X chromosome inactivation of the human TIMP gene

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ABSTRACT

X chromosome inactivation results in the cis-limited inactivation of most, but not all, genes on one of the two X chromosomes in mammalian females. The molecular basis for inactivation is unknown. In order to examine the transcriptional activity of human X-linked genes, a series of mouse-human somatic cell hybrids under positive selection for the active or inactive human X chromosome has been created. Northern blot analysis of RNA from these hybrids showed that the human *MIC2* gene, which is known to escape X inactivation, was transcribed in hybrids with either the active or inactive X chromosome. In contrast, the human *TIMP* gene was only transcribed in hybrids with an active human X chromosome. Further analysis using the polymerase chain reaction showed that there was at least one-hundred fold less transcription of the *TIMP* gene from the inactive X than from the active X chromosome. These findings demonstrate that the human *TIMP* gene is subject to X inactivation at the level of transcription, and illustrate the usefulness of the polymerase chain reaction to study the extent of X-linked gene repression by the process of X inactivation.

INTRODUCTION

Genes located on the mammalian X chromosome are subject to a unique form of gene regulation known as X chromosome inactivation (1). In females, one of the pair of X chromosomes is inactivated early in development, resulting in the chromosome becoming heterochromatic, late-replicating and, for the most part, genetically inert (2, 3). Of the over 140 different expressed loci which have been assigned to the human X chromosome (4), there is evidence for approximately forty of them being subject to X chromosome inactivation (5). Although transcriptional inactivation is presumed to be the basis for all X-inactivation, it has only been shown directly for two genes, *HPRT* (6) and DXS435E (7). The basis for inactivation of other X-linked genes remains unknown. There are five X-linked genes which are known to escape the process of X chromosome inactivation, *STS*, *XG*, *MIC2*, *ZFX* and *A1S9T* (8–12). Transcription from both the active and inactive X chromosomes has been demonstrated directly for *STS* and *ZFX* (11).

To determine whether genes are subject to X inactivation, we have based a test system on the observation that the human X chromosome retains its active or inactive state when isolated in somatic cell hybrids made with rodent cell lines (13–16). Thus human-rodent hybrids with active or inactive human X chromosomes provide a means of directly analyzing the transcriptional activity of genes on the X chromosome (7). In the present study, we have made use of hybrids under positive selection for the retention of either the active or inactive human X chromosome to analyze the transcriptional activity of the human *TIMP* gene compared with that of the human *MIC2* gene, which is known to escape X inactivation (10).

TIMP, the X-linked gene encoding a tissue inhibitor of metalloproteinases, has not been analyzed previously for inactivation and has not been associated with any known disease phenotype to date. Because *TIMP* maps to the proximal short arm of the human X chromosome (17), a region known to contain at least one locus that escapes X inactivation (18), it is possible that *TIMP* might also escape inactivation. Furthermore, *TIMP* can be transcriptionally activated by serum, growth factors or phorbol esters (19), which allows examination of the effect of gene induction in conjunction with X inactivation.

Using Northern blot analysis, we have detected both *MIC2* and *TIMP* transcripts in the hybrids containing an active X, but only *MIC2* transcripts in the hybrids with an inactive human X chromosome. Thus the human *TIMP* gene is not transcribed from inactive X chromosomes and must, therefore, be subject to the process of X inactivation. Further analysis of the RNA from these hybrids by polymerase chain reaction amplification demonstrated that transcription of the *TIMP* gene on the inactive X chromosome is repressed by a factor of at least one-hundred fold compared to transcription from the active X.

MATERIALS AND METHODS

Hybrid cell lines and tissue culture

The derivation and characterization of the cell lines used has been described previously. The human-mouse somatic cell hybrids AHA-11aB1 and t60-12 (20) contain the X chromosome as their

