Am. J. Hum. Genet. 65:699–708, 1999

Polymorphic X-Chromosome Inactivation of the Human *TIMP1* Gene

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Summary

X inactivation silences most but not all of the genes on one of the two X chromosomes in mammalian females. The human X chromosome preserves its activation status when isolated in rodent/human somatic-cell hybrids, and hybrids retaining either the active or inactive X chromosome have been used to assess the inactivation status of many X-linked genes. Surprisingly, the X-linked gene for human tissue inhibitor of metalloproteinases (*TIMP1*) is expressed in some but not all inactive X-containing somatic-cell hybrids, suggesting that this gene is either prone to reactivation or variable in its inactivation. Since many genes that escape X inactivation are clustered, we examined the expression of four genes (*ARAF1, ELK1, ZNF41,* and *ZNF157*) within ~100 kb of *TIMP1.* All four genes were expressed only from the active X chromosome, demonstrating that the factors allowing *TIMP1* expression from the inactive X chromosome are specific to the *TIMP1* gene. To determine if this variable inactivation of *TIMP1* is a function of the hybrid-cell environment or also is observed in human cells, we developed an allele-specific assay to assess *TIMP1* expression in human females. Expression of two alleles was detected in some female cells with previously demonstrated extreme skewing of X inactivation, indicating *TIMP1* expression from the inactive chromosome. However, in other cells, no expression of *TIMP1* was observed from the inactive X chromosome, suggesting that *TIMP1* inactivation is polymorphic in human females.

Introduction

X-chromosome inactivation is the transcriptional silencing of one of the two X chromosomes in mammalian females, which achieves dosage compensation for X-linked gene products, between males and females (Lyon 1961). Within each cell, one X chromosome is chosen, early in development, to be inactivated, and this epigenetic silencing is maintained in a clonal fashion throughout subsequent somatic-cell divisions (Davidson et al. 1963). The exceptional stability of this silencing is demonstrated by tumors that maintain clonal X inactivation, with only one allele expressed after >100 cell divisions (Linder and Gartler 1965). The inactive X chromosome is hypermethylated and nuclease insensitive and has delayed replication timing compared with the active chromosome (Gartler and Goldman 1994). *XIST* is expressed only from the inactive X chromosome, and the XIST RNA is localized to the inactive X chromosome (Brown et al. 1992). The histones associated with the inactive X chromosome tend to be hypoacetylated (Jeppesen and Turner 1993), and there is preferential association of histone macroH2A1 (Costanzi and Pehrson 1998). It is believed that these factors contribute, either alone or cooperatively, to the stable silencing of the chromosome.

Although the majority of X-linked genes are believed to be subject to the dosage-compensation mechanism of X inactivation, there are numerous genes that escape this silencing and that are expressed from both the active and inactive X chromosomes (Disteche 1995). The X-inactivation status of human X-linked genes has been determined in a number of ways. Direct evidence for X inactivation includes mosaic expression of X-linked gene products in heterozygous females or equal levels of gene expression in males and females (reviewed in Brown and Willard 1993). Rodent/human somatic-cell hybrids retain the X-inactivation status of their human X chromosome (Migeon 1972) and are therefore frequently used to assess the expression state of individual genes from the active or inactive X chromosome (Brown et al. 1997; Esposito et al. 1997). Previous analyses of the inactivation status of X-linked genes, using somatic-cell hybrids, have been concordant with prior evidence for X-inactivation status (Migeon 1972; Schneider-Gadicke et al. 1989; Fisher et al. 1990). Surprisingly, some genes are expressed in some but not all of the inactive X-containing hybrids (Brown et al. 1997). One of the genes showing this variable expression is the human tissue inhibitor of metalloproteinases-1 (*TIMP1* [MIM

Received April 24, 1999; accepted for publication June 30, 1999; electronically published July 29, 1999.

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0002-9297/1999/6503-0015$02.00

305370]), one member of a family of proteins that inhibit metalloproteinases by binding to their active sites (Denhardt et al. 1993).

