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 concepts 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 nonsimple 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. 1C and 3B). 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

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

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, years</th>
<th>% rings*</th>
<th>Presence of XIST</th>
<th>Severe phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RNA†</td>
<td>DNA‡</td>
</tr>
<tr>
<td>1 (SV)</td>
<td>10</td>
<td>17</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2 (BT)</td>
<td>3</td>
<td>60</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3 (AE)</td>
<td>6</td>
<td>25</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4 (AL)</td>
<td>6</td>
<td>85</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>5 (DC)</td>
<td>52</td>
<td>20</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6 (SB)</td>
<td>33</td>
<td>21</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7 (DM)</td>
<td>2½</td>
<td>20</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8 (TT)</td>
<td>2</td>
<td>74 (59)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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 tsA159ats31B (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.
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 M1C2 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 (C11 and C12) 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.

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 X chromosome in many cells with one ring smaller than the other (Fig. 1C). For three of the females whose ring chromosome included the selectable UBEI 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. 3B). 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
clones had a P3 transcript (Fig. 5C). 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. 5B. 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. 3B).

**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. 3B); 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
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 PHKAI) are expressed from the ring chromosomes of subjects BT and AL, indicating that these chromosomes are active.

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