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Deficient transcription of *XIST* from tiny ring X chromosomes in females with severe phenotypes

(X chromosome inactivation/Turner syndrome)

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ABSTRACT **The severe phenotype of human females whose karyotype includes tiny ring X chromosomes has been attributed to the inability of the small ring X chromosome to inactivate. The *XIST* locus is expressed only from the inactive X chromosome, resides at the putative X inactivation center, and is considered a prime player in the initiation of mammalian X dosage compensation. Using PCR, Southern blot analysis, and *in situ* hybridization, we have looked for the presence of the *XIST* locus in tiny ring X chromosomes from eight females who have multiple congenital malformations and severe mental retardation. Our studies reveal heterogeneity within this group; some rings lack the *XIST* locus, while others have sequences homologous to probes for *XIST*. However, in the latter, the locus is either not expressed or negligibly expressed, based on reverse transcription–PCR analysis. Therefore, what these tiny ring chromosomes have in common is a level of *XIST* transcription comparable to an active X. As *XIST* transcription is an indicator of X chromosome inactivity, the absence of *XIST* transcription strongly suggests that tiny ring X chromosomes in females with severe phenotypes are mutants in the X chromosome inactivation pathway and that the inability of these rings to inactivate is responsible for the severe phenotypes.**

Although most conceptuses with X chromosomal monosomies are found among spontaneous abortions, some survive fetal life. In fact, most survivors are remarkably healthy individuals whose intelligence is within the normal range (1, 2). Their phenotypes usually include short stature and failure to maintain normal ovarian structure and function. Commonly, but not invariably, they have dysmorphic features such as widely spaced nipples, narrow palate, small mandible, webbed neck, and lymphedema—a constellation of abnormalities referred to as Turner syndrome. Turner syndrome is also associated with karyotypes that include 46 chromosomes with one normal X chromosome and a second X that is structurally abnormal—i.e., having deletions or reduplications of the long arm. Occasionally the abnormal X is one with breaks in both short and long arms that have led to the formation of a ring X chromosome. The relatively benign nature of the anomalies associated with an X monosomy or the presence of a structurally abnormal X is explained by the fact that females normally have only a single active X chromosome (3). The abnormal (genetically deficient) X is usually inactive due to selection favoring cells in which the normal X is active, and therefore the phenotypes of such females are similar to those with a true X monosomy. On the other hand, some females who are mosaic, 45,X/46,X,r(X), and whose ring X chromosome is tiny (i.e., Fig. 1) are much more severely affected than those with a nonmosaic 45,X karyotype. They have severe mental retardation and developmental delay, growth retardation present at birth, and multiple congenital anomalies including facial dysmorphism (coarse features, epicanthal folds, upturned nares, long philtrum, hypertelorism, strabismus), soft tissue syndactyly of upper and lower limbs, and increased frequency of heart defects (ventricular septal defects, mitral valve stenosis) (reviewed in ref. 2). It has been suggested that small ring X chromosomes are more detrimental than large ones because they are unable to inactivate, and therefore some genes (those within the ring) would be expressed from both X ring and normal X chromosomes (2, 4, 5). Recently, it has been shown that the *XIST* locus on the X chromosome is uniquely transcribed on the inactive X chromosome (6–10). It has been proposed that this locus is required for a chromosome to become inactive (6, 10). In addition, the presence of *XIST* transcripts in spermatocytes has been considered indicative of X chromosome inactivity at some stages of spermatogenesis (11, 12). To test the hypothesis that at least some of these tiny ring X chromosomes are unable to inactivate, we examined females with mental retardation and congenital abnormalities for the presence and expression of the *XIST* locus on their tiny ring X chromosome. Our results show that deficient transcription of this locus is characteristic of the tiny ring X chromosomes we studied and strongly suggest that these chromosomes are active.

