Am. J. Hum. Genet. 55:497–504, 1994

The Severe Phenotype of Females with Tiny Ring X Chromosomes Is Associated with Inability of These Chromosomes to Undergo X Inactivation

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Summary

Mental retardation and a constellation of congenital malformations not usually associated with Turner syndrome are seen in some females with a mosaic 45,X/46,X,r(X) karyotype. Studies of these females show that the XIST locus on their tiny ring X chromosomes is either not present or not expressed. As XIST transcription is well correlated with inactivation of the X chromosome in female somatic cells and spermatogonia, nonexpression of the locus even when it is present suggests that these chromosomes are transcriptionally active. We examined the transcriptional activity of ring X chromosomes lacking XIST expression (XISTE^-), from three females with severe phenotypes. The two tiny ring X chromosomes studied with an antibody specific for the acetylated isoforms of histone H4 marking transcribed chromatin domains were labeled at a level consistent with their being active. We also examined two of the XISTE^- ring chromosomes to determine whether genes that are normally silent on an inactive X are expressed from these chromosomes. Analyses of hybrid cells show that TIMP, ZXDA, and ZXDB loci on the proximal short arm, and AR and PHKA1 loci on the long arm, are well expressed from the tiny ring X chromosome lacking XIST DNA. Studies of the ring chromosome that has XIST DNA but does not transcribe it show that its AR allele is transcribed along with the one on the normal X allele. These findings provide compelling evidence that (1) ring X chromosomes associated with severe phenotypes are unable to undergo X chromosome inactivation; (2) they represent chromosomal mutations affecting *cis* inactivation; and (3) the severe phenotype is due to functional disomy resulting from lack of dosage compensation for genes present within the ring chromosome.

Received March 3, 1994; accepted for publication May 6, 1994.

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0002-9297/94/5503-0011$02.00

Introduction

Inactivation of one of the two X chromosomes in mammalian females compensates for the sex difference in X chromosome dosage (Lyon 1972). Females with two active X chromosomes have not previously been observed among live births or abortuses, and it has been assumed that failure to inactivate one X chromosome would be lethal early in embryogenesis. However, candidates for having more than one functional X chromosome have recently been ascertained; their karyotypes are mosaic, with one cell line having an X monosomy (45,X) and the other having 46 chromosomes, including a tiny ring X (Kusnick et al. 1987; Van Dyke et al. 1991, 1992; Lindgren et al. 1992; Dennis et al. 1993) or marker X chromosome (Wolff et al. 1993), and they have significant mental retardation and severe congenital malformations (Van Dyke et al. 1991, 1992). Usually, females with an X monosomy or other structurally abnormal X chromosomes (isoX chromosomes, X deletions, or large ring X chromosomes) have the relatively benign condition known as *Turner syndrome,* presumably because these abnormal X chromosomes are usually inactive in all cells, and the normal X chromosome is always the functional one. Kusnick et al. (1987), Van Dyke et al. (1991, 1992), and Lindgren et al. (1992) have proposed that the severe phenotype associated with tiny ring X chromosomes is due to the chromosomes' inability to inactivate because they lack the X inactivation center. The XIST locus, residing in the region of the putative X inactivation center, in Xq13.2, is expressed only from the inactive X chromosome and is considered a prime player in the initiation of mammalian X inactivation (Borsani et al. 1991; Brockdorff et al. 1991; Brown et al. 1991, 1992; Ballabio and Willard 1992). We have previously reported studies of the XIST locus in ring chromosomes from eight females who were ascertained because of mental retardation and congenital malformations (Migeon et al. 1993). Southern blot and PCR analyses of DNA from hybrids containing the ring X chromosome, and FISH analysis of diploid cells containing these rings, show that the XIST locus is either absent—or, if present, is not transcribed or transcribed at barely detectable levels. Therefore, what these ring X chromosomes have in common is a level of XIST transcription comparable to that of an active X chromosome (Mi-

geon et al. 1993). Because the inactive X chromosomes in female somatic cells (Brown et al. 1991; Ballabio and Willard 1992) and in male germ cells (McCarrey and Dilworth 1992; Richler et al. 1992; Salido et al. 1992) characteristically express XIST, the lack of XIST transcription from these ring X chromosomes in females with severe phenotypes strongly suggests that they are active chromosomes. Inactive X chromosomes can be distinguished at metaphase by their lack of histone H4 acetylation, a marker for active chromatin (Jeppesen and Turner 1993), and they do not express most genes that are transcribed from the active X. In contrast, we find that the tiny ring X chromosomes from three patients with severe phenotypes are active chromosomes, on the basis of their level of histone H4 acetylation and/or expression of loci that are silent on inactive X chromosomes.