Genes expressed from both the inactive and active X chromosomes are often located in clusters along the X chromosome, suggesting a regional basis for the establishment of X-inactivation patterns (Disteche 1995; Miller and Willard 1998). Most of the ~30 genes known to escape inactivation are located either in the pseudoautosomal and flanking regions of distal Xp or in the proximal short arm of the X chromosome. These regions of the inactive X chromosome behave more like the active chromosome, replicating earlier and retaining more acetylation of histones than does the remainder of the inactive X chromosome (Schempp and Meer 1983; Jeppesen and Turner 1993). Human *TIMP1* is in a gene-rich region containing *ARAF1, ELK1, ZNF41*, and *ZNF157* within 100 kb (Coleman et al. 1994; Knight et al. 1994; Derry et al. 1995; Brandau et al. 1998). We therefore assessed whether this entire region was expressed from the inactive X chromosome, by examining the expression of these genes flanking *TIMP1*.

Expression from the inactive X chromosome may also be the result of reactivation of a gene normally subject to X inactivation. Reactivation of the entire inactive X chromosome occurs naturally during oogenesis, and single genes have been shown to reactivate with age in mice. Experimentally, reactivation of single genes or regions of the X chromosome can be increased by cell fusion and/or treatment with demethylating agents (reviewed in Gartler and Goldman 1994). The maintenance of X inactivation does appear to be less stringent in rodent/human hybrids compared with human fibroblasts (Kahan and DeMars 1975; Ellis et al. 1987), and the observed expression of *TIMP1* from the inactive X chromosome may be due to the hybrid-cell environment. To determine if *TIMP1* expression from the inactive X chromosome occurs in human cells as well as in hybrid cells, an expressed polymorphism in the *TIMP1* gene was identified and was used to assess expression in heterozygous female cells previously shown to have extreme skewing of X inactivation.

Material and Methods

Somatic-Cell Hybrids

Human/rodent somatic-cell hybrids were kindly provided by H. F. Willard (Case Western Reserve University) or S. Hansen (University of Washington) or were generated by somatic-cell fusion as described elsewhere (Hansen et al. 1988; Brown and Willard 1989; Willard et al. 1993). Fourteen independent hybrids were used to study the expression of genes in the region surrounding *TIMP1*. Six hybrids retained an active X chromosome, and eight hybrids had an inactive X chromosome, including the four inactive X-containing hybrids that displayed *TIMP1* expression. The hybrids contained entire human X chromosomes—except for t81-az1D, which had a shortened human X chromosome that was deleted for the majority of the long arm (Brown et al. 1991). There were no autosomes in common among all of the inactive X-containing hybrids that expressed *TIMP1*.

Reverse Transcriptase–PCR Analysis (RT-PCR)

Cells were harvested at confluence with trypsin-EDTA (0.25%). RNA was prepared by means of a standard acid-guanidinium extraction protocol (Chomczynski and Sacchi 1987). RNA concentration was determined spectrophotometrically, and 2–5 μg of RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), by random hexamer priming (Brown et al. 1990). The PCR primers used to amplify products for the genes at Xp11.23 are listed in table 1, with the corresponding amplification conditions. PCR was performed by means of GIBCO-BRL *Taq* polymerase, 1 μM primer, 20 μM of each dNTP, and 100–200 μg of template, in a Techne thermocycler. As controls, all primer pairs were shown to be human specific, with no amplification of mouse and hamster cDNA, and all cDNAs were demonstrated to be free of DNA contamination, by amplification of RNA without reverse transcription, by means of the *XIST* 1:2r primers that amplify DNA (Brown et al. 1992). The presence or absence of *XIST* expression, which is transcribed only from the inactive X chromosome, confirmed the inactivation status of the human X chromosome in the hybrids and demonstrated the presence of amplifiable cDNA in the inactive X–containing hybrids.

TIMP1 *Polymorphism Assay*

To identify expressed polymorphisms, human *TIMP1* cDNA (EMBL A10416 [EMBL Outstation, European Bioinformatics Institute]) was compared with all sequences in GenBank (version 101), by means of the BLASTN program (BLAST). Of 17 sequences for this region, 4 contained a C instead of a T at base pair 434 in exon 5. A constant primer (CA1) and a pair of allele-specific oligonucleotide primers were designed with the variant base at the 3 end of the second primer. PCR conditions were optimized to allow allele-specific amplification (table 1 and fig. 2). The primers do not amplify mouse DNA or mouse cDNA and give a smaller product when amplifying human cDNA, because they span an intron. The amplification products were visualized on 2% agarose gels stained with ethidium bromide. The female cells were shown to have extreme skewing of X inactivation (>90% one allele active), by one of several assays that rely on either an expressed polymorphism (*XIST* [Rupert et al. 1992]) or methylation differences near a polymorphic site (androgen re-