MATERIALS 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. Their karyotypes in each case revealed mosaicism that included a 45,X cell line and a second cell line with a normal X chromosome and one small ring X chromosome. One of the females (subject 8, TT) had two ring chromosomes in many of her cells, presumably one derived from the other (see Figs. 1*C* and 3*B*). The ring X chromosomes were identified in each case by *in situ* hybridization with a DNA probe for the X centromere or by chromosome "painting" with an X library probe. We also studied the ring chromosome from a female with a small ring X chromosome that was not associated with a severe phenotype. The clinical

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Abbreviations: FISH, fluorescence *in situ* hybridization; RT, reverse transcription; YAC, yeast artificial chromosome.

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Table 1. Characteristics of females with small ring X chromosomes

Subject	Age, years	%, rings*	Presence of *XIST* RNA†	DNA‡	FISH	MR	CM	Ref.
Severe phenotype								
1 (SV)	10	17	−		−	+	+	2
2 (BT)	3	60	−	−	−	+	+	5
3 (AE)	6	25	−		−	+	+	2
4 (AL)	6	85	±		+	+	+	13
5 (DC)	52	20	−		+	+	+	2
6 (SB)	33	21	−	+		+	+	4
7 (DM)	2½	20	−	+	+	+	+	4
8 (TT)	2	74 (59)	+		+, −§	+	+	2
Turner phenotype only								
9 (DS)	17	65	+		+	−	−	2

FISH, fluorescence *in situ* hybridization; MR, mental retardation; CM, congenital malformations; −, absence of locus or RNA; +, consistent signal, comparable to control specimens with normal inactive X; ±, very faint signal in some specimens. Blank indicates not done.

*Percent cells with one ring in specimens analyzed for RNA (for subject 8, percent cells with two rings is shown in parentheses).

†RNA was assayed by reverse transcription (RT)–PCR.

‡DNA was assayed by PCR and Southern blot analysis of hybrids containing only the ring X.

§Denotes heterogeneity of rings in female with two rings. Larger ring has *XIST* locus; smaller ring lacks the locus. The transcript must come from the larger ring (see text).

characteristics and karyotypes of all the females have been reported elsewhere (1, 2, 4, 5, 13) (Table 1).

Cell Cultures. Blood specimens were used as a source of DNA; lymphoblast or skin fibroblast cultures were used for RNA, karyotyping, *in situ* hybridization, and preparation of somatic cell hybrids.

Clonal Cultures. Clones were obtained from suspensions of fibroblasts plated at 10 cells per dish and isolated after 10 days with cloning cylinders. The clones were karyotyped to identify those with ring chromosomes in 90–100% of the cells.

Hybrid Cells. Hybrids were prepared from lymphoblast or fibroblast cultures by fusion with the mouse cell line tsA1S9az31B (14), and selection was carried out at 39°C, the nonpermissive temperature (14). Hybrids containing the ring X chromosome and not the normal X were obtained from three of these females and were identified by their polymorphic *DXS255* alleles.

DNA Analysis. DNA from hybrid cells was analyzed by Southern blot hybridization with the *XIST* cDNA probe 14A from exon 6 (6). DNA from these cells was also analyzed with PCR primers 1 and 3 for the region of *XIST* that includes exon 6, as well as *XIST* primers for the 5′ end (31 sense and 29r antisense) and for the 3′ region d (5r sense and 18r antisense) (8).

RNA Analysis. RNA (5 μg) obtained from lymphoblasts, fibroblasts, and hybrid cells (15) was analyzed for expression of the *XIST* locus by RT–PCR (16). The RNA was transcribed with the downstream *XIST* primer and 100 units of Moloney murine leukemia virus reverse transcriptase (BRL) in 20-μl

