VARIABLE X INACTIVATION OF THE HUMAN *TIMP1* GENE

by

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ABSTRACT

X inactivation silences most of the genes on one of the two X chromosomes in mammalian females, presumably to achieve dosage compensation of X-linked gene products. The human X chromosome preserves its activation status when isolated in rodent/human somatic cell hybrids. Surprisingly, the human tissue inhibitor of metalloproteinases-1 gene (*TIMP1*) is expressed in some but not all inactive X-containing hybrids, suggesting that this gene is either prone to reactivation or variable in its inactivation. Although many genes that escape X inactivation are clustered along the X chromosome, genes flanking *TIMP1* (*ARAF1*, *ELK1*, *ZNF41*, and *ZNF157*) were expressed only from the active X (Xa) chromosome, demonstrating that the factors allowing *TIMP1* expression from the inactive X (Xi) are specific to the *TIMP1* gene. This variable X inactivation of *TIMP1* is not limited to the hybrid cell environment because *TIMP1* expression from the Xi was demonstrated in female cells.

As TIMP1 and its target metalloproteinases are involved in many biological processes, women with elevated *TIMP1* expression may exhibit different disease susceptibilities, *TIMP1* expression levels were analyzed. The range of *TIMP1* RNA levels from the Xa precluded analysis of the contribution of the Xi to total *TIMP1* RNA levels in females, so I examined expression in Xi hybrids. *TIMP1* expression levels varied more widely from the Xi than the Xa, suggesting variable retention of the epigenetic silencing mechanisms. I examined methylation and expression patterns and found that *TIMP1* was generally unmethylated when expressed. Methylation was associated with unstable expression because only 58% of clones derived from a methylated *TIMP1+* culture retained expression. One of the 33 *TIMP1-* subclones reactivated *TIMP1*, suggesting another epigenetic feature differs in the Xi hybrids expressing TIMP1. The acetylation status of histone H3 was examined and intriguingly,

in the clones from Xi hybrids with *TIMP1* expression, the *TIMP1* promoter was always hyperacetylated regardless of current expression status. This was not a reflection of chromatin configuration because the promoter remained nuclease insensitive in all silent clones, similar to the methylation results. These results establish a hierarchy to the epigenetic silencing of TIMP1, with histone acetylation preceding expression whereas methylation and chromatin structure are concordant with expression.

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1 INTRODUCTION

It is the regulated patterns of gene expression, not the genes themselves that define the various cell types found in higher organisms. Although the completion of the Human Genome Project is a remarkable achievement, it is only when the complex functions and interactions of the identified genes are determined that we will begin to truly understand human biology. The inactivation of the second X in human female cells is an extraordinary example of co-ordinated gene regulation and investigations into the epigenetic mechanisms involved will lead to a better understanding of stable transcriptional silencing. Because the *TIMP1* gene has variable X inactivation, being expressed from some inactive X (Xi) chromosomes but silenced on others, it offers a unique system to examine the complex gene regulation critical for normal development and functioning of higher organisms.

1.1 Dosage Compensation

Mary Lyon first proposed that one X chromosome in mammalian females is silenced (Lyon 1961). Previous investigations had suggested that the dense, darkly stained body present in female cells was an X chromosome (Ohno and Hauschka 1960) and that X0 mice are normal, fertile females (Welshons and Russell 1959), indicating that mice only require one X for normal development. By adding her observations that females heterozygous for sex-linked coat colour mutants are mottled, with patches of normal and mutant colour, Mary Lyon hypothesized that one X chromosome in females is genetically inactivated at random to allow dosage compensation of X-linked gene products between males and females (Lyon 1961).

The sex chromosomes of mammals are heteromorphic, differing in morphology and gene content but are partly homologous. The X and Y chromosomes are thought to have evolved from an autosome pair. The pseudoautosomal regions at the termini of the X and Y chromosomes still recombine during male meiosis but elsewhere on the sex chromosomes, X-Y recombination has been suppressed. These nonrecombining regions of the X and Y chromosomes have become highly differentiated during evolution (reviewed in Lahn and Page, 1999). The large X chromosome comprises approximately 5% of the haploid genome or about 2000 genes in humans, using the current estimates of total gene number but the small Y chromosome has gradually lost most of the ancestral genes (Lahn and Page 1999). In the sex-determining system of mammals, females have 2 X chromosomes and males have one X and a Y. Therefore, females have two doses of the majority of X-linked genes compared to only one in males. To ensure that the sexes have similar amounts of X-linked gene products, female mammals suppress expression on one of two X chromosomes. In the somatic cells of mammals, the X to be inactivated is selected at random. After the inactive state has been established, the same X chromosome remains inactive throughout further cell divisions, leading to clusters of cells with the same active X chromosome in the adult. Females that are heterozygous for an X-linked gene become mosaics of two types of cells, with one or the other allele active (Lyon 1999). These mosaic patches explain the mottled phenotype of mice heterozygous for X-linked coat colour mutants. Likewise, the variegated look of calico cats arises from the females being heterozygous for an X-linked colour gene. Human examples are less obvious but females heterozygous for X-linked skin disorders show similar mosaic patterns. In hypohidrotic ectodermal dysplasia (HED; OMIM 305100), hemizygous males have a significant lack of sweat glands and

heterozygous females have a patchy distribution of sweat pores (reviewed in Traupe 1999).

Other heterogametic organisms have evolved different dosage compensation strategies. In *Drosophila melanogaster*, male flies hypertranscribe their single X to match the level of expression from the X chromosome pair in females. The *Caenorhabditis elegans* hermaphrodites (XX) halve transcription on both X chromosomes to equal the expression level in X0 male worms (Meller 2000). Female birds are the heterogametic sex (ZW) and the lack of dosage compensation with the ZZ male was presumed to explain avian sexual differentiation (Nanda et al. 2000). However, a recent study showed equivalent expression in 6 of 9 Z-linked genes in male and female chick embryos, suggesting that birds do have dosage compensation (McQueen et al. 2001) but the mechanism used remains unknown. While the three (known) strategies affect the X chromosome differently, they seem to have evolved through the adaptation of pre-existing gene regulation tools (Meller 2000). Mammalian X inactivation has an additional layer of complexity because the 2 X chromosomes within one nucleus are treated differently. Therefore, the cell machinery must view the X chromosomes separately, similar to the imprinted gene clusters where the gene expression of each homologue is determined by parent of origin. Discovering how one X chromosome is distinguished from the other and how it is inactivated will help determine the mechanism of gene expression regulation.

1.1.1 Mechanism of Dosage Compensation

An Xi chromosome is a cytologically obvious heterochromatic body in interphase nuclei, called a Barr body, and has a characteristic bend at Xq13 during metaphase (Flejter et al. 1984). At the molecular level, the Xi is hypermethylated at promoters, nuclease insensitive, has delayed replication timing (Gartler and Goldman 1994) and

contains histones that are hypoacetylated (Jeppesen and Turner 1993) compared with the active X (Xa) chromosome. Additionally, *XIST*, the functional RNA pivotal in X inactivation initiation, coats the Xi (Clemson et al. 1996) and there is a preferential accumulation of the macroH2A histone variant on the Xi (Costanzi and Pehrson 1998). These features associated with the Xi have been well characterized but the molecular mechanisms converting one of the two female X chromosomes into the mature Xi are only partially understood, as are the genetic and developmental controls. X inactivation has been divided into 3 stages: initiation, spreading, and maintenance.