Subjects, Material, and Methods

Subjects

The subjects of this study were females ascertained because features of Turner syndrome, or mental retardation, and/or multiple congenital abnormalities led to a karyotype analysis (Migeon et al. 1993). The clinical characteristics and karyotypes of the three females with severe phenotypes have been reported elsewhere: TT (Van Dyke et al. 1992), BT (Lindgren et al. 1992), and AL (Dennis et al. 1993). In each case the karyotype was mosaic and included a 45,X cell line and a second cell line with a normal X chromosome and one small ring X chromosome (See fig. 1 *a–f*). One of them, (TT) had two ring chromosomes in many of her cells (table 1 and fig. 1*e*). The ring X chromosomes were identified by in situ hybridization, with a DNA probe for the X centromere or chromosome painting with an X library probe. The genetic content of these rings is under study, and in general they contain contiguous DNA extending from Xp11 on the short arm to Xq13-21 on the long arm. Table 1 summarizes characteristics of these chromosomes. The ring from BT and the smaller ring in TT lack XIST sequences. In contrast, the ring from AL includes the XIST locus, but the XIST transcript is barely detectable (Migeon et al. 1993). We also studied the XISTE^+ ring chromosome from MB, a female with Turner syndrome only. Although her ring X chromosome was comparable in size to those of the other females, it was not associated with a severe phenotype. In addition, we studied hybrid cells derived from SM, another female with Turner syndrome only; this hybrid contained an inactive X linear chromosome that lacks the distal part of the long arm, expresses XIST, and is late replicating (data not shown).

Cell Cultures

Lymphoblast or skin fibroblast cultures were used for karyotyping and acetylated histone H4 studies and for DNA-PCR and reverse transcription–PCR (RT-PCR) analyses. Somatic cell hybrids containing the ring X chromosome from BT or the inactive X from SM were used for some RNA studies (see Hybrid Cells).

Clonal Cultures

To enrich for ring chromosomes, clones were obtained from suspensions of fibroblasts plated at 10 cells/dish and isolated after 10 d, with cloning cylinders. The clones were karyotyped to identify those with ring chromosomes in 90%–100% of the cells.

Hybrid Cells

The BT hybrids were derived from lymphoblasts by fusion with the mouse cell line tsA1S9az31B, which has a temperature-sensitive mutation at the Ube1x locus, and selection for expression of the human UBE1 gene was carried out at 39°C, the nonpermissive temperature (Brown and Willard 1989). The BT X^r hybrid that contained the ring X chromosome and not the normal X was identified by its polymorphic DXS255 and AR alleles and by the absence of distal short-arm and long-arm markers by using Southern blot hybridization and PCR analysis (see Results). We studied, as a control for studies of the BT X^r hybrid, a hybrid derived from SM lymphoblasts that contained an X chromosome carrying a long-arm deletion that includes distal Xq13-Xqter (SM X^i hybrid).

Histone H4 Studies

Unfixed metaphase preparations of primary fibroblasts and lymphoblastoid cell lines were labeled by indirect immunofluorescence with rabbit antiserum R5/12, specific for histone H4 acetylated at lysine-12 (Turner and Fellows 1989), as described elsewhere (Jeppesen et al. 1992; Jeppesen and Turner 1993). All steps were carried out at room temperature, 22°C. Cells that were attached to slides by cytocentrifugation were treated with KCM (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl [pH 8], and 0.5 mM EDTA 0.1% [v/v], Triton X-100) for 15 min to solubilize membranes. The cells were incubated for 2 h with a 1:100 dilution of antiserum in KCM containing 10% (v/v) normal goat serum (NGS). After slides were washed with KCM, primary antibody binding was detected with fluorescein isothiocyanate–conjugated (FITC) affinity-purified goat anti-rabbit immunoglobulin (Sigma) diluted 1:20 in KCM, 10% NGS. The secondary antibody incubation was carried out for 30 min, and slides were again washed with KCM. Postfixation with formaldehyde, Hoechst 33258 counterstaining, and fluorescence microscopy were all as described elsewhere (Jeppesen et al. 1992). To inhibit deacetylation of H4 occurring during metaphase arrest, and hence to ensure that even weakly acetylated H4 domains should be immunolabeled, metaphase cells were accumulated from some cultures in the presence of 4 mM sodium butyrate (Jeppesen and Turner 1993).