Table 1

PCR Primers and Amplification Conditions

Gene	Primers[a]	Product Size (bp)	PCR Conditions	Reference
TIMP1	C1A: CCCTGATGACGAGGTCGGAA C1B: AGATCCAGCGCCCAGAGAGA	147	1.5 mM $MgCl_2$; 94°C, 1 min; 54°C, 1 min; 72°C, 2 min (30 cycles)	Brown et al. (1990)
ARAF1	B: TCAGCAAAATCTCCAGCAAC 3: TGGAGATGGAGGAGCTCCCA	482	2.5 mM $MgCl_2$; 94°C, 1 min; 60°C, 1 min; 72°C, 2 min (40 cycles)	Brown et al. (1997)
ELK1	A: GGACCTAGAGCTTCCACTCA B: AGAGCATGGATGGAGTGACC	388	1.5 mM $MgCl_2$; , 6% DMSO,[b] 94°C, 1 min; 56°C, 1 min; 72°C, 2 min (35 cycles)	Brown et al. (1997)
ZNF41	A: TTTTAGAAGAACTGTGGCAAG B: CTCAGGATGAATTTTCTGATG	300	1.0 mM $MgCl_2$; 94°C, 1 min; 54°C, 1 min; 72°C, 2 min (40 cycles)	Derry et al. (1995)
ZNF157	A: GCCTCTCACTGAAGACTTC B: AGCAAGACATGAACAGCCC	280	2.0 mM $MgCl_2$; 94°C, 1 min; 58°C, 1 min; 72°C, 2 min (35 cycles)	Derry et al. (1995)
XIST	C9-4: AGCTCCTCGGACAGCTGTAA C7B2 (revised): CTCCAGATAGCTGGCAACC	240	1.5 mM $MgCl_2$; 94°C, 1 min; 54°C, 1 min; 72°C, 2 min (30 cycles)	Duncan et al. (1993)
XIST 1:2r (DNA)	3′: GAAGTCTCAAGGCTTGAGTTAGAAG 5′: TTGGGTCCTCTATCCATCTAGGTAG	220	1.5 mM $MgCl_2$; 94°C, 1 min; 54°C, 1 min; 72°C, 2 min (30 cycles)	Brown et al. (1992)
TIMP-C	CA1: GGGTTCCAAGCCTTAGGGGA C: CTGTTCCAGGGAGCCACG	385 (DNA), 183 (cDNA)	1.5 mM $MgCl_2$; 94°C, 1 min; 58°C, 30 s; 72°C, 1 min (30 cycles)	
TIMP-T	CA1: GGGTTCCAAGCCTTAGGGGA C: CTGTTCCAGGGAGCCACA	385 (DNA), 183 (cDNA)	1.5 mM $MgCl_2$; 94°C, 1 min; 58°C, 30 s; 72°C, 1 min (30 cycles)	EMBL Outstation, European Bioinformatics Institute[c]

[a] Underlining denotes primers newly designed on the basis of the sequence provided by the reference cited; other primers are directly from the references listed.

[b] Dimethyl sulfoxide.

[c] Accession number A10416.

Table 2

Expression of the Five Genes in Various Somatic Cell Hybrids

Hybrid Cell Line[a]	Expression[b]					
	TIMP1	*ARAF1*	*ELK1*	*ZNF41*	*ZNF157*	*XIST*
Active X:						
AHA-11aB1	+	+	+	+	+	−
t60-12	+	+	+	+	+	−
A23-1aC15	+	+	+	+	+	−
tHM-34-2A-41B	+	+	+	+	+	−
GM06318D[a]	+	+	+	+	+	−
CHO-01416-M[a]	+	+	+	+	+	−
Inactive X:						
t11-4Aaz5	−	−	−	−	−	+
t48-1a-1Daz4a	−	−	−	−	−	+
tHM-34-2A-3az1a	−	−	−	−	−	+
X8-6T2S1[a]	−	−	−	−	−	+
t86-B1maz1b-3a	+	−	−	−	−	+
t81-az1D	+	−	−	−	−	+
t75-2maz34-1a	+	−	−	−	−	+
CHO–01416-O7[a]	+	−	−	−	−	+