1.1.2 Initiation

There is one Xa for every set of autosome pairs and the presence of two Xa is lethal in diploid female embryos (Takagi and Abe 1990). Diploid individuals do sometimes have extra X chromosomes (XXX, XXY, etc.) but they have only a single Xa. Cases of tetraploidy, 4 sets of chromosomes, have 2 Xa; interestingly, triploid individuals fall in the middle with either one or two X chromosomes remaining active (Migeon et al. 1979; Vogel et al. 1983). Therefore, there must be a counting mechanism to recognize the number of chromosomes (Lyon 1999). One hypothesis is that limited amounts of a blocking factor bind to one X chromosome per diploid cell and shield it from inactivation. Excess X chromosomes are not protected and therefore undergo X inactivation (Avner and Heard 2001).

The initiation of X inactivation originates from a single X inactivation centre (XIC), whose presence was demonstrated in X-autosome translocations. When the X chromosome is split in these translocations, only one of the X chromosome segments shows cytological evidence of X inactivation. This also suggests that the inactivation process travels along the chromosome *in cis* since physically separated segments fail to inactivate (Rastan 1983). By analyzing different translocations and X chromosome

deletions, the XIC was mapped to a candidate region of ~ 1 Mb within band Xq13 (reviewed in Heard et al. 1997). One gene within the XIC is the *Xi specific transcript* (*XIST*), transcribed exclusively from the inactive chromosome (Brown et al. 1991). The gene product is a 17 kb spliced, polyadenylated RNA that remains in the nucleus and seems to be the primary signal for X inactivation (Avner and Heard 2001). Both *XIST* and its mouse homologue, *Xist*, are ubiquitously expressed in female adult tissues and the RNA associates with the Xi in interphase nuclei (Brown et al. 1992; Clemson et al. 1996).

The initial stages of X inactivation have been studied primarily in the mouse for technical and ethical reasons. Targeted deletions and transgenics of *Xist* have shown that it is essential for inactivation in *cis*. A deletion of the 5' end of the gene prevented *Xist* transcription and the targeted chromosome could no longer be inactivated (Penny et al. 1996). Furthermore, the RNA product itself is required because female mice heterozygous for an internal deletion in the *Xist* gene, from part of exon 1 to exon 5, undergo primary nonrandom inactivation of the wild-type X chromosome suggesting that the deleted chromosome could not be inactivated even in the presence of a functional promoter (Marahrens et al. 1998). Several transgenic experiments with additional copies of *Xist* show that it is also sufficient to serve as an XIC and/or induce some properties of inactive chromatin, in autosomes as well as the sex chromosomes (Heard et al. 1996; Heard et al. 1999; Lee et al. 1999b).

It is difficult to pinpoint the onset of X inactivation because initiation differs between tissue types with a developmentally regulated pattern that appears to follow cellular differentiation (Monk and Harper 1979). In cells leading to the embryo, X inactivation occurs at the late blastocyst stage (McMahon et al. 1983). At the beginning of female embryogenesis, neither X chromosome is inactivated, which is presumably tolerated due

to the small number of genes expressed in early development. *Hprt* and *Pgk* transcripts from both X chromosomes have been detected from the two-cell stage to the blastocyst (Latham and Rambhatla 1995; Singer-Sam et al. 1992). *Xist* is also expressed at low levels from each chromosome at this stage. Female embryonic stem (ES) cells are often used as a model system to study initiation because they start X inactivation when differentiated. *Xist* RNA initially appears as two small dots at the site of transcription. Differentiation leads to an accumulation of *Xist* RNA on the future Xi before the low level *Xist* expression from the Xa is silenced (Panning et al. 1997). The increased *Xist* RNA on the Xi is due to an increase in stability of the transcript (Panning et al. 1997; Sheardown et al. 1997). Detection of additional 5' sequence in the early unstable transcript was initially proposed to result from an alternate upstream promoter (Johnston et al. 1998) but is now believed to reflect the presence of anti-sense transcripts (Lee et al. 1999a). *Tsix* is a 40 kb RNA transcribed from the anti-sense strand of *Xist* that spans the whole *Xist* locus from 15 kb downstream to the putative unstable promoter. Before the onset of X inactivation, *Tsix* is expressed from both X chromosomes but once inactivation begins, it is expressed only from the future Xa (Lee et al. 1999a). Given that loss of *Tsix* expression corresponds with an upregulation of *Xist* RNA, *Tsix* may be destabilizing the *Xist* transcript but the exact mechanism is unknown. In differentiated female ES cells, a 65 kb deletion that included the last 2 *Xist* exons and the start of *Tsix* led to the inactivation of the mutated X, even when it was the only X present, contradicting the rule of one active X within a diploid cell (Clerc and Avner 1998). Hence, this deletion may span the binding site for the putative blocking factor so the factor can no longer bind to protect the chromosome from X inactivation (Avner and Heard 2001).

Once the *Xist* RNA starts to accumulate on the future Xi, the genes on this chromosome are transcriptionally silenced and replication timing shifts to late S-phase. Additional epigenetic modifications, such as promoter methylation and hypoacetylation of histones, presumably help transform the *Xist* RNA-coated chromosome into a stably inactive and condensed chromatin state (Avner and Heard 2001). Once X inactivation is established, the XIC and *XIST* are no longer needed because genes subject to X inactivation remain transcriptionally silent despite secondary loss of the XIC (Brown and Willard 1994). This is further supported by transgenic work with an inducible *Xist* cDNA construct. In undifferentiated ES cells, *Xist* expression leads to long range transcriptional repression but silencing requires ongoing *Xist* expression. However, inactivation in differentiated ES cells is accompanied by chromatin modifications, making the inactivation irreversible and independent of *Xist* (Wutz and Jaenisch 2000).

While X inactivation is generally considered to be a random process, non-random X inactivation can happen in a variety of ways. In mice, the alleles of the X-controlling element (*Xce*) within the Xic influence the choice of X inactivated (Johnston and Cattanach 1981). An equivalent system has not yet been identified in humans and skewing of inactivation ratios of humans appears to be primarily due to selection against cells expressing a particular allele after random X inactivation (Migeon 1998). Extreme skewing is also observed with X chromosome rearrangements, presumably to minimize the potential genetic imbalance associated with the chromosomal abnormality. Non-random X inactivation can also arise from imprinting mechanisms where the parent of origin determines which chromosome will be inactivated. Preferential inactivation of the paternal X chromosome is found in the extraembryonic tissues of mouse (Takagi and Sasaki 1975) and in all marsupial tissues (Cooper et al. 1971). The paternal X chromosome may be predisposed to inactivation because it arrives in a partially

condensed state due to passage through the male germline (Heard et al. 1997) or the maternal X may carry an imprint that resists X inactivation (Okamoto et al. 2000). The initial analysis of human extraembryonic tissues suggested that the paternal X might be preferentially inactivated (Goto et al. 1997; Harrison and Warburton 1986). Analysis of G6PD heterodimers present in chorionic villus cells suggested that either the maternal or paternal X could be inactivated (Migeon et al. 1985; Mohandas et al. 1989). Recently, more extensive studies have confirmed a tendency towards skewed X inactivation patterns in the trophoblast but without parental bias (Looijenga et al. 1999)(M. Penaherrera, personal communication). The imprinted X inactivation of extraembryonic tissues and marsupials may represent the original form; and random X inactivation, which provides protection of mosaicism for deleterious X-linked traits, may have evolved to accompany differentiation in the inner cell mass unique to eutherian embryos (Graves 1996).