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only human chromosome. The X chromosome in these hybrids is genetically active and early-replicating (12). The hybrids t11-4Aaz5 and t48-1a-1Daz4A were isolated after selection in azaguanine and thioguanine (at 2×10^{-4} M; 6×10^{-5} M respectively) to select against the active X chromosome. The inactive state of the X chromosome in these hybrids has been confirmed by replication studies, analysis of X-linked enzymes, and DNA methylation studies (12). The t60-12, t11-4Aaz5 and t48-1a-1Daz4A hybrids were created by fusion of human cell lines with the temperature-sensitive (ts) murine cell line, tsA1S9. In such hybrids, the murine ts mutation can be complemented by a locus on the human X chromosome, *A1S9T*, that escapes inactivation (12). Thus, survival at 39°C is dependent on the presence of either an active or inactive human X chromosome (12, 20). These cell lines were grown at 39°C to select for retention of the X chromosome, while AHA-11aB1 was maintained at 37°C in medium supplemented with HAT (10^{-4} M hypoxanthine; 1.6×10^{-5} M thymidine; 4.2×10^{-4} M aminopterin) to select for retention of the active X chromosome. All lines were maintained in α-MEM medium supplemented with glutamine and 7.5% fetal bovine serum (FBS), except for the human fibroblast strains which were supplemented with 15% FBS, glutamine and non-essential amino acids.

For induction studies, subconfluent cell monolayers were treated for six hours with 12-O-tetradecanoylphorbol 13-acetate (TPA; 100ng/ml) in α-MEM supplemented with 2% FCS to induce *TIMP* expression (19). Non-induced cells were incubated in the same medium without TPA prior to RNA isolation.

RNA isolation and analysis

RNA was prepared using the guanidinium-thiocyanate method (21). 15 μg of total RNA was electrophoresed in 1.2% agarose gels in the presence of formaldehyde and transferred by capillarity in 20×SSC to nitrocellulose membranes. Membranes were prehybridized and hybridized at 42°C in 50% formamide, 5×SSC, 5×Denhardt solution, 0.1% sodium dodecyl sulphate, 7mM Tris pH 7.5, and 0.2 mg/ml sheared salmon sperm DNA. Blots were washed to a stringency of 0.1×SSC/0.1% SDS at 42°C. The cDNA probe, pNT3, was used to detect human *MIC2* transcripts (22). *TIMP* RNA was detected using the human-specific *TIMP* cDNA probe, p91023B (23), or the mouse-specific *TIMP* cDNA probe, pTIMP-8 (24). Isolated probe inserts were labelled by the random priming method (25).

For analysis of gene expression by polymerase chain reaction (PCR), 5 μg of RNA was reverse-transcribed with 200 U of M-MLV reverse transcriptase (BRL, Gaithersburg, MD) in 50mM KCl, 20 mM Tris/Cl pH 8.4, 2.5 mM $MgCl_2$, 1 mM each nucleotide, 20 U RNasin (Boehringer Mannheim), 2 μg BSA, and 7.5 μM random hexamer primers (Pharmacia) for 60 minutes at 42° (26). Aliquots from 250 ng to 1 ng of this reaction were amplified for 30 cycles in a Perkin-Elmer thermocycler with Promega Taq polymerase (conditions as described by Promega). A cycle consisted of a one minute denaturation at 94°C, a one minute annealing at 54°C and a four minute elongation at 72°C. The primers used for the *MIC2* amplification were 5′-ACCCAGTGCTGGGGATGACT-3′ and 5′-TCTCCATGT-CCACCTCCCCT-3′, two sequences spanning 360 bp of the *MIC2* cDNA (22). For *TIMP* PCR analysis the primers used were 5′-AACTGCAGGATGGACTCTTG-3′ from the 5′ untranslated region, and 5′-TGCAGGCTTCAGCTTCCACG-3′ which spans the first and second exons (27), resulting in a 147 bp amplification product. 20 μl of the 100 μl PCR reaction was electrophoresed on a 2% TAE (0.4 M Tris/Cl; 0.013 M sodium acetate; 0.002 M EDTA; pH 8.0) agarose gel, and visualized under ultraviolet light after staining with ethidium bromide.

DNA isolation and analysis

DNA was isolated by phenol extraction, or according to non-organic DNA isolation protocols (ONCOR, Gaithersburg, MD), and restricted with XbaI. Conditions for restriction enzyme digestion, gel electrophoresis, Southern blotting, prehybridization and hybridization were as previously described (28). Isolated probe inserts were labelled by the random priming method (25). The final wash was at 65°C in 0.1×SSC and 0.1% SDS. For analysis of the genomic *TIMP* gene, a lambda phage containing the TIMP gene was isolated from the Los Alamos National Laboratory X chromosome-specific library LAOXNL01 (obtained from the N.I.H. Probe and Library Repository at the American Type Culture Collection, Rockville, MD) by screening with the *TIMP* cDNA probe, p91023B. A 3.9 kb XbaI fragment free of human repetitive DNA from the 5′ portion of the gene was subcloned from this phage into the Xba1 site of pTZ. The resulting probe, TIMP-3.9X, was used for analysis of genomic DNA.