[a] Hybrid with a hamster parental line.
[b] A plus sign (+) denotes presence of expression, and a minus sign (−) denotes absence of expression.

ceptor [Allen et al. 1992] or fragile X [Carrel and Willard 1996]). The same sample of cells was used in the *TIMP1* polymorphism assay and to determine the extent of skewing for X inactivation. Causes for the skewed inactivation patterns included X-autosome translocations (in 09, GM01813, and GM02859A); X-chromosome rearrangements (in SA70, described by Leppig et al. 1993); X-linked disease carriers (e.g., Wiskott-Aldrich syndome, in 07); XIST mutations (in HSC593 and 60 [both of which are from a single female], described by Rupert et al. 1992; Plenge et al. 1997); or lymphoblasts derived from normal females (in GM07059 and GM07348), which may have become clonal either during derivation or in culture (Migeon et al. 1988*b*).

Results

Expression of TIMP1 *and Flanking Genes in Hybrid Cells*

Expression of human *TIMP1* was examined in six active X– and eight inactive X–containing human/rodent somatic-cell hybrids. cDNA was amplified by RT-PCR with primers for *TIMP1,* and amplification of *TIMP1* was observed in all six active-X hybrids, as well as in four of eight inactive-X hybrids (table 2). To determine if the expression from the inactive X chromosome extended beyond *TIMP1,* we analyzed the expression of genes surrounding *TIMP1,* within the gene-rich region of Xp11.23 (fig. 1*A*) (Coleman et al. 1994; Knight et al. 1994; Derry et al. 1995; Brandau et al. 1998). RT-PCR was performed with primers for *TIMP1, ARAF-1* (a raf-related gene located 20 kb distal to *TIMP1*), *ELK1* (a transcription factor and proto-oncogene 50 kb proximal to *TIMP1*), and two zinc-finger genes (*ZNF41* and *ZNF157*), which are located <100 kb from *TIMP1.* Although *TIMP1* is found within intron 5 of *SYN1* (Derry and Barnard 1992), *SYN1* expression was not examined, since this tissue-specific gene is not expressed in fibroblasts (Yang-Feng et al. 1986) and, therefore, was not expected to be expressed at significant levels in the somatic-cell hybrids. As shown in figure 1*B* and as summarized in table 2, *ARAF1, ELK1, ZNF41,* and *ZNF157* were expressed only in hybrids that retained a human active X chromosome. No expression of these genes was observed in the inactive X–containing hybrids, including those in which *TIMP1* expression had been detected.

Expression of TIMP1 *from the Inactive X Chromosome in Human Cells*

When *TIMP1* cDNA sequences in the GenBank DNA-sequence repository were compared, a polymorphism was found, eliminating the need for the traditional labor-intensive methods of mutation detection and sequencing. The polymorphism is a T→C base-pair change in exon 5, part of the coding region, but the transition is silent, because it is the third base of a codon and both alleles code for phenylalanine. This polymorphism has been independently identified (Hardcastle et al. 1997). The base-pair difference was exploited to develop a PCR-based assay (fig. 2*A*) to analyze *TIMP1* expression in human females. By means of a constant primer and two allele-specific primers, conditions were optimized to provide amplification of the allele matching the primer, without spurious amplification of the opposite allele. The sensitivity of the assay was determined by mixing