1.1.3 Spreading

Once the *Xist* RNA stabilizes, it coats the future Xi *in cis*, rapidly inducing gene silencing. This process is poorly understood and there are currently more questions than established mechanisms, including how the *Xist* RNA spreads over one X chromosome and how the accumulation of RNA leads to chromatin remodelling. The *Xist* RNA may associate with an anchoring protein to localize it to the Xi (Panning et al. 1997). The *roX* (RNA on the X chromosome) transcripts involved in *Drosophila* dosage compensation interact with regulatory and chromatin proteins to change chromatin structure (reviewed in Avner and Heard 2001). Similar to the *XIST/Xist* RNAs, the *roX1* and *roX2* RNAs are large, spliced, polyadenylated transcripts without significant open reading frames (reviewed in Kelly and Kuroda, 2000). The hnRNPC1/C2 proteins have been shown to interact with *XIST* RNA (Brown and Baldry 1996) but the significance is

unclear. A more promising association is with macroH2A histone, an unusual histone variant that contains a large non-histone region and a section that resembles a full-length H2A (Pehrson and Fuji 1998). MacroH2A is preferentially concentrated on the Xi in mammals (Costanzi and Pehrson 1998) and *XIST* RNA co-precipitated with antibodies against macroH2A (Gilbert et al. 2000). There are two distinct domains capable of directing macroH2A to the Xi: the histone domain alone and a 190 amino acid stretch in the non-histone region if it is fused to the core histone H2A or H2B (Chadwick et al. 2001). However the protein associations shown so far have been in somatic cells and may be a consequence of stable silencing rather than participating in the spread of X inactivation. In fact, accumulation of the histone macroH2A in ES cells occurs several days after differentiation, subsequent to gene silencing (Mermoud et al. 1999). On the other hand, the role of macroH2A in maintenance is also questionable because it no longer associates with the Xi after *Xist* deletion and yet the chromosome remains inactive (Csankovszki et al. 1999). If hnRNPC1/C2 and macroH2A are involved in the spreading and/or function of *XIST*, the mechanisms are uncertain and other proteins are being actively pursued (Baldry, personal communication).

In addition to the proteins proposed to interact with the *XIST* RNA, there may be 'way stations' or DNA elements that spread an X inactivation signal along the entire X chromosome (Gartler and Riggs 1983). X inactivation can spread into an autosomal region when *Xist/XIST* is inserted as a transgene or attached by translocation, albeit less efficiently (Avner and Heard 2001). Therefore, DNA sequences that promote spreading are not confined to the X chromosome but may be increased or organized to promote the chromatin configuration required for stable silencing. Mary Lyon (1999) has proposed that LINE-1 elements, mammalian-specific retrotransposons, are the hypothetical way stations. Both the human and mouse X chromosomes stain brightly

with antibodies to L1, suggesting that they are L1-rich and failure of X inactivation spread into various autosomal segments correlates with cytogenetic bands that are L1-poor (Bailey et al. 2000; Lyon 1998). Sequence analysis confirms that the human X chromosome has twice as many L1 elements as autosomes and the increase in L1 content is due to the younger subclasses of L1M1 and L1P4, indicating that the L1 expansion occurred at the time of eutherian radiation and possible beginning of X inactivation (Bailey et al. 2000). In rodent somatic cells, *Xist* RNA exhibits a banding pattern on metaphase chromosomes with preferential association with G-light (G+C rich) bands (Duthie et al. 1999). Although this appears to contradict L1 involvement because L1s are preferentially found in G-dark (A+T rich) bands, Bailey et al. (2000) found that regional L1 clustering on the X chromosome is independent of G+C content. Thus, *in silico* analysis supports the idea that LINE elements may promote X inactivation spread along the chromosome, but the extent of involvement and the mechanisms are unclear. The L1s could be the attachment sites for nucleoprotein complexes or contact with the *XIST* RNA may trigger the cell defence system to recognize the L1s as repeat elements and silence them and the intervening sequences by converting to heterochromatin (Lyon 1998).

1.1.4 Maintenance

Once the X chromosome is silenced, the Xi takes on its characteristic heterochromatic appearance and gains: delayed replication timing; hypermethylation at promoters; nuclease insensitivity (Gartler and Goldman 1994); hypoacetylation of histones (Jeppesen and Turner 1993); localization of the *XIST* RNA (Brown et al. 1992) and association of macroH2A (Costanzi and Pehrson 1998) as well as a peripheral location in the nucleus (Dyer et al. 1989). It is believed that these epigenetic factors, either alone or co-operatively, maintain the stable silencing of the Xi. "Epigenetic" refers

to a heritable (mother cell to daughter cell) instruction that produces a change in gene expression without a change in the DNA sequence. The amazing array of different cell types within one organism is due to differential expression patterns determined by epigenetic instructions superimposed on the same primary nucleotide sequence. In the early stages of development, every cell is toti-potent and can generate any cell type but during development, cells become more restricted in their possibilities. Genes destined to be silenced are marked by an epigenetic signal leading to the establishment of a heritable but potentially reversible inactive conformation of the gene. Transcriptionally inactive chromatin is generally condensed, late replicating and hypermethylated with less acetylation of histones, which is very similar to the Xi and suggests that the same silencing mechanisms may be used. The two epigenetic features specific to X inactivation, *XIST* RNA and macroH2A localization, can be lost in somatic cells without loss of X inactivation so these specialized features may only be needed to mark the future Xi and initiate the silencing process. Similar epigenetic features are found at other transcriptionally silenced chromatin, not just the Xi, so the conclusions reached by studying X inactivation may be applicable to the wider fields of differentiation and development. My thesis examines epigenetic features between Xi with various states of *TIMP1* expression to establish a hierarchy to the silencing process. Thus, I will describe each heterochromatic component separately.

1.1.4.1 Replication Timing

One of the hallmark epigenetic features associated with the Xi is delayed replication timing, which occurs in late S-phase, and seems to be an integral part of the inactivation process. A late replicating chromosome is one of the first distinguishing characteristics of the Xi observed at two days after ES cell differentiation, accompanying *Xist* RNA accumulation and gene silencing (Keohane et al. 1996). The correlation between X inactivation and delayed replication timing is so strong that late replication is often used

to identify the Xi and to determine the expression status of autosomal genes translocated onto the X chromosome (Heard et al. 1997).