RESULTS

Under appropriate selective conditions, tsA1S9-human somatic cell hybrids specifically retain one or more copies of the inactive human X chromosome in all cells (12). In this study, we have used such inactive X hybrids to ask whether two human X-linked genes, *MIC2* and *TIMP*, are transcribed from the inactive X chromosome. RNA from four different hybrids, two containing only an active X and two containing an inactive X (in the absence of an active X) has been analyzed for the presence of *MIC2* and *TIMP* transcripts.

The four hybrid cell lines were first analyzed by chromosome and DNA analysis to ensure that the human X chromosome was present. In a Southern blot hybridized with a human *TIMP* genomic probe (Figure 1) the band detected (3.9 kb) was of the size expected for a XbaI genomic digest with this probe. Both the active (lanes 5 and 6) and inactive (lanes 3 and 4) X hybrids contain an equivalent amount of human X sequences. The hybrid DNAs (lanes 3−6) show a fainter signal than human female DNA (lane 1), as expected since the hybrids have only one human X chromosome in the presence of approximately 90 mouse chromosomes. Karyotypic analysis of the chromosomes in the four hybrids confirmed the existence of a normal human X chromosome in all cells (data not shown).

As a control to assess the validity of a somatic cell hybrid system for detection of transcription from an inactive X, the expression of the human *MIC2* gene was analyzed by Northern blot hybridization of RNA from the two active X hybrids, the two inactive X hybrids, and mouse and human control lines (panel A, Figure 2). Mouse control RNA (lane 2) showed no signal, while human RNA (lane 1) and RNA from both the inactive X hybrids (t48-1a-1Daz4A and t11-4Aaz5; lanes 3 and 4) and the active X hybrids (AHA-11aB1 and t60-12; lanes 5 and 6) contained a transcript of ~1.3 kb, the expected size for the *MIC2* transcript (22). There was no observable difference between cells treated with TPA and those left untreated (lanes 'a' versus 'b'). These data indicate that the *MIC2* gene is transcribed from both active and inactive X chromosomes, consistent with previous data

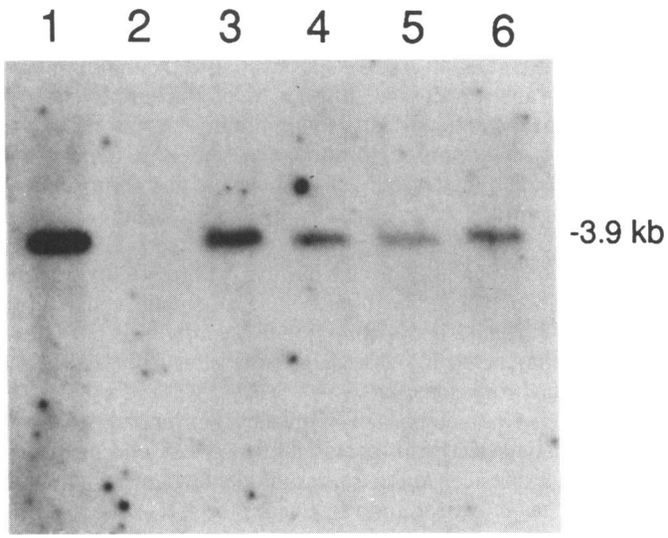

FIG. 1. Presence of human X-linked *TIMP* gene in somatic cell hybrids. DNA was isolated from (1) human fibroblasts; (2) mouse line tsA1S9; (3) t48-1a-1Daz4A (inactive X hybrid); (4) t11-4Aaz5 (inactive X hybrid); (5) AHA-11aB1 (active X hybrid); (6) t60-12 (active X hybrid). DNA was digested with XbaI, transferred to Hybond nylon membrane and hybridized with the human TIMP3.9X probe.