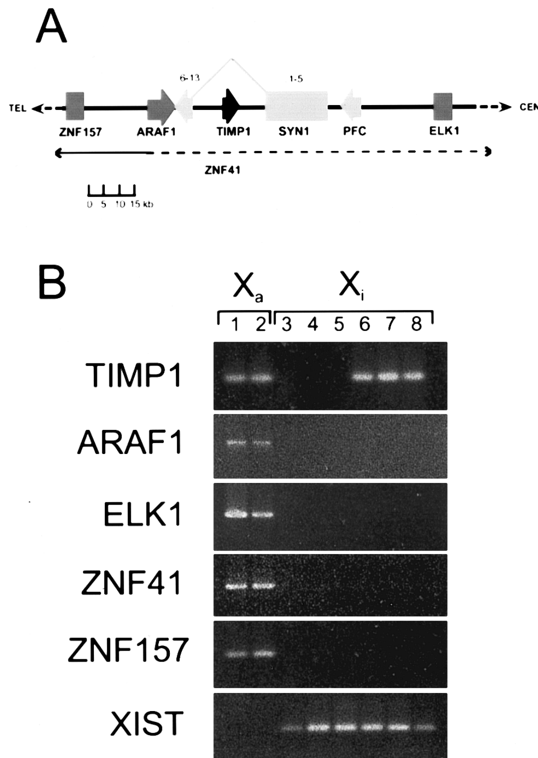

Figure 1 Expression analysis of genes in Xp11.23. *A,* Schematic map of genes flanking *TIMP1. ZNF41* is present in a YAC spanning the region represented by the arrow; however, the unbroken portion of the arrow represents the most likely area for *ZNF41,* since an overlapping YAC that was negative for *ZNF41* was shown to be deleted in this region (Brandau et al. 1998). *B,* Products from amplification of cDNA derived from the hybrids, listed with human-specific primers for the genes shown. Hybrids retaining the active X chromosome (X_a) or inactive X chromosome (X_i) are denoted as follows (numbers denote lanes): 1 = A23-1aC15; 2 = tHM-34-2A41B; 3 = t11-4Aaz5; 4 = t48-1a-1Daz4a; 5 = tHM-34-2A-3az1a; 6 = t75-2maz34-1a; 7 = t86-B1maz1b-3a; 8 = CHO-01416-07.

the DNA from homozygous females in different ratios (fig. 2*B*), and an allele present in ⩾10% of the total DNA was consistently detected (fig. 2*B*). In a sample of unrelated human females, males, cell lines, and somatic-cell hybrids, the different alleles had almost equal frequencies (98 C and 100 T, of 198 X chromosomes tested).

Female cell lines with previously demonstrated extreme skewing of X inactivation were used to assess expression of *TIMP1* from the inactive X chromosome. As shown schematically in figure 2, if *TIMP1* were subject to inactivation, only the one allele on the active X chromosome would be expressed. Expression of both alleles in a female with extreme skewing of X inactivation would demonstrate that *TIMP1* is also being expressed from the inactive X chromosome. Examples of female cells with and without *TIMP1* expression from the inactive X chromosome are shown in figure 2*C:* panels 1 and 2 show the results of analysis of homozygous females, demonstrating the specificity of the assay, and panels 3–6 show results obtained from heterozygous females, as shown by the presence of two alleles when DNA is amplified. When cDNA was amplified, only one allele was observed in GM07059 and female 09, demonstrating that *TIMP1* was only expressed from the active X chromosome in these cells. However, two *TIMP1* alleles were detected after amplification of cDNA from HSC593 and 60 and from female 07, showing that *TIMP1* was expressed from both X chromosomes and was not subject to X inactivation in these cells. The extreme (>90%) skewing of X inactivation for these females is shown in figure 2*D*. For sample 4 in figure 2*D,* the female was heterozygous for androgen receptor, as shown by the presence of two alleles in uncut DNA. Amplification after digestion with the methylation-sensitive enzyme *Hpa*II, which cuts DNA from the unmethylated, active X chromosome, shows predominantly the upper band. A very faint band is seen for the other allele, suggesting either a very small proportion of cells with the other X chromosome active or incomplete digestion; however, this band is of much lower intensity than the weaker band seen in *TIMP1* cDNA. For sample 6 in figure 2*D,* the female was heterozygous for *XIST,* as has been described elsewhere (Rupert et al. 1992; Plenge et al. 1997). In cDNA, only one allele is amplified, establishing that the same X chromosome is active in all cells. In summary, *TIMP1* expression was seen from the inactive X chromosome in three individuals, whereas no expression from the inactive X chromosome was detected in five females (table 3).