Replication of mammalian DNA is not linear from one end of a chromosome to the other but the chromosome is divided into multiple replication units (Hand, 1978). Although each subsection replicates at a defined time (Latt, 1974), the order of replication is not rigid and there can be cell and tissue variation in timing (Zannis-Hadjopoulos and Price 1999). In general, transcriptionally active genes replicate early in S-phase whereas silent genes replicate later (reviewed in Gilbert 2001). It is not yet clear if replication timing is a consequence of transcription or whether replication timing influences gene activity. Genes replicating early in S-phase may have a higher probability of binding transcription factors and/or structural proteins required for expression (Haaf 1995). A change in the replication timing of a tissue-specific gene appears to reflect the commitment of that gene to transcriptional competence or silence rather than transcriptional activity (Goldman et al. 1984). Similarly, the replication asynchrony of the X chromosomes reflects the opportunities for transcription, with the inactivated X replicating later than the active X in human, mouse, and marsupial female cells.

The original cytogenetic observations of replication timing differences between the two X chromosomes in female cells have been confirmed by analysis of single genes (reviewed in Heard et al. 1997). Both molecular and cytogenetic techniques are used to assess replication timing at individual loci. The molecular methods label newly synthesized DNA with 5-bromo-2-deoxyuridine (BrdU), sort the cells into different fractions of S-phase based on DNA content, and isolate the BrdU-labelled DNA. These sequences are subsequently analyzed by Southern (D'Andrea et al. 1983), single nucleotide primer extension, (Xiong et al. 1998), or general PCR amplification (Hansen

et al. 1996) to determine at what point in S-phase specific loci are replicated. Replication status can also be easily observed in interphase nuclei using fluorescent in situ hybridization (FISH) with single gene probes. An unreplicated locus is characterized with a single hybridization signal whereas a replicated locus gives a doublet (Boggs and Chinault 1994). The *HPRT*, *PGK1*, *FMR1*, *IDS*, and *FRAXA* genes are expressed only from the active X and all demonstrate replication asynchrony between the two X chromosomes (Boggs and Chinault 1994; Hansen et al. 1996; Schmidt and Migeon 1990). When a gene is not expressed from the active chromosome because it is mutated (*FMR1*), silent on the Xa (*XIST*), or not normally expressed in the cells examined (*F9, F8C*), both homologues replicate in the second half of S-phase (Schmidt and Migeon 1990; Torchia et al. 1994). This suggests that the replication timing of individual X-linked genes seems to reflect their transcriptional activity. However, further molecular and FISH analyses of *XIST* replication timing indicate that the replication timing of a gene is determined by the dominant pattern of the region. The silent *XIST* allele on the active X replicates before the expressed Xi homologue (Gartler et al. 1999; Xiong et al. 1998) and this "backward" pattern may reflect the replication requirements of other genes in the same domain (Hansen et al. 1996). Therefore, the time of a gene's replication may not be determined by its individual transcriptional activity but by the surrounding replication unit (Gartler et al. 1999).

The eukaryotic genome is divided into $10^4 - 10^6$ replication units (replicons) of 50 – 250 kb, each with an origin of replication. DNA synthesis extends along two diverging replication forks and presumably ends when two adjacent forks meet and fuse. The initiation of replication is controlled by *cis* sequences at the start of DNA synthesis, where *trans*-acting factors bind. Eukaryotic origins of replication have been studied extensively in yeast, primarily due to the ease of identifying autonomously replicating

sequences (ARS) by cloning into a vector with a selectable marker and subsequent transformation of cells. The cells that survive selection must have a sequence that confers the ability to replicate efficiently in yeast. Due to the conserved nature of replication, mammalian cells are also expected to start at specific DNA sequences but the sequences have been difficult to identify since assays are challenging, and because contradictory results are found with different techniques. When replication bubbles are identified through 2D electrophoresis, initiation of replication seems to take place throughout 4 to 55 kb zones with no clear preference for one particular site. When newly synthesized DNA is analyzed, initiation events seem to occur at specific sites of 0.5 to 2 kb, similar to the origins in yeast (DePamphilis 1999). The mammalian origin recognition complex is partially reassembled during the cell cycle, which could allow a change in origin use from one cell cycle to the next (reviewed in Cimbora and Groudine, 2001).

Clusters of 23-100 adjacent replicons are activated together at different times throughout S-phase and they may share nuclear position, secondary structure, or common DNA sequences (Heun et al. 2001). A condensed chromatin structure could restrict access of initiator proteins to chromosomal sites (Gilbert 1998). Nuclear position and local protein environments may influence both gene activity and replication timing. Inactive genes are found near centromeric heterochromatin and activated genes are located away from these areas (Francastel et al. 1999). Early-replicating sequences are distributed throughout interphase nuclei whereas later-replicating sequences are located at the periphery, suggesting that nuclear position may establish replication timing (reviewed in Cimbora and Groudine 2001). Similarly, the Xa and Xi occupy distinct nuclear positions but it is not yet clear if nuclear position is a consequence of replication timing or replication timing is influenced by position. DNA replication may initiate at CpG islands because these GC-rich sequences are found in short newly synthesized DNA

strands (Delgado et al. 1998) which could help explain why transcription and replication are tightly linked. Transcriptionally active CpG island promoters are generally unmethylated (see below) and methylation of these sequences correlates with gene repression as well as delayed replication timing. DNA demethylation following treatment with 5-azacytidine reactivates silent genes and advances replication time so that the two X chromosomes appear to now replicate synchronously (Jablonka et al. 1985).

Late replication timing is one of the first epigenetic feature associated with X inactivation and it may set up heterochromatin formation, particularly if required enzymes or structural proteins are only available in late S-phase. The coating of the future Xi with *XIST* RNA may influence replication timing by decreasing the availability of initiation sites. Assaying replication timing alongside other epigenetic features of genes that are variably expressed from the Xi should clarify whether delayed replication timing is a primary cause or a secondary indication of gene silencing.

1.1.4.2 Nuclease sensitivity and chromatin conformation

DNA is complexed with histones and non-histone proteins in a highly structured way, which provides a method to organize transcription in addition to fitting large amounts of DNA in a small space and helping chromosome segregation. Most of the DNA in interphase nuclei is extended and diffuse throughout the nucleus (euchromatin) but some DNA remains highly condensed throughout the cell cycle (heterochromatin). Constitutive heterochromatin is usually transcriptionally inactive and mainly consists of repetitive DNA whereas the Xi is an example of facultative heterochromatin, silenced DNA sequences that also exist in active copies. Despite no dramatic volume differences between Xa and Xi chromosomes, three-dimensional analysis of X chromosomes in interphase supports differential packaging of the Xi because Xa territories are flatter with

a more irregular surface compared to the smoother and rounder Xi territories (Eils et al. 1996).