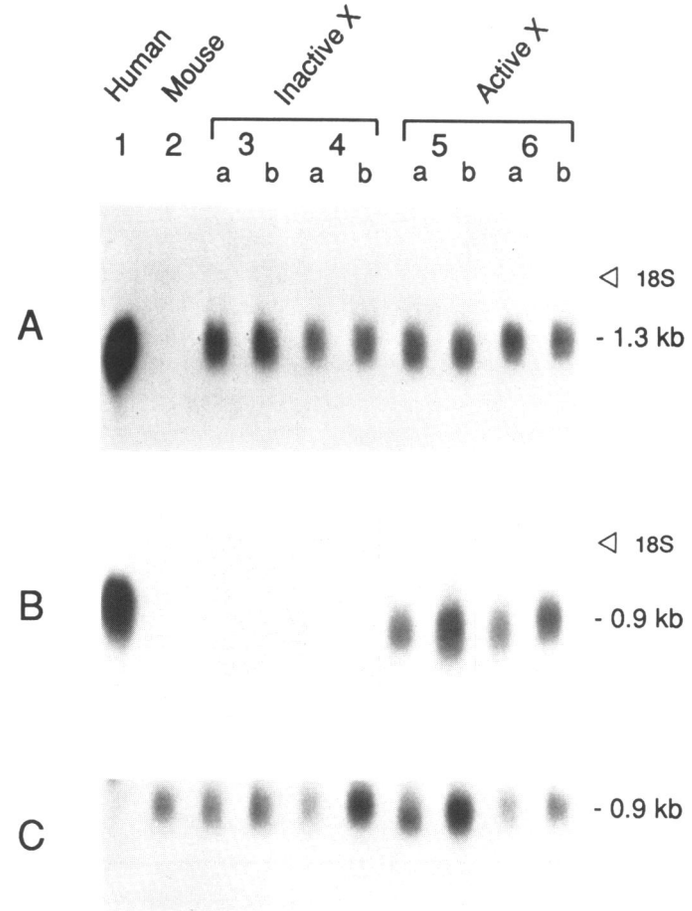

FIG. 2. Northern blot analysis of hybrids with active and inactive human X chromosomes. RNAs were isolated from (1) human fibroblasts; (2) mouse line tsA1S9; (3) t48-1a-1Daz4A (inactive X hybrid); (4) t11-4Aaz5 (inactive X hybrid); (5) AHA-11aB1 (active X hybrid); (6) t60-12 (active X hybrid). Lanes labelled 'a', as well as lanes 1 and 2, contain RNA from cells that were not treated with TPA, while RNA in the 'b' lanes was from cells treated with TPA. The RNA was transferred to nitrocellulose and hybridized with: A. the human *MIC2* cDNA probe, pNT3; B. the human specific *TIMP* cDNA probe, p91023B; and C. the murine specific *TIMP* cDNA probe, pTIMP-8.

demonstrating that the *MIC2* cell surface antigen is expressed from both active X and inactive X hybrids (10).

To determine whether the human *TIMP* gene was expressed from an inactive X, a similar RNA analysis was carried out (panel B, Figure 2). When the *TIMP* cDNA clone p91023B was used under conditions that allow species-specific hybridization, the human control RNA (lane 1) exhibited a strong signal of ~800−900 bp in size, while the mouse control RNA (lane 2) showed no signal. RNA from the active X hybrids (lanes 5a and 6a) showed a basal signal, consistent with the known expression of *TIMP* in cultured fibroblasts (27). This signal was increased in RNA isolated from cells treated with TPA (lanes 5b and 6b), indicating induction of *TIMP* expression by TPA (19). In contrast, the inactive X hybrids did not show a detectable hybridization signal either with (lanes 3b and 4b) or without (lanes 3a and 4a) treatment with TPA. As an added control, the same blot was reprobed with the mouse *TIMP* cDNA, to demonstrate that all the hybrid lanes have intact RNA. All the lanes except the human lane showed a signal indicative of the murine *TIMP* transcript (panel C, Figure 2).

To further assess the transcriptional activity of the X chromosome in these hybrids, the RNA was amplified by the polymerase chain reaction using species-specific primers for each gene. Amplification was performed with both pairs of primers in one reaction to control for reaction conditions. An amplification product of 360 bp, the size expected for the *MIC2* primers, was detected in the cDNA from both the active and inactive X containing hybrids and the human control (Figure 3, lanes 1, 3−6), but not in the mouse cDNA (lane 2), consistent with the Northern blot hybridization analysis. The smaller 147 bp amplification product, of the size expected for the *TIMP* primers, was also observed in the human (lane 1) but not the mouse (lane 2). However, only the active X hybrids (lanes 5 and 6) yielded such a product. The inactive X hybrid lanes (3 and 4) have no band apart from *MIC2*. There does not appear to be any significant difference in signal intensity between the TPA-induced and uninduced RNAs (lanes 'a' versus 'b'), presumably because the amount of *TIMP* RNA is not rate-limiting in this analysis.