Discussion

To gain insight into the mechanisms involved in the maintenance of X inactivation, *TIMP1* expression from the inactive X chromosome was analyzed in somatic-cell hybrids and females with extreme skewing of X inactivation. It had previously been shown that *TIMP1* was expressed in two of five inactive-X hybrids (Brown et al. 1997), and the present study examined four additional inactive-X hybrids, including ones derived from hamster (table 2). When both studies are combined, four of nine inactive-X hybrids expressed *TIMP1,* and the variable inactivation of *TIMP1* was not confined to one rodent parental line. We postulated that the *TIMP1* expression from the inactive chromosome might be restricted to the hybrid system, since reactivation (sporadic expression from the inactive X chromosome) of other genes is observed at an elevated frequency in hybrids (Kahan and DeMars 1975; Gartler and Goldman 1994). *TIMP1* expression from the inactive X chromosome was therefore investigated by examination of heterozygous

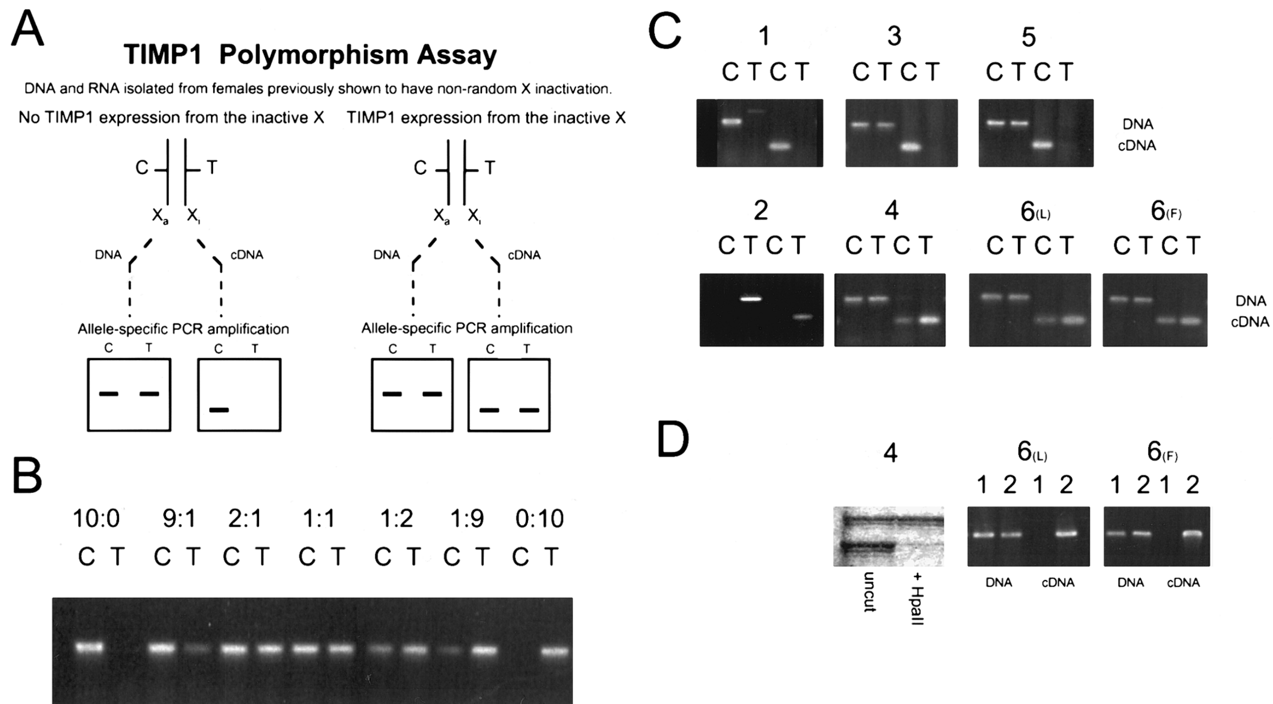

Figure 2 *TIMP1* polymorphism assay and results. *A,* Schematic outline of the *TIMP1* polymorphism assay. Oligonucleotide primers were designed and optimized to provide allele-specific amplification. We analyzed females with extreme skewing of X inactivation so that the same X chromosome would be active in all cells. DNA was first tested to find heterozygous females. In these informative females, cDNA was then amplified. The resulting products differ, in size, between DNA and cDNA, since the primers span a small intron. In this diagram, the active X chromosome is shown to have the C allele, and the inactive X chromosome has the T allele. If only the C allele was amplified in cDNA, there was no *TIMP1* expression from the inactive X chromosome. If both the C and T alleles were seen in cDNA, *TIMP1* was expressed from both X chromosomes. *B,* Specificity of the allele-specific primers, as demonstrated by the amplification of only one allele in the homozygous samples (*end lanes*). When these DNA samples were mixed in the various proportions as listed, 10% of one allele could be detected in the presence of the other allele. *C,* Results for females with extreme skewing of X inactivation, demonstrated by other assays (numbers denote gels): 1 = female homozygous for C (GM07023); 2 = female homozygous for T (AG); 3 = heterozygous female, *TIMP1* inactivated (09); 4 = heterozygous female, *TIMP1* expressed from both X chromosomes (07); 5 = heterozygous female, *TIMP1* inactivated (GM07059); 6 = heterozygous female, *TIMP1* expressed from both X chromosomes. L = lymphoblast (HSC593); F = fibroblast (60). *D,* Confirmation of X-inactivation patterns (>90% skewing), for panels 4 and 6. For female 07 (*gel 4*), androgen-receptor methylation assay is shown. Both alleles are seen in uncut DNA. After digestion with the methylation-sensitive enzyme *Hpa*II, amplification of the bottom allele is essentially eliminated, demonstrating that it is unmethylated—and therefore active—in the majority of the cells. For female HSC593 (L) and 60 (F) (*gel 6*), the results of *XIST* allele-specific PCR amplification are shown. Both alleles are present in DNA, but only one allele is amplified in cDNA, showing that the same X chromosome is inactive in all cells.