Transcription Studies: RNA Analysis

RNA (5 μg) obtained from lymphoblasts, fibroblasts, or hybrid cells (Chomczynski and Sacchi 1987) was analyzed

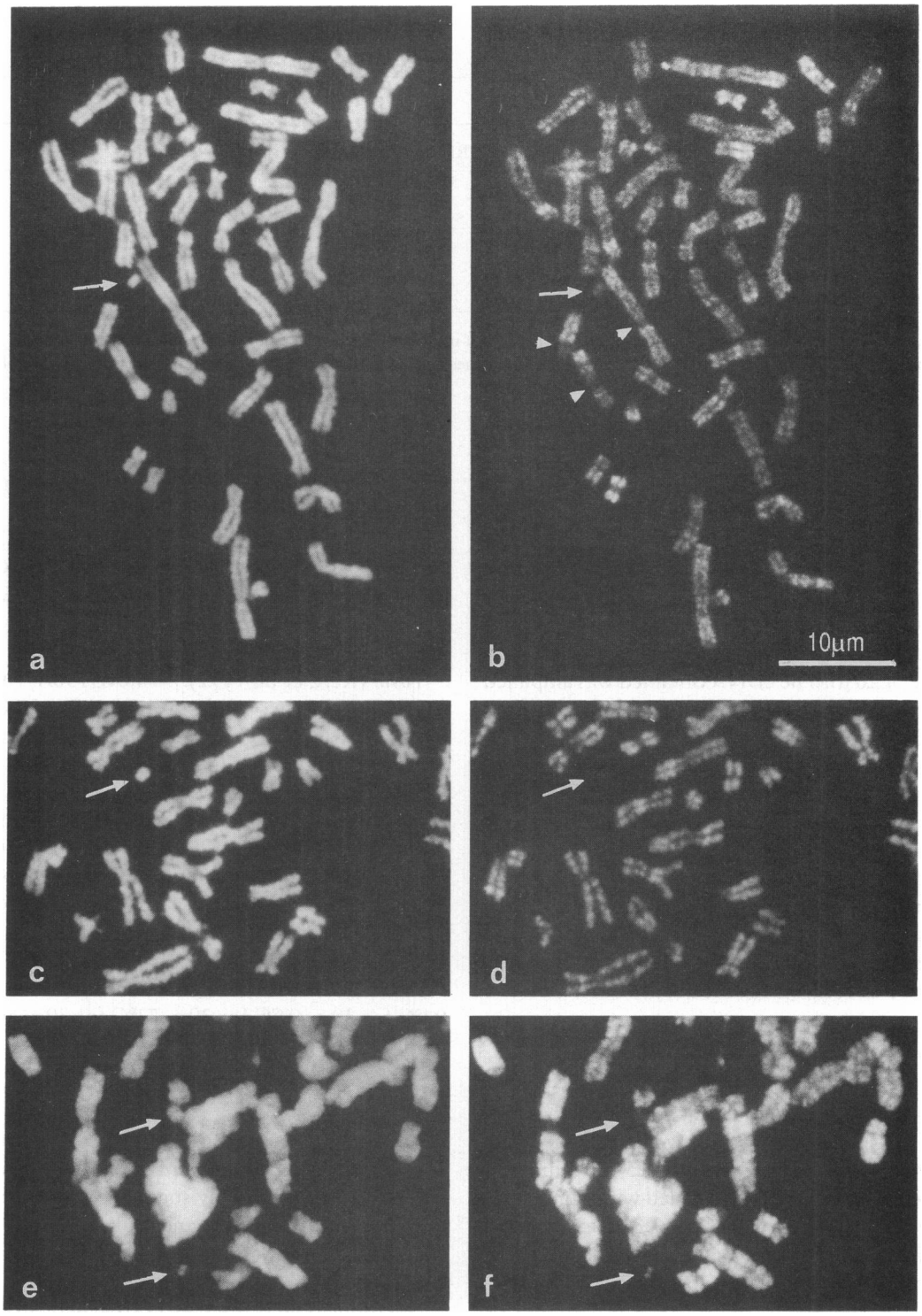

Figure 1 Histone H4 acetylation status of ring X chromosomes, determined by immunofluorescence. *a,* Primary fibroblast metaphase spread from subject AL, fluorescently stained with the DNA fluorochrome Hoechst 33258, showing ring (*arrow*). *b,* Same chromosomes labeled by indirect immunofluorescence (i.e., FITC) with rabbit antiserum R5/12, specific for histone H4 acetylated at lysine-12. The ring chromosome (*arrow*) shows a moderate level of H4 acetylation, less than the strongest bands on other chromosomes but above the background level of centromeric heterochromatin, some examples of which are shown (*arrowheads*). *c,* Partial metaphase from a lymphoblastoid line derived from subject MB, stained with Hoechst 33258, showing ring. *d,* Same chromosomes immunolabeled with R5/12. The ring chromosome (*arrow*) is labeled at background level only, indicating that it is inactive. *e,* Partial metaphase from lymphoblasts derived from subject TT, stained with Hoechst 33258, showing her two rings (*arrows*). *f,* Same chromosomes labeled with R5/12. The larger ring (*upper arrow*) is unlabeled, whereas the tiny ring (*lower arrow*) is labeled, which is consistent with its being active. Cells from MB and TT (but not from AL) were grown in the presence of sodium butyrate to enhance the signal from weakly acetylated H4 domains.