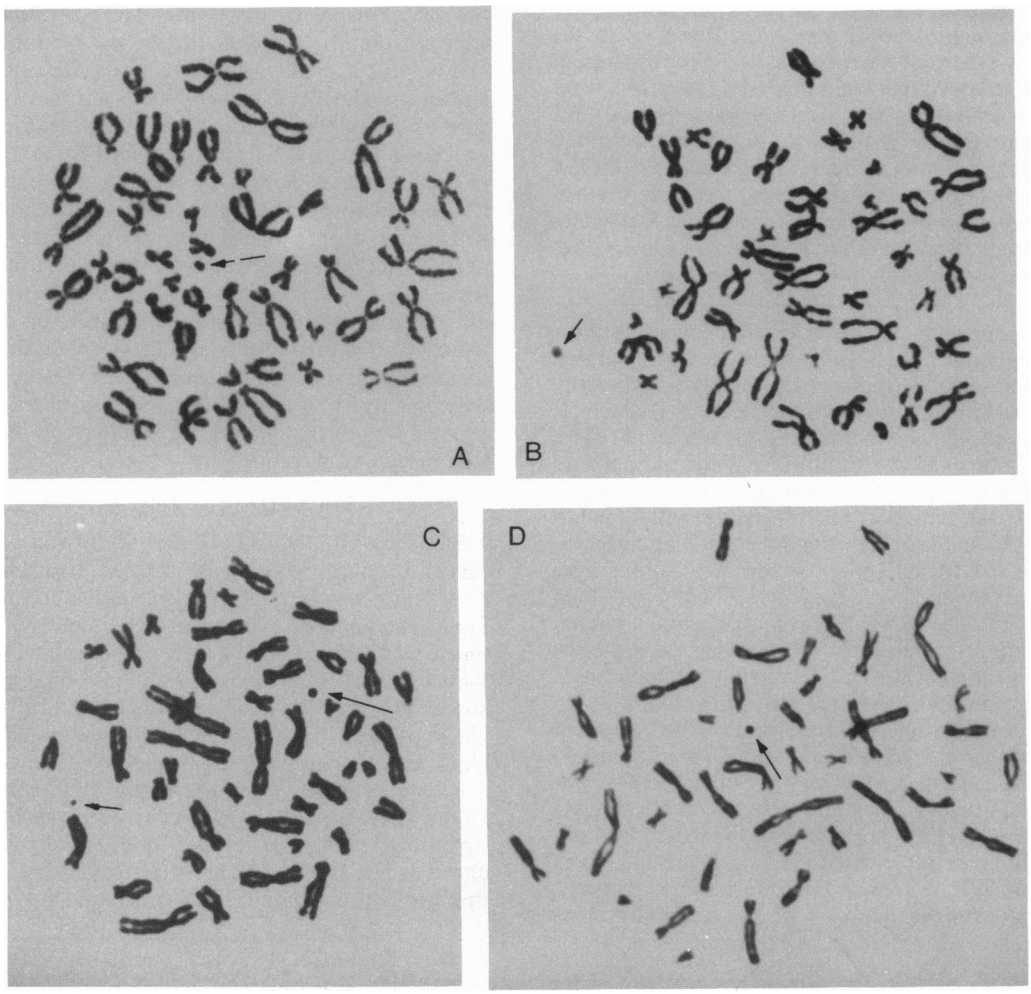

FIG. 1. Metaphases showing small ring chromosomes (arrow) from AL (*A*), SB (*B*), TT (*C*), and DM (*D*) (subjects 4, 6, 8, and 7, respectively). Note two rings in *C*: both were present in 59% of metaphases.

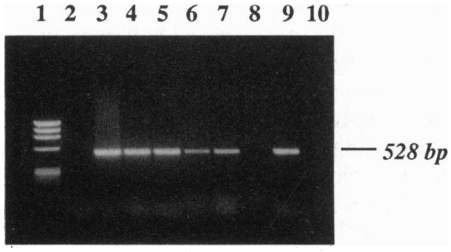

FIG. 2. Heterogeneity of ring hybrids revealed by PCR analysis using primers for the 5′ region of *XIST*. Lane 1, size markers; lane 2, mouse fibroblasts; lane 3, normal male; lane 4, normal female; lane 5, 4X female; lane 6, ring hybrids from DM; lane 7, SB; lane 8, BT; lane 9, hybrid with inactive X; lane 10, blank. The presence of the 528-bp product in the DM and SB ring hybrids (lanes 6 and 7), but not in the BT ring hybrid (lane 8), indicates heterogeneity with respect to presence of *XIST* sequences.

