown by the presence of the product of only one allele at one or several X-linked gene loci (for example, see Nagel et al., 1995).

Since X inactivation is as likely to involve the maternal X as the paternal X, it is possible to calculate the number of stem cells in a particular cell lineage at the time X inactivation takes place by analyzing the range in the proportion of cells with paternal X inactivation. The greater the range, or skewing from the 50% expected, the fewer the stem cells. Thus, if there were only one stem cell, 100% of cells in that lineage would have the same X inactivated. If there were only two stem cells, the paternal X would be inactive in 100% of cells in one-fourth of females, on average; the maternal X would be inactive in 100% of cells in another one-fourth; and so on. In this way, Puck et al. (1992) estimated that mature T lymphocytes are derived from a pool of only about 10 bone marrow stem cells.

Skewed X Inactivation

Skewed X inactivation is clinically important because it can lead to the expression of X-linked recessive disorders in females who are heterozygous for the mutant allele. Skewed X inactivation is common in female fetuses or newborns who have uniparental disomy of chromosome 15 and whose mothers' pregnancies were marked by confined placental mosaicism, with meiotic origin of the trisomic cell line (Chapter 21). The cause is presumed to be a reduction in the size of the pool of early embryonic cells (Lau et al., 1997).

Migeon et al. (1989) observed skewed X inactivation in females heterozygous for the incontinentia pigmenti mutation, which is lethal in male embryos. They suggested that cells in which the mutant allele is the active one tend to die, leaving only cells in which the normal allele is active in XX embryos; XY embryos, having no normal allele, die. Cell lethality of a mutant allele may also account for the skewed X inactivation that Pegoraro et al. (1997) observed in

16 females in one family. In each of the 16 females, the paternal X was the active X chromosome in more than 95% of the cells. Linkage analysis localized the mutation to Xq28, and molecular studies showed it to be an 800-kb deletion just distal to the *G6PD* locus. The females carrying the deletion were normal except for a doubling of the incidence of spontaneous abortion (to 32%). These presumably represented XY fetuses with the deletion, and the skewed X inactivation again arose because of a cell-lethal karyotype, or genotype. Exclusive (highly skewed) inactivation of the same X chromosome is characteristic of most X;autosome translocations and *XIST* mutations, as discussed below; skewing also occurs in monozygotic twins.

The Critical Region for X Inactivation: X Inactivation Center and the *XIST* Gene

In female carriers of an unbalanced X;autosome translocation (in which only one of the two reciprocal products is present), the Xcen–q13 region is always included in the translocation chromosome. This chromosome, not the normal X, undergoes inactivation in these cells. The shortest possible segment of Xq remaining after deletion or translocation that is still capable of inactivation is the distal end of Xq13, suggesting that this region contains an essential center of X inactivation (Therman et al., 1979). This has been verified by molecular methods (Brown et al., 1991), and positional cloning has been used to identify the gene responsible, called *XIST*. The *XIST* gene does not have a protein product. Instead, the large *XIST* RNA transcript remains in the nucleus, coats the X chromosome from which it is transcribed, and inactivates it (Brown et al., 1992). This involves a major alteration in the chromatin structure, mediated by DNA methylation, which leads to the binding of a methylation-specific DNA-binding protein that recruits a histone deacetylase. Histone H4 on the inactive X thus shows a lack of acetylation (Jeppesen and Turner, 1993). A different histone, called macroH2A1, is preferentially concentrated in the nucleosomes of the inactive X, as shown by combining FISH (using an X-specific probe) with immunofluorescence (using antibodies to macroH2A1). The immunofluorescence identified a *macrochromatin body* (MCB) in a very large percentage of XX cells but in less than 1% of XY cells. Three MCBs were seen in XXXXY cells. MCBs are visible even in cell types in which Barr bodies are not distinguishable, which could make them useful in searching for X chromosome mosaicism (Costanzi and Pehrson, 1998).