Heterochromatic regions are generally transcriptionally inactive and it is widely accepted that DNA folded into the higher order structures are relatively inaccessible to the transcriptional machinery. The condensation of chromatin can be analyzed through cytogenetic staining or nuclease digestion. Heterochromatin was first discovered by its differential staining but this visual system assesses the overall structure of large regions and does not give much information about individual loci, therefore nuclease digestion is used to analyze particular sequences. Transcriptionally active chromatin is more susceptible to DNaseI digestion, presumably because the DNA is more accessible to the enzyme. Furthermore, specific sites in active chromatin are hypersensitive and are the first sequences digested at low enzyme concentrations. These hypersensitive sites are generally found at promoters and regulatory sequences of active genes and appear to be free of nucleosomes, most likely displaced by regulatory proteins like transcription factors or ATP-dependent chromatin-remodelling complexes. A relaxation of chromatin structure occurs before transcription, indicated by DNaseI sensitive chromatin. This suggests that it is a requirement, not a simple consequence, of transcription. Therefore, chromatin must be accessible to enzymes and regulatory proteins before transcription occurs. However, not all accessible DNA is actively transcribed, similar to the accommodating state of early replication timing where a permissive state allows transcription but does not guarantee the gene will be expressed (reviewed in Jones and Wolffe 1999).

Generally, genes on the Xa are more sensitive to digestion by exogenous nucleases than the inactivated copies. *HPRT* (Lin and Chinault 1988), *PGK* (Riley et al. 1984), and *G6PD* (Wolf and Migeon 1985) all have 5' hypersensitive sites near the promoter

present only on the Xa. The overall chromatin structure of the active *HPRT* gene was approximately two-fold more sensitive to DNaseI digestion than the equivalent sequences on the inactive chromosome (Lin and Chinault 1988).

1.1.4.3 Histone Acetylation

Areas of relaxed chromatin structure are preferentially stained with antibodies to acetylated histones (Hebbes, 1988), suggesting that the addition of acetyl groups to the lysine residues of histone tails is associated with transcription. The fundamental structural unit of chromatin is the nucleosome, consisting of approximately 146 bp of DNA wound around a roughly heart-shaped octamer of the four core histones H2A, H2B, H3, and H4. The amino-terminal domains of the histones are called tails because they extend beyond the nucleosome core. The N-termini of histones H3 and H4 are remarkably conserved across species, and each contain multiple conserved lysine residues that serve as sites of post-translational acetylation (Turner 1991). Acetylation of H3 occurs at lysine (K) 9, 14,18, and 23 whereas K5, 8, 12 and 16 are acetylation sites for H4 (reviewed in Roth et al. 2001), with acetylation of K16 commonly enriched in transcriptionally active and potentially active chromatin (Johnson et al. 1998).

Although histone acetylation may reflect acetylation of other critical non-histone proteins (Pazin and Kadonaga 1997), the correlation of transcriptionally competent chromatin with domains of histone acetylation suggests that acetylated chromatin may be a requirement for transcription. The N-terminal tails of the core histones are positively charged and could interact with the negatively charged DNA. Acetylation of lysine residues may reduce this charge and the strength of electrostatic contacts, leading to a release of histone-DNA interaction. However, mutants with neutral residues do not model the effects of acetylation *in vivo* (Zhang et al. 1998) and the crystal structure of the nucleosome shows the tails free in solution, not making firm contacts

with the DNA (Luger et al. 1997). Consequently, the tails may not interact with the DNA but may be important for internucleosomal interactions in higher order chromatin structure. Thus, histone acetylation may disrupt higher order chromatin packaging and provide the first step toward a more permissive, transcriptionally competent substrate (Chen et al. 2001b). The charged histone tails may prevent transcription factor binding and acetylation may remove this restriction because proteolytic removal of the tail and histone acetylation led to comparable transcription factor access (Wolffe and Guschin 2000). Although it is unknown whether acetylation relaxes the chromatin conformation or maintains the previously opened configuration, acetylation and general nuclease sensitivity are now recognized as hallmark features of transcriptionally competent chromatin.

The condensed structure of the Xi also has decreased acetylation of the core histones. After immunolabelling of metaphase chromosomes with antibodies specific to the acetylated form of histone H4, both the Xi and constitutive heterochromatic regions stain weakly, indicating that the histones of the Xi have decreased acetylation (Belyaev et al. 1996b; Jeppesen and Turner 1993; Wakefield et al. 1997). Similar results have been obtained with antibodies to acetylated H3 and H2A (Belyaev et al. 1996a; Boggs et al. 1996). The lack of antibody staining reflects less acetylation, not technical problems in heterochromatic regions because antibodies to non-acetylated H3 stain the 2 X chromosomes equally (Boggs et al. 1996). Decreased histone acetylation on the Xi has been observed in several mammalian species: mouse, human (Belyaev et al. 1996a; Jeppesen and Turner 1993), hamster (Belyaev et al. 1996b), and the Tammar wallaby (Wakefield et al. 1997). The conservation of this epigenetic feature from human to marsupials suggests that it may have a critical role in X inactivation.

The decrease in H4 acetylation at the promoters of inactivated genes has been observed in mice (O'Neill et al. 1999) and humans (Gilbert and Sharp 1999) by analyzing individual X-linked genes with chromatin immunoprecipitation (ChIP). The cytogenetic technique examines average acetylation over large chromosomal regions but the ChIP technique allows analysis of specific sequences, revealing complex acetylation patterns. The promoters of the *OCRL*, *PGK1, POLA, SOX3, IDS, XIST, HPRT,* and *NDP* genes were examined on both active and inactive X chromosomes. The promoters of these genes were hyperacetylated when the genes were expressed, but hypoacetylated when the genes were silent (Gilbert and Sharp 1999), indicating that decreased histone acetylation at the promoter correlates with X inactivation of individual genes. Analysis of the *OCRL*, *ZFX*, and *XIST* genes beyond the promoter region shows a low level of acetylated histone H4 present in the bodies of genes on both X chromosomes regardless of the gene's expression status, suggesting that the gene-body acetylation is independent of transcription (Gilbert and Sharp 1999). The low level acetylation may reflect background acetylation due to the dynamic nature of histone acetylation with a half-life of three to seven minutes for acetylated lysines of H4 in active domains (Covault et al. 1982). The steady-state balance of histone acetylation is maintained by histone acetyltransferases (HATs) and deacetylases (HDACs) (Kuo and Allis 1998). The marked hyperacetylation or hypoacetylation observed at X-linked promoters suggests these states are actively maintained and are of functional significance.