To compare the amounts of transcription between the active and inactive X chromosomes, different amounts of cDNA were used in the PCR amplification (Figure 4). The first six lanes show a dilution series of cDNA from the active X hybrid t60−12. Between 50 and 250 ng of starting cDNA, there is little if any difference in signal intensity for both the *MIC2* and *TIMP* amplification signals. When 5.0 or 2.5 ng of cDNA was used in the amplification, a faintly visible band is seen for both *TIMP* and *MIC2*. With only 1 ng of starting cDNA, no bands were visible under these conditions. For the inactive X hybrid t11−4Aaz5, the MIC2 band was similar to that seen for the active X. However no band corresponding to *TIMP* was seen at any dilution. Similar results were seen with the other two active and inactive X hybrids used in this study (data not shown). These data indicate at least a one hundred-fold difference in the level of *TIMP* RNA between the active X and inactive X hybrids.

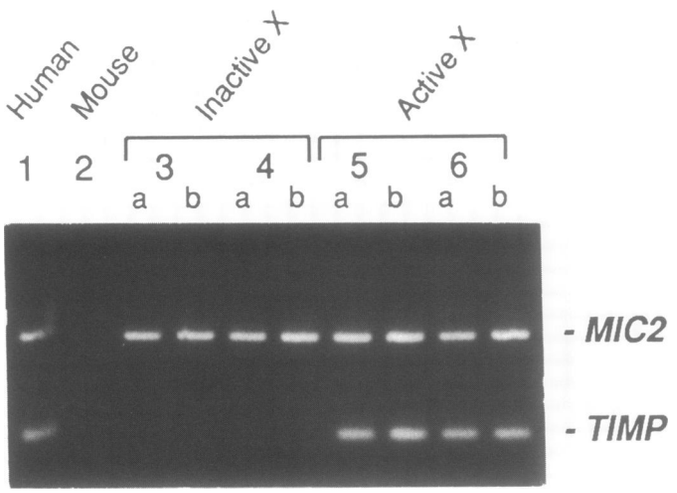

FIG. 3. PCR amplified products from 50 ng cDNA reverse-transcribed from the RNA from (1) human fibroblasts; (2) mouse line tsA1S9; (3) t48-1a-1Daz4A (inactive X hybrid); (4) t11-4Aaz5 (inactive X hybrid); (5) AHA-11aB1 (active X hybrid); (6) t60-12 (active X hybrid). Lanes labelled 'a', as well as lanes 1 and 2, contain RNA from cells that were not treated with TPA, while RNA in the 'b' lanes were from cells treated with TPA. 20 μl of the 100 μl PCR amplification reaction were run on a 2% TAE gel and stained with ethidium bromide. The band marked *TIMP* is about 150 bp in size, while that marked *MIC2* is 360 bp.

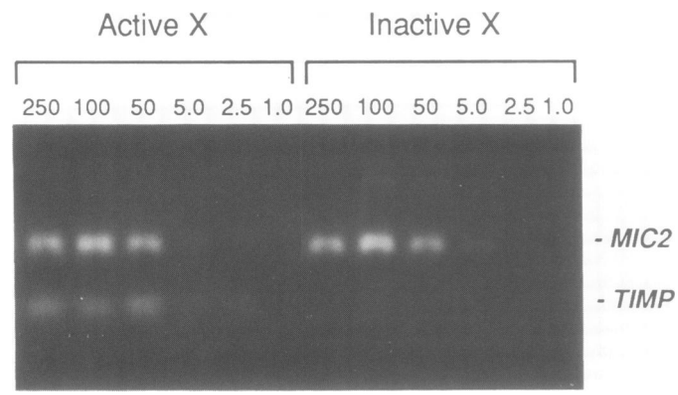

FIG. 4. PCR amplified products from a dilution series of cDNA reverse-transcribed from t60-12 (active X) and t11-4Aaz5 (inactive X) after induction with TPA. Amounts of cDNA, in ng, in each reaction are as labelled. The band sizes, and gel running conditions are as for Figure 3.