Table 1

Characteristics of Females and Ring X Chromosomes Analyzed

	PHENOTYPE		XIST			TRANSCRIPT			
SUBJECT: CELL	Severe	Turner Only	DNA	RNA	αAcH4	PHKA1	AR	TIMP	ZXDA/ZXDB
BT: X^r Hybrid	+	...	−	−	nd	+	+	+	+
AL: clone	+	...	+	−	+	ni	+	ni	ni
TT: lymphoblasts[a]	+	...	+, −	+	−, +	ni	ne	ni	ni
MB: lymphoblasts	...	+	+	+	−	ni	ne	ni	ni
SM: X^i hybrid	...	+	+	+	nd	−	−	−	−

NOTE.—+ = assay positive; − = assay negative; nd = not done; ni = not informative; and ne = not expressed in lymphoblasts.

[a] These cells have two rings; one has the XIST locus, and the other does not (Migeon et al. 1993). The ring that lacks XIST is the one that has acetylated histone H4 (αAcH4).

for expression of the loci XIST, phosphorylase kinase A (PHKA1), metallothionine protease tissue inhibitor (TIMP), and two zinc-finger proteins (ZXDA and ZXDB), by using RT-PCR (Wang et al. 1989). One cycle of reverse transcription was carried out at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min with 100 U M-MLV reverse transcriptase (BRL) and the appropriate 3′ primer in a total reaction volume of 20 μl. The cDNA obtained was amplified with both primers for 35 cycles by using the Gene Amp RNA PCR Kit (Perkin Elmer Cetus). The conditions for amplification are listed below with the primers. The amplification products were electrophoresed in a 2% NuSieve: agarose (1:1) gel and viewed under UV light after staining with ethidium bromide. To distinguish ZXDA from ZXDB, the common amplification product was digested with *Ava*I or *Dde*I, respectively (Greig et al. 1993). The housekeeping genes P3 at Xq28 (Filippi et al. 1990) and MIC2 at Xp23 (Brown et al. 1990) expressed from the active X were used as controls for the quality of RNA.

RNA and DNA Analysis of the Androgen Receptor (AR) Locus

A polymorphic trinucleotide repeat at the AR locus (Edwards et al. 1992) was detected using the primers listed below. The PCR assay was carried out in 25-μl volumes by using the downstream primer end-labeled with gamma ^{32}P-ATP, and products were separated on 6% urea-polyacrylamide sequencing gels as described elsewhere (Warren et al. 1992). Polymorphisms were scored from autoradiographs.

The primers, synthesized by the Johns Hopkins Genetics Core Facility, are listed 5′–3′. All programs began with 4 min denaturation at 94°C followed by 35 cycles at conditions specified below and ended with 10 min final extension at 72°C.

XIST (Brown et al. 1991).—PCR primers are from the region of XIST that includes exon 6: XIST-1, GAAGTCTCAAGGCTTGAGTTAGAAG; and XIST-3, ACATTTTTCTCTAGAGAGCCTGGC. Conditions were as follows: 94°C for 1 min, 54°C for 1 min, 72°C for 4 min, and 705-bp product.

PHKA1 (Lafreniere et al. 1993).—Primer 1, GGCCTGGCTGAGTGTTCATT; and primer 2, CGGGTTGATTGTGCATGGAA. Conditions were as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and 180-bp product.

TIMP (Kere et al. 1992).—sWXD136A, AGATAGCCTGAATCCTGCC; and sWXD136B, CTGGGTGGTAACTCTTTATTTC. Conditions were as follows: 94°C for 1 min, 54°C for 1 min, 72°C for 4 min, and 106-bp product.

ZXDA and ZXDB (Greig et al. 1993).—Primer 1, CTCTTACAAGCTCAAGAGGC; and primer 2, ACATGAACCTCCGGTCATCG. Conditions were as follows: 94°C for 30 s, 58°C for 30 s, 74°C for 2 min, and 510-bp product.

AR (Edwards et al. 1992).—Primer 1, TCCAGAATCTGTTCCAGAGCGTGC; and primer 2, GCTGTGAAGGTTGCTGTTCCTCAT. Conditions were as follows: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 261–312-bp product.

Results

Our previous observations that the tiny ring chromosomes associated with severe phenotypes have few or no XIST transcripts strongly suggested that they were active chromosomes. Jeppesen and Turner (1993) have shown that the inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation and that H4 acetylation could be used as a cytogenetic marker for gene expression. Therefore, we examined the ring chromosomes from affected female AL and control MB (Turner syndrome only) for their ability to bind antibodies specific for the acetylated isoforms of histone H4. We also examined cells from TT (severe phenotype), who has two ring X chromosomes. The larger ring expresses XIST at a level comparable to that of the normal inactive X, whereas the smaller one does not have the XIST locus (Migeon et al. 1993).