A direct link between transcriptional activity and histone acetylation was established by the discoveries that transcriptional activators associate with HATs (Brownell et al. 1996) and transcriptional repressors recruit HDACs (Taunton et al. 1996). The connection between acetylation and transcription is strengthened by the discovery of a vast range of transcription-related proteins with intrinsic HAT activity (reviewed in

Gregory 2001). The HAT isolated in *Tetrahymena* is homologous to Gcn5p, a yeast transcriptional cofactor (Brownell et al. 1996). HAT activity is required for gene regulation by Gcn5 *in vivo* because mutants that abolish the HAT activity in Gcn5 significantly weaken its ability to activate transcription (Wang et al. 1998). Recruitment of HAT activity to the active promoters may account for the increased acetylation when the gene is expressed. Conversely, the decreased acetylation at the promoters of inactivated genes may be due to the presence of a deacetylase that is removing acetyl groups from the histones. The mammalian HDAC1 is similar to the yeast transcriptional regulator Rpd3p (Taunton et al. 1996) and more HDACs have been identified. Transcriptional repression by a sequence-specific DNA-binding factor can be mediated by the recruitment of a deacetylase to the promoter region (Pazin and Kadonaga 1997). The differential acetylation states of the promoters on the active and Xi-linked genes are most likely maintained by the presence of HATs or HDACs respectively. Yet this HAT/HDAC recruitment can not be driven by a sequence recognition site because the same promoter sequences have opposite acetylation activities on the two X chromosomes. Inactivated genes are generally methylated at the promoter (see below) and methylated CpG sites bind the methyl-binding protein MeCP2, which can recruit histone deacetylases (Jones et al. 1998; Nan et al. 1998). This interaction is supported by the acetylation status of the *FMR1* gene on the active X where normal cells expressing *FMR1* are hyperacetylated but fragile X-syndrome cells with the 5' CGG repeat expansion are methylated, hypoacetylated and non-expressing (Coffee et al. 1999), suggesting that the methylation has helped to recruit the HDAC. Although CpG methylation and related binding proteins may help to maintain promoter hypoacetylation on the Xi throughout cell divisions, it is not the initial cue in early development because hypoacetylation of the Xi precedes the methylation of CpG islands in differentiated ES cells undergoing X inactivation (Keohane et al. 1996).

1.1.4.4 Methylation

Transcriptional repression is strongly associated with DNA methylation, the covalent addition of a methyl group to the carbon 5 position of a cytosine ring within the context of a CpG dinucleotide (Robertson and Jones 2000). A methyl group is added to cytosine residues in newly synthesized DNA by methyltransferases (DNMTs) rather than incorporating a methylated base directly into the DNA. Approximately 70% of the CpG dinucleotides in mammals are methylated, but CpG distribution is non-random and the majority of the genome is markedly deficient in CpGs (Cooper and Krawczak 1989). CpG sites have been shown to act as hotspots for mutation and may contribute to 30% of all point mutations in the germline (Cooper and Krawczak 1989). Deamination of cytosine forms uracil, which is easily recognized and repaired, but deamination of 5-methylcytosine forms thymine (Robertson and Jones 2000) leading to C to T transitions. CpG islands are short stretches of DNA that possess the CpG frequency expected from a random distribution of nucleotides, given the observed GC content of the DNA. These clusters of CpGs are often found in the promoters and first exons of genes and are generally unmethylated within the promoters of housekeeping genes (Jones and Wolffe 1999). Methylation of CpG islands at promoters is associated with selective silencing of genes on the Xi (Riggs and Pfeifer 1992) or genes silenced by genomic imprinting (Razin and Cedar 1994), and the level of transcriptional repression is correlated with the density of DNA methylation (Hsieh 1994).

The first identified methyltransferase, *DNMT1*, has a five to 30 fold preference for hemi-methylated substrates and is therefore considered a maintenance methylase (Bestor 2000). Another group of methyltransferases, *Dmnt3a* and *Dnmt3b*, was discovered in EST databases. These enzymes act equally at hemi-methylated and unmethylated DNA, suggesting that they are the elusive *de novo* methyltransferases (Okano et al. 1999). It is unknown how *Dnmt3a/3b* create the initial methylation

patterns, particularly since they do not target specific sequences, so there may be additional methyltransferases. Moreover, the features that delineate a region for *de novo* methylation have not yet been discovered (reviewed in Bestor, 2000). Once a DNA methylation pattern is set, it is faithfully inherited due to the preference of the maintenance methyltransferase for hemi-methylated sites. The replication of symmetrically methylated DNA molecules leads to two hemi-methylated DNA strands. The maintenance methyltransferase detects each methylated site in the parental strand and adds a methyl group to the corresponding CpG in the newly synthesized strand. An unmethylated CpG will be left alone but a methylated CpG site on the parent strand will be modified on the complementary strand. Therefore, DNA methylation is an epigenetic self-perpetuating mechanism for the transfer of chromatin structure information to the daughter cells (Haaf 1995).

DNA methylation may directly repress transcription by creating steric hindrance at transcription factor recognition sites. However, this mechanism requires that a CpG be present in the binding site, a condition not met by all transcription factors. Although methylation hinders the binding of some transcription factors (*e.g.* CREB), others, notably Sp1, can act independent of the methylation state of the template (Holler et al. 1988). If methylation functions through a finite set of recognition elements for individual transcription factors, its effect would be limited. Alternatively, transcription could be controlled globally with a system that recognizes methylated DNA, not specific sequences. Therefore, methylation may indirectly modify transcriptional activity through accessory proteins. The identified methyl-binding proteins, MeCP1 and MeCP2, are specific for symmetrically methylated CpGs and bind independent of the underlying DNA sequence. MeCP2 can bind single methylated CpG pairs whereas MeCP1 prefers densely methylated DNA (reviewed in Dragich et al. 2000). These proteins could

compete with transcription factors at binding sites or restructure DNA into a closed chromatin configuration that is incompatible with transcription. The MeCP2 protein has a methyl-binding domain and a transcriptional repression domain that recruits histone deacetylases via the corepressor Sin3a. Furthermore, the transcriptional repression is relieved by the deacetylase inhibitor trichostatin A, indicating that deacetylation of histones is an essential component of this silencing mechanism (Jones et al. 1998; Nan et al. 1998). This suggests that MeCP2 links DNA methylation-dependent transcriptional silencing and histone deacetylation. The requirement of altered chromatin structure for repression is illustrated by the microinjection of methylated or unmethylated constructs of the Herpes simplex virus thymidine kinase gene into *Xenopus* oocytes. Immediately after injection, both templates are transcribed equally but as chromatin is assembled, the methylated DNA is progressively repressed (Kass et al. 1997).

It is widely accepted that methylation contributes to silencing and the mechanisms are being elucidated but the primary role of methylation in eukaryotes is still unclear. Maintenance of methylation patterns is crucial because a deficiency of *Dnmt1* leads to embryonic lethality in mice (Li et al. 1992). However, the early death could result from disrupting any one of the proposed roles for methylation. Methylation is believed to have evolved in bacteria as a defence against invading DNA. Prokaryotes have specific methylases that act at short palindromic sequences and the corresponding methylation sensitive restriction enzymes cut the sequences if they are unmethylated, as in the case of foreign DNA. Although mammalian cells appear to have created new uses for this epigenetic mark, methylation also acts as a host defence against parasitic mobile elements (Walsh et al. 1998). Transposons account for more than 40% of the mammalian genome and are relatively rich in CpG dinucleotides. Methylation provides short-term protection by silencing the promoters of transposons and endogenous

retroviruses and enduring inactivation by accumulating C to T mutations (Bestor 2000). Mouse embryos without *Dnmt1* activity express normally silent retrotransposons, presumably due to the demethylation of the LTR sequences, indicating that methylation actively contributes to retrotransposon repression (Walsh et al. 1998).