DISCUSSION

X chromosome inactivation resulting in dosage compensation between males and females was first proposed by Lyon in 1961 (1). While it seems likely that most X-linked genes are subject to inactivation in females, at least five genes are known to escape inactivation. Genes which escape inactivation may provide information on the process of inactivation and may be involved in the phenotypic effect of X chromosome aneuploidies (29). Since genes that escape inactivation map to at least three different intervals on the short arm of the human X chromosome (4, 11, 18), it is of interest to directly assess the inactivation status of all X-linked genes, particularly those on the short arm.

Most of the existing evidence for genes being subject to X chromosome inactivation is based on expression analyses, primarily enzyme assays, antibody reaction or complementation (eg. 30–33) and does not address the level at which inactivation occurs. One method to assess X inactivation at the transcriptional level is to analyze dosage differences of transcripts between males, females, and individuals with multiple X chromosomes. This approach was used to show that the *ZFX* gene is not transcriptionally inactivated (11). However, this approach may not be straightforward or generally applicable, as the expression of at least some genes from the inactive X is reduced compared to the active X (34,35). Since the inactive X chromosome remains inactive when isolated in mouse/human somatic cell hybrids (10–15), the analysis of expression in such hybrids provides a means of independently assessing expression from active and inactive X chromosomes.

The activity of a number of X-linked genes has been assessed using somatic cell hybrids containing inactive X chromosomes. However, expression at the transcriptional level in such hybrids has only been examined for a few genes (6, 7, 11), due, at least in part, to the relative difficulty of obtaining hybrids with an inactive X passively retained in a high frequency of cells. To overcome this difficulty, we have exploited the *A1S9T* locus (12), which provides a selectable marker for the inactive human X chromosome, so that hybrids containing only the inactive X can be directly selected for without requiring reactivation of a locus on the inactive X (13). In this study, we have used two inactive X hybrids created with the tsA1S9 cell line, along with two active X hybrids, to analyze the transcription of two X-linked genes. The *MIC2* gene, which is known to escape X inactivation, was analyzed to confirm that transcription from the inactive X could be observed in hybrid lines. Both the active and inactive X hybrids expressed a human-specific RNA corresponding to the *MIC2* transcript (24). The amount of transcript did not appear to differ significantly between the active and inactive X hybrids. The transcription of the *TIMP* gene was assessed using these same RNAs. There was no prior evidence to indicate whether or not *TIMP* would be inactivated. In contrast to the results obtained with *MIC2*, the human-specific *TIMP* transcript was observed only in active X hybrids and not in the uninduced or TPA-induced inactive X hybrids. These data suggest that TIMP is subject to X inactivation in the two inactive X hybrids analyzed, and further, that the basis for X inactivation is transcriptional. Treatment with TPA, a phorbol ester, increased the expression from the active X, but did not induce expression from the inactive X, suggesting that the transcriptional basis of inactivation cannot be overriden by such induction signals.

The technique of polymerase chain reaction amplification of reverse-transcribed RNA has been used to detect low levels of transcription (36, 37), often below that which could be detected by Northern blot analysis. We therefore applied this technique to our study of the transcriptional inactivation of the *TIMP* gene. Amplified product was detected in reverse-transcribed RNA from active X containing hybrids even when diluted one-hundred fold, while none was visible from the inactive X hybrids, suggesting that *TIMP* gene expression is at least two orders of magnitude greater from the active X than from the inactive X chromosome. This finding, if confirmed for other X-linked loci, suggests that repression of transcription by the X inactivation signal(s) is highly effective.

The *TIMP* gene has been assigned to Xp11.23-p11.3 (17), a region that overlaps the location of the non-inactivated *A1S9T* gene in Xp11.1-p11.3 (18). *TIMP* is the most proximal gene on the short arm of the X that has been demonstrated to undergo

X inactivation and thus may set a distal limit on the extent of a possible non-inactivated segment in band Xp11 (38).

This work confirms that somatic cell hybrids containing active or inactive X chromosomes can be used to study the transcriptional activity of cloned X-linked genes (7), and further examines the degree of transcriptional inactivation using the polymerase chain reaction. Assessment of the transcriptional basis for inactivation and the extent of repression by the X inactivation process should be useful in considering models for the mechanism(s) of X chromosome inactivation.

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