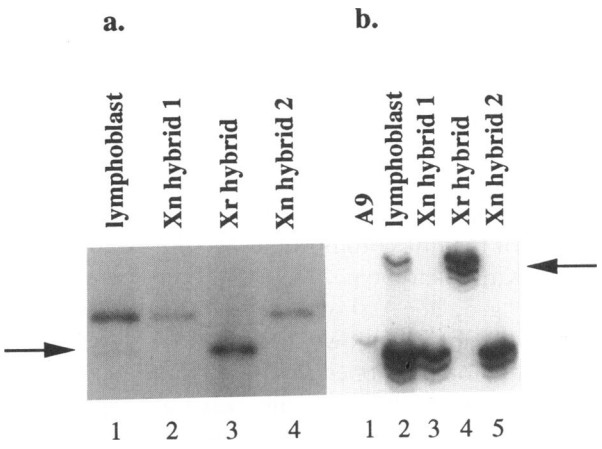

Figure 2 Evidence that the BT X^r hybrid contains the ring X chromosome and not the normal one. *a*, DXS255 locus: Southern blot of DNA from BT lymphoblasts (lane 1) showing both 7- and 8-kb alleles; hybrids containing normal X (Xn hybrids; lanes 2 and 4) with only the ~8-kb (larger) allele; and the hybrid containing the ring chromosome (Xr hybrid; lane 3) with only the ~7-kb (smaller) allele (*arrow*). Note that in lymphoblasts the ~7-kb allele (arrow) is less intense because it comes from the ring chromosome, which is not present in many cells. *b*, AR locus: PCR analysis of DNA from BT lymphoblasts (lane 2) with both alleles; hybrids containing normal X (Xn hybrids; lanes 3 and 5) with only the smaller allele; and the hybrid containing the ring allele (Xr hybrid; lane 4) with only the larger allele. Products were separated on a urea-polyacrylamide gel.

Unfixed metaphase spreads containing these ring chromosomes were immunolabeled with a rabbit antiserum that is specific for histone H4 acetylated at lysine-12; this position is acetylated predominantly in the more highly acetylated isoforms of H4 (Turner and Fellows 1989). It has also been shown that untranscribed chromatin domains, such as pericentromeric heterochromatin and the major part of the normal inactive X chromosome, are not measurably immunolabeled by R5/12 (Jeppesen et al. 1992; Jeppesen and Turner 1993). The tiny ring X chromosome in AL fibroblasts shows moderate overall labeling at a level significantly above that of pericentromeric heterochromatin in the same spread (fig. 1*a* and *b*). The smaller ring from subject TT that lacks the XIST locus was well immunolabeled in the presence of butyrate (fig. 1*e* and *f*), consistent with its being an active chromosome.

In contrast, there was a virtual absence of H4 acetylation in the $XIST^{E+}$ rings studied, even in the presence of sodium butyrate. These include the larger ring in TT lymphoblasts (fig. 1*e* and *f*) and the ring in lymphoblasts from MB with Turner phenotype only (fig. 1*c* and *d*). That the bulk of histone H4 is underacetylated indicates that these chromosomes are inactive and is consistent with our findings that they transcribe XIST (table 1).

If the tiny rings that have acetylated histone H4 were in fact active chromosomes, then they should transcribe loci that are expressed from an active X. Our efforts to examine this were impeded by the paucity of informative expressible loci. An informative locus needs not only to reside within the ring chromosome, but also to be silent when on the normal inactive X and to be transcriptionally active in the tissue under study. Also, only loci with detectable polymorphic alleles can be examined in the presence of the normal X. The loci that we studied were AR, PHKA1, TIMP, ZXDA, and ZXDB; all are subject to X inactivation, as they are not expressed from inactive X chromosomes. Because only the AR locus has a known common polymorphism, all the others could be studied only using hybrid cells.

The BT X^r hybrid contains the ring X chromosome, but not the normal one. Evidence for this comes from analysis of polymorphic loci that can distinguish the ring from its normal homologue in BT diploid cells (fig. 2). Figure 2*a* shows that when Southern blots containing *Eco*RI-digested DNA from BT lymphoblasts (fig. 2, lane 1) were probed with M 27 β (DXS255 locus) (Fraser et al. 1987), there is an intense ~8-kb fragment from the normal X, as well as a faint ~7-kb fragment from the ring X. The signal from the ring X (fig. 2*a*, lane 1; see arrow) is relatively weak because, whereas all cells had a normal X, only a few of them had the ring X. As expected, DNA from the BT X^r hybrid (fig. 2*a*, lane 3) yielded only the ~7-kb fragment. In addition, Figure 2*b* shows that this hybrid has only the larger AR allele (fig. 2*b*, lane 4), which is the less intense allele in her lymphoblast DNA (fig. 2*b*, lane 2; see arrow) and not the smaller allele that comes from the normal X (fig. 2*b*, lanes 3 and 5). Also, FISH analysis with X library probes revealed a ring and no intact X chromosome (data not shown).