reaction mixtures; the cDNA obtained was amplified with both of the primers and 2.5 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus) in 100-μl reaction mixtures for 35 cycles. The amplification products were electrophoresed in a 2% NuSieve/agarose (1:1) (FMC) gel and stained with ethidium bromide. The primers are the same as those used for DNA analysis and they probe the regions a, b, and d (8). The housekeeping genes *P3* at Xq28 and *MIC2* at Xp23 were used as controls for the quality of the RNA. Both controls are expressed from the active X in the specimens analyzed, as neither locus is present on the ring chromosomes.

***In Situ* Hybridization.** Metaphase chromosomes were hybridized *in situ* with two yeast artificial chromosomes (YACs) containing the entire cDNA of the human *XIST* gene: YAC B245H8 (250 kb) is nonchimeric, extends from the 5′ region of *XIST* and includes the downstream *LAMRP4* locus. YAC A39G7 (450-kb insert) includes the entire *XIST* locus and some upstream sequences (17). YAC DNA was amplified with primers (Cl1 and Cl2) from within the *Alu* consensus sequences (18) and was biotinylated with a Bio-Nick kit (BRL). Approximately 250 ng of labeled amplification product was combined with 5 μg of Cot-1 DNA (BRL) and 2 μg of unlabeled yeast DNA. Alternatively, total YAC DNA was labeled and 300 ng was used as probe with suppression. The signal was detected as described by Walker *et al.* (19). For each subject, 10–20 metaphases which included the ring were scored for the presence or absence of signal on the ring.

RESULTS

Table 1 presents results of our analysis of the *XIST* locus in females with tiny ring chromosomes. All but one (subject 9, DS) had mental retardation as well as congenital malformations. In each case the ring chromosomes (Fig. 1) contain only small amounts of the X chromosome—with breakpoints within Xp11 and Xq13–21, based on molecular analysis (data not shown). Subject 8 (TT) had two tiny ring X chromosomes in many cells with one ring smaller than the other (Fig. 1*C*). For three of the females whose ring chromosome included the selectable *UBE1* locus in Xp11 (14), we were able to isolate the ring chromosome in somatic cell hybrids so as to facilitate analysis of its DNA by Southern or PCR analysis. The ring chromosomes in two of the hybrids [from subjects 6 (SB) and 7 (DM)] had sequences homologous to the *XIST* cDNA probe 14A on Southern blots, indicating that at least exon 6 was present. In addition, the predicted PCR products were obtained by using primers for exon 6 and the 5′ and 3′ regions of the locus. In contrast, the ring in the third hybrid (from subject 2, BT) was negative for *XIST* by Southern blot and PCR analysis (Fig. 2, lane 8). Therefore, by DNA analysis, we observed heterogeneity with regard to the presence of the *XIST* locus in these tiny ring chromosomes.

Using YAC clones containing the *XIST* locus for FISH to metaphase chromosomes from lymphoblasts or fibroblasts, we confirmed this heterogeneity. The ring chromosomes in subjects 1–3 (see Table 1), when analyzed by hybridization with YAC B245H8, containing the entire *XIST* locus and downstream sequences, had no hybridization signal on the ring but did show a clear signal on the normal X chromosome. In contrast, the ring chromosomes from subjects 4–8 hybridized with both YAC B245H8 and YAC A39G7. The two rings from subject 8 (TT) differed in that only the larger one consistently hybridized with *XIST*-containing YACs (e.g., Fig. 3*B*). Therefore, the results based on analysis of hybrids and *in situ* hybridization studies showed that not all of the tiny ring chromosomes analyzed lacked the region of the *XIST* locus.