females with extreme skewing of X inactivation. These females had the same X chromosome active in all cells, so that the presence of two alleles in the cDNA is indicative of expression from the active X chromosome as well as from the second, inactive X chromosome. *TIMP1* expression from the inactive X chromosome was observed in three of the eight informative human females and cell lines analyzed (table 3). This frequency is similar to that observed in the hybrids, and, in the two cases in which human cell lines and the inactive X–containing hybrids derived from them were examined, the expression from the inactive X chromosome was concordant, providing further evidence that the hybrid system accurately reflects expression of X-linked genes in humans. Therefore, other genes expressed variably in the hybrid system (Brown et al. 1997) may also be polymorphic in humans. Indeed, polymorphic inactivation does not seem to be limited to the *TIMP1* gene, since variable inactivation has been described in females with choroideremia (Carrel and Willard, in press). Therefore, *TIMP1* may belong to a new class of X-linked genes that are subject to inactivation in some individuals but that escape inactivation in others.

We do not yet know the mechanism leading to polymorphic expression from the inactive X chromosome. The variability could involve a feature inherent in the *TIMP1* gene, another X-linked feature that functions in the *cis*-limited inactivation process, or some *trans*-acting factor. Expression of *TIMP1* was observed from inactive X chromosomes with both the C and T alleles, sug-

Table 3

Expression of *TIMP1* in Human Female Cells with Nonrandom X Inactivation

Individual	Cell Type	*TIMP1* Allele(s)	
		DNA	cDNA
09	Peripheral blood	C+T	C
07	Peripheral blood	C+T	C+T
GM07059	Cultured lymphoblasts	C+T	C
GM07348	Cultured lymphoblasts	C+T	C
SA70	Cultured lymphoblasts	C+T	T
HSC593	Cultured lymphoblasts	C+T	C+T
60	Cultured fibroblasts	C+T	C+T
GM02859	Cultured fibroblasts	C+T	T
GM01813	Cultured fibroblasts	C+T	C+T

gesting that one *TIMP1* allele is not predisposed to expression from the inactive X chromosome. Imprinting has been shown to be polymorphic among individuals, with monoallelic expression in some individuals and biallelic expression in others (Jinno et al. 1995; Giannoukakis et al. 1996). It is possible that similar *trans*-acting factors are involved in the biallelic expression of *TIMP1* and imprinted genes, since X inactivation and imprinting may involve similar epigenetic mechanisms of silencing (Tilghman and Willard 1995). Imprinting has also been observed to change between developmental stages (Jinno et al. 1995) and tissues (Ohlsson et al. 1994), but the expression of *TIMP1* from the inactive X chromosome was not limited to one cell type in the one individual examined, since both lymphoblasts (HSC593) and fibroblasts (60) of that individual had biallelic expression. Furthermore, if X-linked or *trans*-acting factors were involved in this variable expression from the inactive X chromosome, then all the genes susceptible to expression from the inactive X chromosome would be expected to be expressed in the same inactive-X hybrids, which has not been observed (Brown et al. 1997).