Figure 3 shows that the PHKA1 locus at Xq13 is transcribed from the ring chromosome in the BT X^r hybrid cells, but not from the inactive X in the SM X^i hybrid (fig. 3*a*, compare lanes 5 and 6). Figure 3*b* shows that products were obtained only when reverse transcriptase was included in the reaction. The RNAs that were negative for PHKA1 produced the expected PCR product at the P3 locus (Filippi et al. 1990) (data not shown).

The TIMP locus, on the short arm of the X chromosome proximal to UBE1 and distal to DXS255, is subject to X inactivation and is transcribed in hybrid cells (Brown et al. 1990). The left panel of figure 4 shows that TIMP is expressed in the BT X^r hybrid that contains the ring chromosome and not the normal X (lane 6) and is not transcribed from the inactive X (lane 8).

The duplicate ZXDA and ZXDB loci on the proximal short arm of the X, which code for zinc-finger proteins, are not expressed on an inactive X chromosome, and one can be distinguished from the other on the basis of restriction-site differences (Greig et al. 1993). The right panel of figure 4 shows that in hybrid cells both of these loci are transcribed from the ring chromosome (lanes 6–8) and not from the inactive X (lane 9).

Alleles at the AR locus can be distinguished using primers around the variable CAG repeat in exon 1 (Edwards et

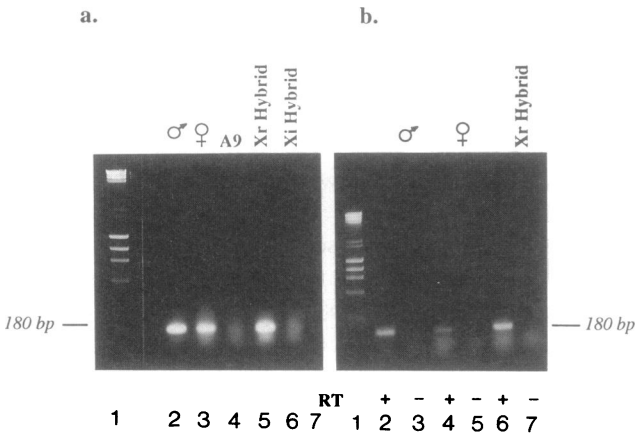

Figure 3 PHKA1 locus expressed from the tiny ring X. *a,* Markers (lane 1), normal male (♂; lane 2), normal female (♀; lane 3), mouse A9 fibroblasts (lane 4), BT X^r hybrid (lane 5), SM X^i hybrid with an inactive X (lane 6), and blank (lane 7). The 180-bp RT-PCR product is seen in RNA from the normal female and male and from the BT X^r hybrid, but not in the RNA from mouse A9 cells or from the SM hybrid with only an inactive X (i.e., SM X^i hybrid). *b,* Assay carried out with reverse transcriptase (*even-numbered lanes*) and without reverse transcriptase (*odd-numbered lanes*). It shows that products were obtained only when reverse transcriptase was included in the reaction. Markers (lane 1), normal male (♂; lanes 2 and 3), normal female (♀; lanes 4 and 5), and BT X^r hybrid (lanes 6 and 7) are shown.

al. 1992), so that the ring allele can be identified even in presence of the normal X chromosome. However, the AR locus is not well expressed in lymphoblasts or blood cells. Figure 5 shows the DNA-PCR products on the left and the allele-specific RT-PCR products on the right. The allele from the ring chromosome in the BT X^r hybrid was identified by DNA-PCR analysis (fig. 5, lane 5). The BT X^r hybrid gives the predicted RT-PCR product (fig. 5; compare lane 10 with lane 5), whereas no RT-PCR product was obtained from the hybrid with an inactive X (SM X^i hybrid; lane 11). We also looked for allele-specific transcripts in two clonal populations of fibroblasts from AL; these were enriched for the ring chromosome, as >90% of these cells had a 46,X,r(X) karyotype. DNA-PCR analysis of AL fibroblasts (fig. 5, lane 4) shows that they are heterozygous, and RT-PCR analysis shows that both AR alleles are expressed in fibroblast clones 32 and 33 (lanes 8 and 9, respectively). The presence of the two transcripts within clonal populations derived from single cells means that in each cell both X alleles are functional.

Discussion

We have examined the hypothesis that activity of XISTE^- ring X chromosomes is responsible for severe phenotypes; first, we examined them for the presence of active chromatin by using the acetylation of histone H4 as the assay, and, second, we looked for the presence of transcripts from specific loci within the ring chromosomes that should only be expressed from an active X chromosome.