In studies of cleavage-stage human embryos derived from an in vitro fertilization program, RNA transcripts of the *XIST* gene are barely detectable as early as the one-cell stage, and more readily detectable by the eight-cell stage, in both XX and XY embryos. Thus, expression is not limited to XX cells or to a single X (Daniels et al., 1997). However, a marked increase in the *Xist* RNA level occurs in mice just before X inactivation. Sheardown et al. (1997) presented evidence that this is mediated by an increase in *Xist* RNA stability in XX embryos. An alternative explanation is that an antisense RNA is transcribed from the opposite strand of the *Xist* gene, starting from a point 15 kb downstream, called the *Tsix* gene. *Tsix* is expressed from both alleles until just before the onset of X inactivation, when its expression from the future inactive X ceases (Lee et al., 1999). *XIST* RNA is abundant in human testes, though not in leukocytes, suggesting that the inactivation of the single X in spermatocytes is mediated by the same mechanism as that for additional X chromosomes (Richler et al., 1992).

Given the importance of the *XIST* gene for X inactivation, its deletion would be expected to result in failure of inactivation of the remainder of the deleted X. Further, a translocation should lead to failure of inactivation of any segment no longer contiguous with *XIST*. The presence of the region containing the center of X inactivation in virtually every X chromosome fragment in unbalanced karyotypes and the failure to find cases of 46,X,iso(Xp) or 46,X,tel(Xp) suggest that dosage effects of normally inactivated genes on X are lethal (Therman and Sussman, 1990). Tiny ring X chromosomes are informative in this regard. They have a profound phenotypic effect, with severe mental retardation, congenital anomalies, and growth retardation. The expression of the *XIST* gene is absent or greatly reduced in these individuals, supporting the idea that failure of inactivation of one or more genes carried by the ring X is responsible for the phenotype (Migeon et al., 1994).

Once the *XIST* gene has initiated X inactivation, its continued activity is not required. Thus, an isodicentric X chromosome that had lost the *XIST* locus in a leukemic cell line has remained late replicating (Rack et al., 1994). X inactivation appears to be locked in by DNA methylation (Kaslow and Migeon, 1987).

The *XIST* gene is the only gene known to be active on the inactive X and inactive on the active X. Is it also unique in its replication pattern? Boggs and Chinault (1994) used the interphase FISH technique (Chapter 8) to show that the two *XIST* alleles in XX cells replicate very asynchronously, just like the alleles of genes that undergo normal X inactivation, and unlike the synchronous repli-

cation of genes that escape X inactivation, such as *ZFX* and *RPS4X*. Torchia et al. (1994) obtained comparable results using interphase FISH and probes for *XIST*, *FMR1*, and *factor* 8C (hemophilia A) loci and concluded that the active *XIST* allele was early replicating. However, Hansen et al. (1995) obtained different results, using a different method. They grew cells in the presence of bromodeoxyuridme (BrdU) for 1 hour and used flow sorting to separate the cells into G1, S1, S2, S3, S4, and G2/M fractions. Antibodies to BrdU were then used to isolate the DNA in each fraction that had replicated during each interval and gene probes used to determine when a specific gene was replicated. Their results indicated that the active *XIST* gene replicates late and its silent allele replicates early (Fig. 8.2). They confirmed this by using a modification of the FISH technique (Gartler et al., 1999).

Reactivation of the X Chromosome

The inactive X chromosome is reactivated in oocytes at some time before meiosis. Both X chromosomes are transcribed, and neither shows heteropycnotic behavior. In some tissues, a few normally inactive X-linked genes are active in some cells: X inactivation is incomplete, or "leaky." Moreover, the whole inactive X in trophoblastic cells may become reactivated in human-mouse hybrid cells (Migeon et al., 1986). Rarely, a few genes are reactivated spontaneously. However, treatment with the demethylating agent 5-azacytidine can reactivate X-linked genes more consistently (Mohandas et al., 1981).