As the ring chromosomes from some of the mentally retarded females had *XIST* DNA whereas others did not, we examined the RNA expression from the *XIST* locus on the ring chromosomes. RNAs prepared from lymphoblasts, fibroblasts, and hybrids were analyzed by RT–PCR, and the results are given in Table 1 and Figs. 4 and 5. As expected, no RT–PCR product was found in the three females who had no *XIST* locus by DNA or FISH analysis (subjects 1–3, Table

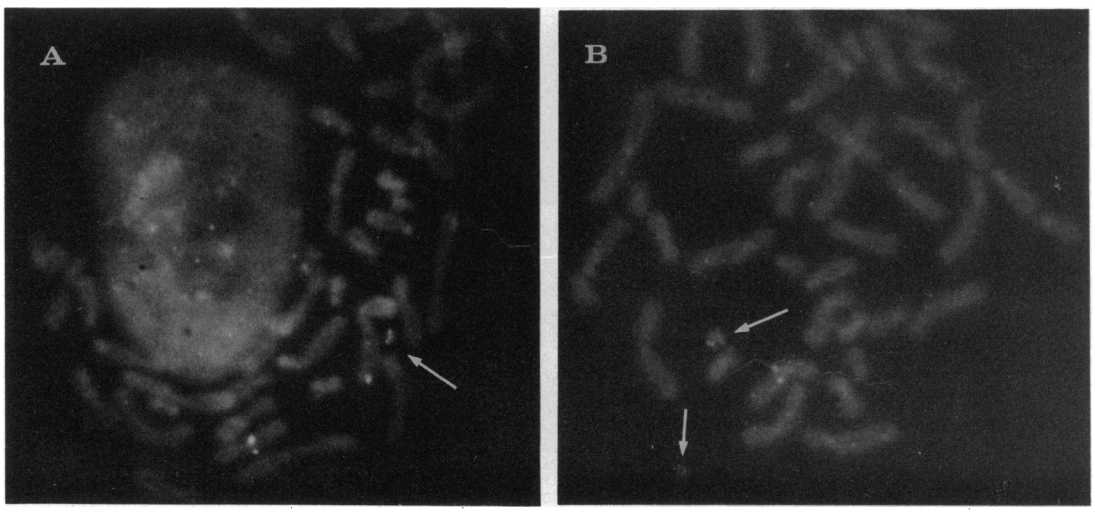

FIG. 3. FISH using *XIST* YAC 953. (*A*) Metaphase from subject 4 (AL) showing two signals on ring and signals on two chromatids of normal X. (*B*) Metaphase from subject 8 (TT) showing signals on X and one of the two rings. The ring chromosomes are indicated by arrows.

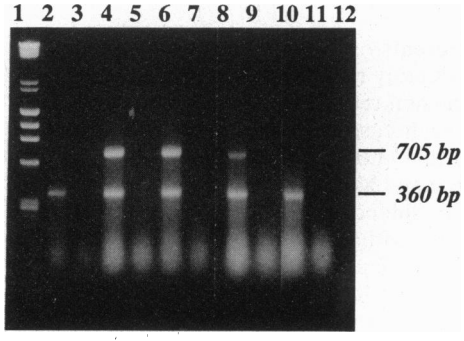

FIG. 4. RT–PCR of RNA from subjects with small ring chromosomes using *XIST* and *MIC2* primers simultaneously. The assay was carried out with reverse transcriptase (even-numbered lanes) and without reverse transcriptase (odd-numbered lanes). Lane 1, size markers; lanes 2 and 3, normal male; lanes 4 and 5, normal female; lanes 6 and 7, 4X female; lanes 8 and 9, lymphoblasts from TT; lanes 10 and 11, lymphoblasts from DC; lanes 12, blank. Note the 705-bp *XIST* product in normal female and 4X female, and in TT, but not in DC, who has only the 360-bp *MIC2* product.