Immunofluorescence using an antiserum specific for the more highly acetylated isoforms of histone H4 shows that the tiny ring chromosomes are active in cells from subjects AL and TT, both of whom show the severe phenotype. These rings show moderate levels of H4 acetylation, above the level of untranscribed pericentromeric heterochromatin, but lower than the brighter immunofluorescent bands

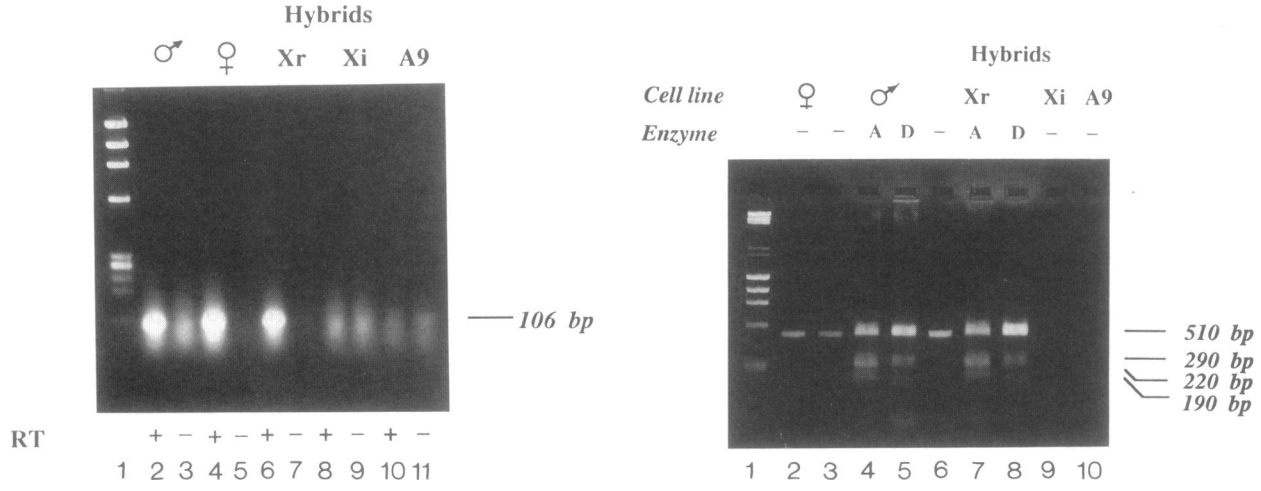

Figure 4 The TIMP locus (*left panel*) and ZXDA and ZXDB loci (*right panel*) expressed from the BT X^r chromosome. Note that the expected RT-PCR products are obtained from the BT ring hybrid (X^r hybrid) and not from the hybrid with the inactive X (X^i hybrid). *Left panel,* Markers (lane 1), normal male (♂; lanes 2 and 3), normal female (♀; lanes 4 and 5), BT X^r hybrid (lanes 6 and 7), SM X^i hybrid with the inactive X (lanes 8 and 9), and A9 mouse cells (lanes 10 and 11). Assays in even-numbered lanes were carried out with RT (+), whereas those in odd-numbered lanes were not (−). *Right panel,* Markers (lane 1), normal female (♀; lane 2), normal male (♂; (lanes 3–5), BT X^r hybrid (lanes 6–8), SM X^i hybrid with the inactive X (lane 9), and A9 mouse cells (lane 10). In lanes 3, 4, 6, and 7 the 510-bp PCR product was digested with either *AvaI* (A) to identify ZXDA (290- and 220-bp cleavage products) or *DdeI* (D) to identify ZXDB (290- and 190-bp cleavage products).

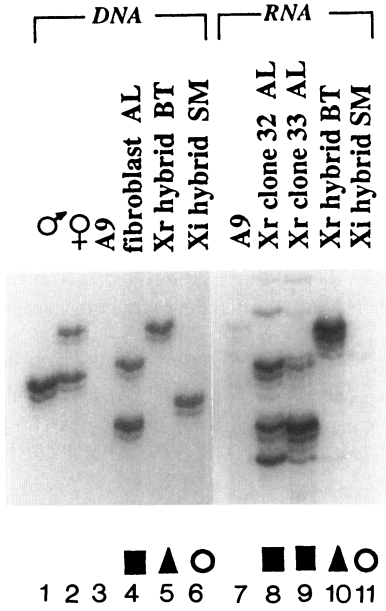

Figure 5 Evidence that the BT X^r hybrid expresses the ring allele and that clonal populations of AL diploid cells express both AR alleles. DNA (*left*) and RNA assays (*right*) were carried out simultaneously and were run out on the same gel. To facilitate comparisons of DNA-PCR and RT-PCR assays, specimens from BT X^r hybrid are denoted by triangles, those from AL cells by squares, and those from SM X^i hybrid by circles. Normal male (♂; lane 1), normal female (♀, lane 2), mouse A9 fibroblasts (lanes 3 and 7), AL fibroblasts (lane 4), AL clones (lanes 8 and 9), BT X^r hybrid (lanes 5 and 10), and SM X^i hybrid with an inactive X (lanes 6 and 11) are shown. Note that (1) the male control has a single allele, and the female control has two alleles; (2) the size of alleles in the RT-PCR assay, on the right is that expected on the basis of the DNA-PCR assay, on the left; (3) the BT X^r hybrid expresses the larger allele, whereas the SM X^i hybrid gives no PCR product; and (4) each AL clone expresses both AR alleles.