Regions That Escape X Inactivation: Functional Map of the X Chromosome

Observations on structural abnormalities of the X chromosome have contributed greatly to understanding of the functional map of the X (Fig. 18.3). A few examples will serve to illustrate this. A tiny deletion of the tip of Xp causes short stature in both females and males; males also have other abnormalities. This suggests the presence of an expressed (noninactivated) gene in this region. A candidate is the *SHOX* gene, which maps to the pseudoautosomal region in humans but is autosomal in mice and might therefore account for impaired growth in XO humans but not in XO mice (Rao et al., 1997). Xp and Xq deletions generally produce similar Turner syndrome phenotypes, apart from short stature

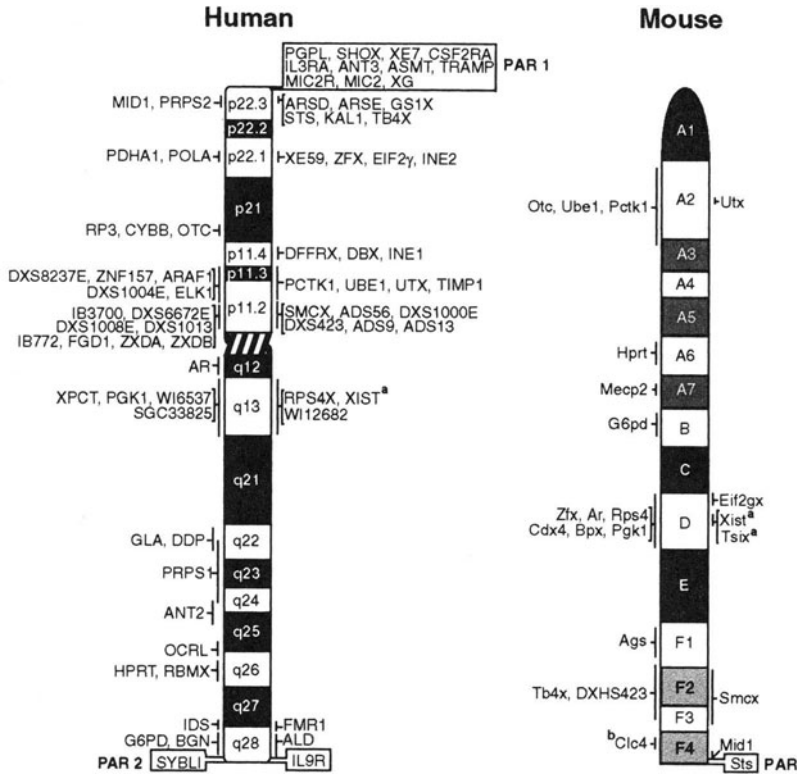

Figure 18.3. Functional maps of the human and mouse X chromosomes. Genes that show X-inactivation are listed to the left of each ideogram, while genes that escape X inactivation are listed to the right of each ideogram (courtesy of CM Disteche).

(Table 19.1), and no specific abnormalities are induced by Xq deletions. Therman and Susman (1990) suggested that a deleted X chromosome has a surplus of the factor needed for inactivation and thus inactivates regions that usually remain active. Against this notion is the fact that the *XIST* RNA normally traverses interstitial regions that remain active to reach more distant regions it does inactivate, so its amount is not critical. Furthermore, if these regions were fully inactivated, the phenotypes of 45,X, 46,X,Xp–, and 46,X,Xq– females should always be indistinguishable, and that is not the case (Table 19.1). The spreading of inactivation into autosome segments can also leapfrog over regions that escape inactivation, for reasons that remain to be discovered. Further study and new approaches are needed to resolve this problem.

Homologous loci of genes that are subject to X inactivation replicate asynchronously, just like the active and inactive X chromosomes themselves (Schmidt and Migeon, 1990). Therefore, synchronous early replication of any region on the two X chromosomes probably indicates that genes in the region have escaped X inactivation. This is certainly true for the genes in the pseudoautosomal region near the tip of Xp: All of them appear to escape inactivation (Fig. 18.3). In addition, several other early-replicating regions on X escape inactivation, including the nonpseudoautosomal region of Xp22.3 and regions Xp11.2–p11.4 and Xq13 (reviewed by Disteche, 1995).