Reversible methylation and demethylation has been proposed to regulate gene expression during normal development. The early embryo is characterized by changing DNA methylation patterns. Many expression-methylation correlations have been described. Because methylation patterns are linked to gene inactivity and are stably inherited, they are an attractive mechanism for epigenetic memory. However, several organisms, including *Drosophila* and *C. elegans*, complete complex developmental patterns without extensive methylation, suggesting that conserved regulatory networks control cellular differentiation. Furthermore, some genes reported to be regulated by reversible methylation did not express inappropriately in *Dnmt1*-deficient mouse embryos (Walsh et al. 1998). Therefore, the associations between lack of methylation and gene activity may describe a consequence of transcriptional activation, not the primary event. Recent evidence suggests that demethylation follows transcriptional factor binding and activation (Lin et al. 2000; Matsuo et al. 1998). While the role of dynamic methylation patterns in directing development is uncertain, methylation is definitely involved in the irreversible promoter silencing of allele-specific gene expression, such as X inactivation and genomic imprinting. The expression patterns of imprinted genes are generally set in the parental germline, apparently marked by methylation. The differential expression of imprinted genes according to parental origin correlates with methylation status. The expression of imprinted genes in mutant mice deficient for *Dnmt1* was altered, indicating that a normal level of DNA methylation is

required for controlling differential expression of the paternal and maternal alleles of imprinted genes (Li et al. 1993).

Disruption of methylation patterns can also cause abnormalities in humans. Mutations in the *DNMT3b* gene occur in patients with a rare autosomal recessive disorder, termed the ICF syndrome, for immunodeficiency, centromeric instability, and facial anomalies (MIM 242860). Rett Syndrome (MIM 312750) is a severe neurological disorder with developmental regression and stereotypical hand movements. It is an X-linked dominant disorder that results from mutations in the *MeCP2* gene (Amir et al. 1999). Alterations in DNA methylation patterns are also observed in many tumours, suggesting repression of tumour suppressors through C to T mutations or epigenetic silencing (reviewed in Jones and Gonzalgo 1997).

The promoters of X-linked genes are methylated on the Xi but not on the Xa, suggesting that DNA methylation contributes to the silencing of the Xi. The methylation status at the CpG islands of the constitutively expressed *HPRT* and *PGK1* genes are the most extensively studied. For both the *HPRT* and *PGK1* genes, high resolution methylation analysis by ligation-mediated PCR shows that the promoters on the Xa are unmethylated while the promoter of the Xi is methylated at most of the CpG dinucleotides (Hornstra and Yang 1994; Pfeifer et al. 1990). This association between inactivation and extensive methylation appears to be functional, not just a secondary consequence of stable silencing because individual X-linked genes reactivate when methylation is removed by treatment with demethylating agents (Mohandas et al. 1981). Methylation appears to have the role of maintaining X inactivation rather than being the primary signal as the methylation of *HPRT* appears late in the inactivation process, approximately two weeks after gene silencing occurs on the Xi in mouse (Keohane et al. 1996). Marsupials do not have hypermethylated promoters on the Xi so the X

chromosome can be inactivated without CpG island methylation but this inactivation is less stable (Kaslow and Migeon 1987), implying that methylation has a stabilizing effect on the inactive state.

Further evidence that methylation is involved in the stable transcriptional silencing of the Xi is the reactivation of X-linked genes by demethylation treatment with 5-aza-cytidine (5-aza-C). 5-aza-C is pyrimidine analog with nitrogen replacing carbon 5 and it can not be methylated when incorporated into the DNA (Haaf 1995). Only a small percentage of incorporated 5-aza-C (~5%) is required for near complete demethylation of genomic DNA (Creusot et al. 1982; Jones and Taylor 1981), suggesting 5-aza-C action is not only at the level of DNA substitution. It may also covalently bind the methyltransferases, sequestering the enzyme and preventing the maintenance of the methylation state (Haaf 1995). 5-aza-C treatment leads to *HPRT*, *PGK* and *G6PD* expression from the Xi (Mohandas et al. 1984; Mohandas et al. 1980). However, lack of DNA methylation may not be the direct mechanism allowing expression from the Xi because 5-aza-C treatment in gerbil cells also causes a dramatic increase in the DNase-I sensitivity of the entire inactive X chromosome as well as an advance in replication timing (Jablonka et al. 1985). The initial events during *HPRT* reactivation are hemi-demethylation and relaxation of chromatin structure, followed by symmetrical demethylation and gene transcription (Sasaki et al. 1992). Transcription factor binding correlates with the appearance of *HPRT* RNA, not the previous relaxation of chromatin structure, indicating that transcription factor binding is not required for the change in chromatin structure after 5-aza-C demethylation treatment (Litt et al. 1997). To determine whether the transcriptional silencing of the *HPRT* gene requires methylation at specific sites or relies on the overall level of promoter methylation, methylation patterns in 5-aza-C treated cells were examined. Reactivation of *HPRT* was associated

with complete promoter demethylation but three CpG sites remained methylated in all silent cells, suggesting that they may be essential to maintain inactivation (Chen et al. 2001a). Although methylation of specific sites may determine the transcriptional activity of *HPRT*, the complete lack of methylation required for reactivation suggests that the absence of promoter methylation is required for the *HPRT* expression. The promoters of genes expressed in most cells are generally unmethylated so a lack of methylation may be essential for expression of all housekeeping genes.

1.2 Expression from the Xi

1.2.1 Escape from X inactivation

The majority of X-linked genes are subject to the dosage compensation mechanism of X inactivation, but there are several genes that escape this silencing and are expressed from both the active and inactive X chromosomes (Carrel and Willard 1999). The lack of X inactivation was anticipated for genes within the pseudo-autosomal region at Xp22.3 as this region contains functional X and Y homologues that pair in meiosis. Predictably, the first genes identified to escape inactivation were located at or near this pseudo-autosomal region. Further analysis has demonstrated that other genes along the chromosome are also expressed from the Xi.

The X inactivation status of human X-linked genes has been determined in several ways. Escape from X inactivation was first demonstrated in humans by a lack of mosaicism for the XG blood-group polymorphisms (reviewed in Disteche 1997). The expression of *STS* from the Xi was indicated by the presence of *STS* enzyme activity in all fibroblast clones from females heterozygous for null mutations because clones without enzyme activity would be expected from heterozygous females if the gene is subject to X inactivation (Shapiro et al. 1979). Genes escaping inactivation can also be identified by a lack of dosage compensation. For example, the amount of *RPS4X*

transcription increases with additional X chromosomes (Just et al. 1992). Human X chromosomes retain their original inactivation status in rodent/human somatic cell hybrids (Migeon 1972) and because the Xa and Xi can be studied independently, polymorphisms are no longer required to distinguish the two X chromosomes. If a gene is subject to X inactivation, its gene product will be detected only from the hybrids retaining the Xa whereas expression is detected in both Xa and Xi hybrids for a gene that escapes X inactivation. The human *UBE1* gene was able to complement the temperature-sensitive cell-cycle mutant murine cell line tsA1S9 with either the Xa or Xi chromosome, revealing that *UBE1* is expressed from both X chromosomes (Brown and Willard 1989). Likewise, *STS* activity was observed in a hybrid retaining a human Xi, providing further evidence that the *STS* gene escapes inactivation (Mohandas et al. 1980). The *ZFX* and *RPS4X* genes were also expressed in Xi hybrids, consistent with other evidence for X inactivation status (Fisher et al. 1990; Schneider-Gadicke et al. 1989), which supports the somatic cell hybrid system hybrid system as an accurate reflection of the expression of X-linked genes in humans. More recently, RT-PCR with human specific primers has been used to survey the inactivation status of many genes along the X chromosome and >10% of X-linked genes appear to escape X inactivation (Brown et al. 1997; Carrel et al. 1999).