seen on some chromosomes (fig. 1*b* and *f*). Although previous results show that untranscribed chromatin domains can be clearly distinguished by their lack of histone H4 acetylation, the significance of different levels of H4 acetylation in transcribed chromatin is not yet understood, but it may reflect the local density of transcribed sequences. A small number of weakly acetylated H4 domains on the normal human inactive X chromosome can be detected by sodium butyrate pretreatment; some of these correlate with regions known to escape inactivation (Jeppesen and Turner 1993). However, immunolabeling at Xq13, from where XIST is transcribed on the inactive X, is extremely weak even in the presence of butyrate and is difficult to detect in most spreads (Jeppesen and Turner 1993). In the experiments described here, the XISTE^+ ring X chromosomes in MB and TT cells occasionally showed very weak immunolabeling near the centromeres (data not shown), which might be detecting XIST expression. However, the fluorescence intensity in these sporadic cases was very low and was very different from the overall and much stronger labeling of the rings in AL cells in the absence of butyrate.

That the BT hybrid containing only the ring chromosome transcribes five loci that are not expressed from normal inactive X chromosomes and that the AL clones have two functional AR alleles indicate that the XISTE^- ring chromosomes that we studied are transcriptionally active. Consistent with the overall immunolabeling of the XISTE^- rings in TT and AL (fig. 1*b* and *f*), the expression studies also suggest that many loci within these rings are active; all the loci examined were expressed, and these loci are situated on the short (TIMP, ZXDA, and ZXDB) as well as the long (AR and PHKA1) arm of the X chromosome.

Because X inactivation is random at onset, it is likely that in these mosaic females the normal X chromosome is inactivated in some cells of the early embryo. Since they would lack an intact functional X chromosome, such cells would be eliminated by cell selection. Consequently, the normal X would be the active one in all surviving cells. Because the normal X and the tiny ring X chromosomes are both active chromosomes, the genes on both of these chromosomes are functional. Therefore, the severe phenotype that is associated with these XISTE^- ring X chromosomes most likely results from the lack of dosage compensation for loci within the chromosome. Functional disomy, for genes in the Xq12-Xq13.2 region, resulting from the duplication of this region on the single X chromosome in two males has been reported recently (Schmucker et al. 1993); the clinical constellation of abnormalities (growth retardation, mental retardation, and dysmorphic features) that are associated with this duplication in these males is similar to that found in the females with tiny ring X chromosomes. One may be able to discriminate prenatally the XISTE^- rings associated with severe abnormalities from those of similar size that will be inactivated, on the basis of whether XIST is expressed. It is highly likely that these tiny ring X chromosomes are active because they lack sequences essential for inactivation of the chromosome. In two of our cases (BT and TT), the ring chromosome lacks the XIST locus, so that clearly the absence of XIST sequences alone may be sufficient to interfere with inactivation of the chromosome. In the third case (AL), the XIST locus is present but is only minimally expressed (see fig. 5*A* in Migeon et al. 1993). The fact that this chromosome has both acetylated histone H4 and an active AR allele strongly suggests that the XIST locus must be well transcribed for the chromosome to be able to inactivate. The association of deficient transcription with failure to *cis*-inactivate raises the possibility of regulatory sequences or other relevant loci downstream of the XIST locus (as it is now defined) that control the XIST locus. Although our preliminary studies suggest that the rings are contiguous segments of the X, it is conceivable that rearrangements have occurred that interfere with the expression of XIST. Further studies of the genetic content of these chromosomes should help elucidate the sequences essential for *cis*-inactivation. These tiny ring X chromosomes are likely to be powerful tools in the search for genes involved in *cis*-X inactivation.

Acknowledgments

We thank Drs. Howard Cooke and Kirby D. Smith for helpful discussions of the manuscript. We gratefully acknowledge Dr. Beth S. Torchia for FISH analysis of MB and hybrid cells and for her help with preparation of the figures, Joyce Axelman for maintaining cultured cells, and Melanie Dunn for Southern blot analysis. We are indebted to Drs. Patricia A. Jacobs, Teresa L. Yang-Feng, Daniel D. Van Dyke, and Lester Weiss for providing specimens from subjects with ring X chromosomes, and to Dr. Bryan M. Turner for a gift of R5/12 antiserum. This work was supported by NIH grants HD29044 and HD05465, both to B.R.M.

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