1). However, even in those specimens with a single ring that had an *XIST* locus by FISH, the RT–PCR signals were consistently absent (subjects 5–7) (Fig. 4, lanes 10 and 11) or very weak (subject 4) (Fig. 5*A*, lane 4) compared with signals from a normal female (Fig. 4, lanes 4 and 5; Fig. 5*A*, lane 3) or from the specimen with two ring chromosomes (subject 8, Fig. 4, lane 8 and 9). The RNA was of good quality, as all *XIST*-negative RNA specimens (that had a normal X) gave a positive signal with primers for the X-linked *P3* locus and/or *MIC2* locus, which are transcribed from the normal active X. Therefore, even though the locus was present on some of the tiny ring chromosomes, it was not significantly expressed.

To exclude the possibility that the absent or minimal levels of *XIST* transcription were attributable to the low frequency of cells containing rings (Table 1), we established clones of single-cell origin from two subjects, nos. 4 and 7, whose rings had hybridized *in situ* with both of the *XIST*-containing YACs but produced none or very little of the *XIST* transcripts (Fig. 5*A*), respectively. Thirteen independent clones from subject 4 had the ring in 100% of their cells, and RNA from these 13 clones had a *P3* transcript (Fig. 5*C*). Eleven of them had no *XIST* RT–PCR product; the other 2 had very small amounts of the expected PCR product, but not enough to be visible in Fig. 5*B*. These results also show a high degree of consistency among clones. Analysis of the fibroblasts cloned from subject 7 which had the ring X chromosome in 90% of the cells also revealed barely visible levels of *XIST* transcripts (data not shown), despite the presence of strong signals for *XIST* DNA *in situ* and by Southern analysis. The only subject who had any significant RT–PCR product was subject 8, whose cells contained two ring chromosomes (Figure 4, lanes 8 and 9). Although we have been unable to isolate either ring in hybrids, it is clear that the transcript comes from the larger ring, as FISH studies show that the smaller ring lacks the *XIST* locus (Fig. 3*B*).

DISCUSSION

To test the hypothesis that activity of tiny ring X chromosomes is responsible for severe phenotypes, we have studied *XIST* expression in the ring chromosomes from eight females ascertained because they manifest mental retardation and congenital malformations. In all of the females with single rings, the *XIST* locus either was absent (Table 1, subjects 1–3) or, if present, was not transcribed (subjects 5–7) or transcribed at barely detectable levels (subject 4). While one of the two ring chromosomes in subject 8 was inactive as judged by *XIST* expression, the other had no *XIST* locus by FISH analysis (Fig. 3*B*); therefore, like the other females with severe phenotypes, she has a tiny ring chromosome that does not seem to be inactivated. The ring in subject 8 which expresses *XIST* is not obviously larger than those from the other females which do not express XIST. Therefore, although the smaller the ring, the greater probability that it lacks essential sequences, clearly it is the genetic content of the ring rather than its size that determines *XIST* expression. In addition, the lack of *XIST* transcription is associated with the severe phenotype, as the small ring in the female with a Turner phenotype [subject 9 (DS)] expressed *XIST* at the level of our normal female controls (data not shown).

It is unlikely that the lack of transcription we observed when the locus was present is attributable to deletion of