X;Autosome Translocations and Spreading of Inactivation (Position Effect)

X;autosome translocations involving every autosome have been observed, although the autosome is either 21 or 22 in one-third of the translocations. A reciprocal t(X;autosome) usually produces a balanced chromosome complement in which there is no loss or gain of either an X or an autosome segment. Meiotic segregation in a balanced translocation carrier (heterozygote) can lead to unbalanced karyotypes (Chapter 16). In adult female carriers of balanced X;autosome translocations, the normal X chromosome is inactivated in almost all cases, at least in lymphocytes. The reason for this may be that inactivation of the autosome segment attached to the portion of the X chromosome with the X-inactivation center would result in functional monosomy for the autosome segment, while failure of inactivation of the other part of the X chromosome would result in functional trisomy for that segment of the X. Thus, while the normal X and the t(X;A) chromosome containing the inactivation center may initially undergo random inactivation, the chromosomally unbalanced cells may die or grow slowly, so that the better-balanced cell line replaces the other line.

In carriers of unbalanced X;autosome translocations, who often are offspring of balanced carriers, the abnormal chromosome is almost always inactivated, presumably because spreading of inactivation to the autosome segment of the single translocation chromosome would prevent functional trisomy of the autosome segment. Whether this functional trisomy is really that detrimental at a cellular level is questionable, and the reason for skewing of X inactivation in these cases is not clear. Nevertheless, this explanation for the observed replication patterns is consistent with the results of more extensive studies which indicate many balanced X;autosome translocation carriers have a minority cell line in which one

of the translocation chromosomes is inactivated. In one case, the normal X was late replicating in all lymphocytes of a woman with a 46,X,t(X;3)(q28;q21) karyotype. However, the normal X was late replicating in only two-thirds of cultured fibroblasts. In the other one-third, the X-chromosome portion but not the autosomal portion of the Xq+ chromosome was late replicating. This indicates that inactivation had not spread to the autosomal segment, thus preventing functional partial monosomy for that segment (Hellkuhl et al., 1982).

Spreading of inactivation into an autosome segment translocated to the X chromosome is a well-known but still poorly understood phenomenon. Allderdice et al. (1978) showed by autoradiographic analysis that inactivation could spread throughout virtually the entire long arm of a chromosome 14 to which the long arm of the X was attached, thus accounting for the relatively normal phenotype of a 46,der(X)t(X;14),der(X)t(X;14),Y male despite the presence of almost three complete copies of chromosome 14. The same method showed that spreading of inactivation was responsible for the absence of Down syndrome in a t(X;21)(q27;q11) translocation carrier (Couturier et al., 1979). Replication studies suggest that spreading of inactivation can leapfrog over autosomal regions that remain early replicating. This is not surprising, since the same is true of the X chromosome, in which early-replicating regions containing genes that escape X inactivation are interspersed with late-replicating, inactive regions throughout its length (Fig. 18.3).

White et al. (1998) used molecular probes to extend this kind of analysis a step further. They studied a woman whose karyotype was 46,X,der(X) t(X;4)(q22;q24). She was phenotypically normal despite duplication (partial trisomy) of the distal half of chromosome 4, which is usually associated with severe retardation of growth and mental development. Spreading of inactivation was demonstrated by showing that three genes and 11 ESTs (expressed sequence tags for anonymous genes) scattered along the 100-Mb segment of 4q were not expressed in a somatic cell hybrid containing only the der(X) translocation chromosome. However, three genes and three ESTs interspersed among the inactive genes were expressed. That is, 14/20 autosomal genes had been inactivated, while 6/20 escaped inactivation, a much higher fraction than that found for X-linked genes. White and associates suggested this was evidence for a difference between autosomal and X chromosomal DNA, but it also indicates a great deal of similarity in the DNA, its organization, and the mechanisms of inactivation of genes on the two types of chromosomes.

Keohane et al. (1999) used FISH with both RNA and DNA probes to show that sites of *XIST* RNA, histone H4 deacetylation, and late replication coincided

and that they marked the boundaries of inactivation in an X;autosome translocation and an autosomal insertion into an X. Using RNA FISH, Duthie et al. (1999) showed that *XIST* RNA is localized mainly to R-bands, not to constitutive heterochromatin, and that the spread of *XIST* RNA into the autosomal segment of an X;autosome translocation matched the spread of inactivation. The DNA demethylating agent 5-azacytidine hampers mitotic condensation of the inactive X (Haaf et al., 1993). It would be interesting if it did the same for inactive autosomal regions in X;autosome translocation chromosomes.

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