The variability of X inactivation could arise through either the loss of maintenance ("reactivation") or an initial "escape" from inactivation. X inactivation is generally stable in somatic cells, with reactivation occurring only at very low frequencies—for example, $<10^{-8}$ for *HPRT* (Kahan and DeMars 1975), which is much below the 30%–50% frequency seen for *TIMP1*. The fact that *TIMP1* expression from the inactive X chromosome was concordant among different tissues in the same individual provides evidence for an early choice of expression status. Genes escaping inactivation are often found in clusters, which implicates regional features permitting their expression (Gartler and Goldman 1994; Miller and Willard 1998), but we determined that variable expression from the inactive X chromosome was limited to the *TIMP1* gene. It is possible that the distinction between genes escaping or subject to X inactivation may not be mechanistic but, rather, temporal. The mouse gene *Smcx* escapes X inactivation in adult tissues, but it is initially inactivated in development (Lingenfelter et al. 1998). Genes that "escape" inactivation may be reactivated early in development, whereas other genes may rarely reactivate and therefore appear fully inactivated. For genes such as *TIMP1,* which show variable expression from the inactive X chromosome, the timing of reactivation may occur between these extremes. In mice, reactivation of several genes has been shown to increase with age (Cattanach 1974; Wareham et al. 1987; Brown and Rastan 1988), but long-term culture of inactive X–containing hybrids shows no evidence for reactivation of *TIMP1.* An age-related increase in reactivation frequency has not been observed for the human *HPRT* gene (Migeon et al. 1988*a*), but *HPRT* may be strongly silenced and therefore may require a period longer than a human life span before it is reactivated. Elevated reactivation frequencies are seen in marsupials and extraembryonic tissues that do not have hypermethylation of their inactive X chromosomes (Migeon et al. 1985; Kaslow and Migeon 1987), indicating that methylation may be critical for the stability of X inactivation. The relatively low CpG density of the *TIMP1* promoter may therefore be contributing to its expression from the inactive X chromosome. This argument of reactivation timing suggests that maintenance of silencing is the critical determinant of expression patterns for X-linked genes, not the initial inactivation event. It will be interesting to analyze the epigenetic factors normally associated with the inactive X chromosome, such as methylation and chromatin structure, to see if they differ when *TIMP1* is expressed from the inactive chromosome.

Whatever the mechanism allowing *TIMP1* expression from the inactive X chromosome, we anticipate that expression levels will vary between individuals, since *TIMP1* inactivation status is not consistent. *TIMP1* therefore seems to be an exception to X inactivation maintaining gene dosage between males and females (Lyon 1962). *TIMP1* expression levels may follow a continuum dependent on whether a female has random inactivation and whether both of her X chromosomes express *TIMP1* when they are inactivated. Furthermore, the amount of *TIMP1* expressed from the inactive X chromosome may be lower than that expressed from the active X chromosome, as has been shown for several genes expressed from the inactive X chromosome (Migeon et al. 1982; Carrel et al. 1996; Sheardown et al. 1996). It is possible that dosage is controlled at the level of translation, so the TIMP1 protein may not vary regardless of RNA levels. However, if the TIMP1 protein is elevated, then the variable expression may be clinically significant, since TIMP1 and the metalloproteinases that it inhibits are widely expressed proteins involved in a

variety of processes. Women with an increased level of TIMP1 might be insulated from conditions arising from decreased TIMP1, including rheumatoid arthritis, but might also be susceptible to other disorders, such as liver fibrosis (Denhardt et al. 1993; Gomez et al. 1997). In addition to identifying possible phenotypic variations and disease susceptibilities due to the polymorphic X inactivation of the *TIMP1* gene, further analysis of this variability will provide a window to study the epigenetic changes underlying the maintenance of X inactivation.

Acknowledgments

We are grateful to Helen McDonald for assistance with the inactivation status of the females and to the members of the Brown lab for helpful discussions. We also thank members of the U.B.C. Medical Genetics clinic (Drs. S. Langlois and F. Dill) for patient samples. This work was supported by Medical Research Council (Canada) grant MT-13690 to C.J.B.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast?Jform=0 (for BLASTN)
EMBL Outstation, European Bioinformatics Institute, http://www.ebi.ac.uk/cgi-bin/emblfetch (for human *TIMP1* cDNA [A10416])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for *TIMP1* [MIM 305370])

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