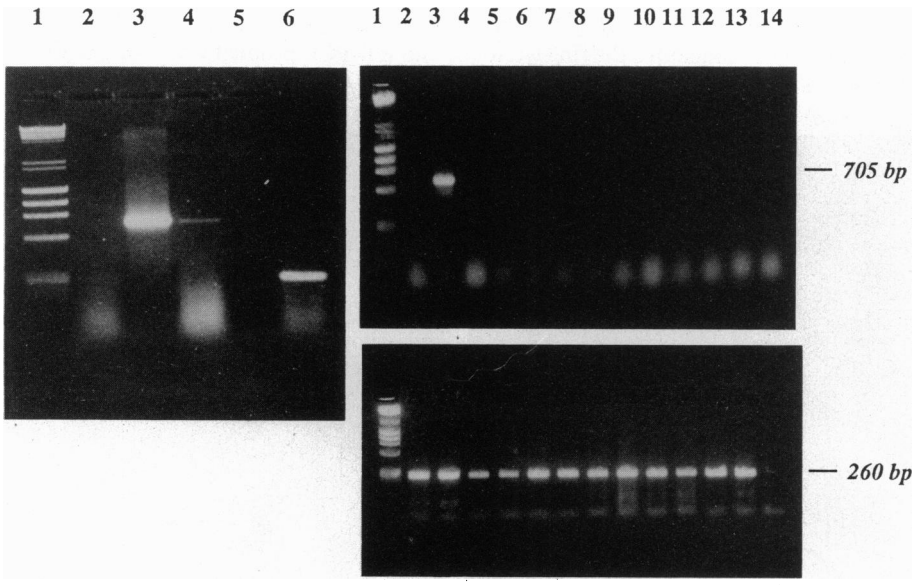

FIG. 5. RT–PCR amplification of RNA of fibroblast clones from subject 4 (AL). (*A*) Primers for *XIST* (lanes 2–4) or for *P3* (lane 6) were used. Lane 1, size markers; lane 2, normal male; lane 3, normal female; lanes 4 and 6, fibroblasts from AL; lane 5, blank. (*B*) *XIST* sequences from RNA from clones derived from AL. Each clone has the ring chromosome in each cell. Lane 1, markers; lane 2, normal male; lane 3, normal female; lanes 4–13, clones. (*C*) Same samples as in *B*, but amplified with *P3* primers.

sequences within the gene as it is now defined (8). Using primers for extreme 5′ and 3′ ends of the cDNA, we have shown that these sequences are present in rings from subjects 6 and 7 which have been isolated in hybrid cells. Such results suggest that *XIST* coding sequences may not be sufficient for X inactivation. Breakpoints in formation of these ring chromosomes may have disrupted neighboring regulatory or enhancer sequences, or there is a second gene in the XIC region essential for *XIST* transcription.

It is very likely that the absence of *XIST* expression means that these tiny rings are active chromosomes. The most salient feature of the *XIST* locus is its pattern of expression, being transcribed only from the inactive X chromosome in both mouse (7, 9) and human (6). It is thus far the only gene that is specifically transcribed from the inactive X. It does not appear to encode a protein, and the RNA remains within the nucleus (8, 20). The presence of *XIST* expression has been well correlated with inactivity of X chromosomes in female somatic cells (6), hybrid cells (6), and spermatocytes (11, 12). Therefore, because *XIST* expression is a specific marker for an inactive X chromosome, the lack of expression strongly suggests many of these tiny ring X chromosomes are active. This conclusion is supported by additional findings. Although studies of DNA replication of small chromosomes are difficult to interpret, they suggest that at least some of our tiny ring chromosomes are early replicating, a hallmark of X chromosome activity (21, 22). Lindgren *et al.* (5) noted that the ring chromosome in subject 2 was early replicating, compatible with our finding that it lacks an *XIST* locus. A third of the ring chromosomes in subjects 6 and 7 have been reported to be early replicating (4). In addition, preliminary studies indicate that tiny rings, deficient in *XIST* activity, contain acetylated histone 4, a cytological marker for an active chromosome (ref. 23; B.R.M. and P. Jeppesen, unpublished observations).

We find that the ring X chromosomes which are active, as defined by deficient *XIST* transcription, are present in females who have mental retardation and multiple congenital abnormalities. Therefore, our findings support the hypothesis that many of the smallest ring X chromosomes lack DNA sequences essential for X inactivation and are consequently active chromosomes, a reasonable explanation for the severe phenotype associated with these ring chromosomes. It is very likely that ring X chromosomes associated with severe phenotypes are mutants in the X inactivation pathway and that they will prove to be a powerful tool in the search for genes involved in X chromosome inactivation.

Note Added in Proof. We have observed that three X-linked loci (*AR, TIMP, and PHKA1*) are expressed from the ring chromosomes of subjects BT and AL, indicating that these chromosomes are active.

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