If the genes have functional Y homologues, expression from both X chromosomes may be required to maintain dosage with the expression from both the X and Y homologues in males. When there is no functional Y homologue, females may have higher amounts of that gene product. Such increased expression of some genes may be necessary for normal female development but may be of no importance for other genes (reviewed in Disteche, 1995). While it is difficult to say how often dosage has an effect, it is clearly important for a subset of X-linked genes. For example, abnormal

duplication of *DAX-1* in males results in sex reversal whereas deletions lead to X-linked congenital adrenal hypoplasia (Goodfellow and Camerino 2001). It can also not be assumed that genes escaping X inactivation express twice as much gene product because expression levels from the Xi can be much lower than those on the Xa. The most popular system used currently to determine the X inactivation status of a gene is to measure expression in somatic cell hybrids with RT-PCR. If a gene is expressed in both Xa- and Xi-containing hybrids, it is considered a gene that escapes X inactivation. The RT-PCR technique is sensitive and can detect very low levels of expression from alleles on the Xi. For example, quantitative measurements of *STS* activity suggest that expression from the Xi is lower compared to the Xa. The *STS* gene is expressed from both X chromosomes and does not have a functional Y homologue. However, female cells did not have the expected two-fold increase of enzyme activity, but only 1.1x more activity than males, suggesting that all *STS* alleles are not equally expressed (Migeon et al. 1982). In mouse, the expression level of *Smcx* from the Xi was highly variable, with 20 – 70% of the Xa expression level (Carrel et al. 1996; Sheardown et al. 1996).

The majority of genes escaping X inactivation are located on the short arm of the chromosome and this may reflect the evolutionary history of the sex chromosomes. It is accepted that the human X and Y derived from autosomes that diverged during evolution due to suppression of X-Y recombination (Ohno, 1967). The original sex chromosomes were probably smaller than the current human X because only the long arm and proximal short arm are conserved between monotremes, marsupials, and eutherians. The remainder of the short arm, or X-added region (XAR) is present only in the eutherian class and is assumed to originate from a translocation of an autosome to the pseudo-autosomal region of both the X and Y chromosomes after the divergence of placental mammals from marsupials (Graves 1996). This is supported by the observation of four

evolutionary strata along the X chromosome, with the oldest portions corresponding to Xq (Lahn and Page 1999). (Figure 1) The fourth stratum arose recently during primate evolution and differentiation may not yet be complete, explaining the higher number of genes that escape X inactivation with functional Y homologues. The genes that escape X inactivation but do not have equivalent Y homologues may be intermediates in the differentiation process of the sex chromosomes and may not yet have acquired the ability to inactivate. Interestingly, *ARAF1*, a gene 25 kb telomeric to *TIMP1*, is found on the marsupial X but *TIMP1* and its surrounding gene, *SYN1*, are autosomal, suggesting that they are part of the most recent fourth addition (Wilcox et al. 1996).

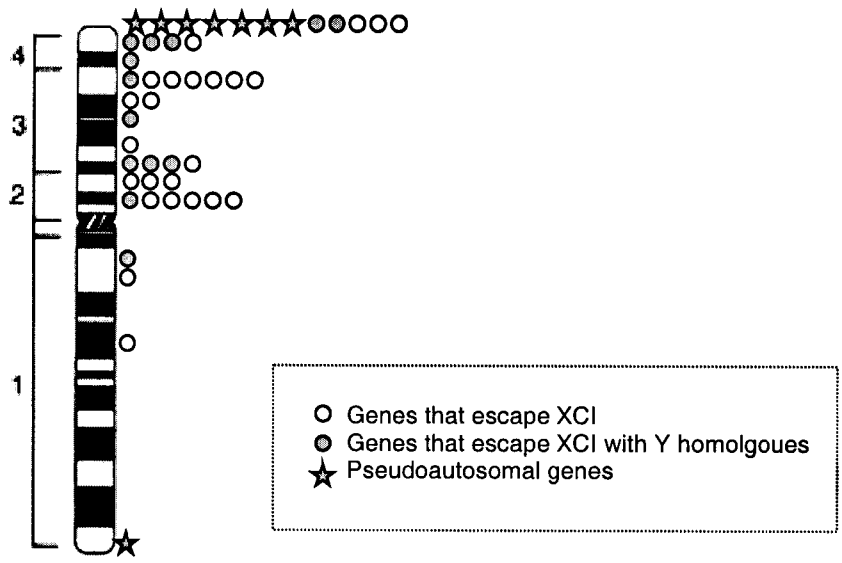

Figure 1.1. Expression from the Xi

An ideogram of the human X chromosome with the cross-hatched constriction showing the position of the centromere. A circle to the right of the ideogram shows the position of a gene shown that escapes X inactivation and the stars are genes in pseudoautosomal regions. A filled symbol denotes a gene with any Y homologue, either functional or non-functional. To the left of the ideogram, there are the approximate evolutionary strata defined by Lahn and Page, 1999. This diagram was adapted from Carrel et al. 1999 and Disteche, 1999.

Mice have fewer genes that escape X inactivation and this may reflect evolutionary differences between the two species. While the relatively simple 4-strata composition of the human X is generally conserved between mammalian chromosomes, the mouse X has been jumbled so that ancestral portions are mixed with newer regions (Disteche 1995) and control of X inactivation may also be altered. Alternatively, mice may appear to have fewer genes expressed from the Xi because comprehensive analysis has not yet been done. A smaller number of genes have been tested and the few examined have been restricted to the homologues of human genes known to escape X inactivation (Carrel et al. 1999). Variability in the number of genes expressed from the Xi could also reflect assay differences as the X inactivation status of mouse genes has been traditionally examined with tissue-based, *in vivo* assays whereas human genes are most often tested *in vitro*. The human *ZFX, RPS4X, and UBE1* genes all escape X inactivation but their mouse homologues do not. The mouse homologues were examined within hamster-mouse hybrids to determine if the *in vitro* system over-estimates expression from the Xi. The *Zfx, Rps4x*, and *Ube1x* genes continued to be subject to X inactivation and expressed only from the Xa, confirming the accuracy of the hybrid system (Salido 1993). This also indicates that the difference in the numbers of genes expressed from the Xi between mouse and human is not an experimental artifact. The lack of escape in mouse may be explained by differences in the Y homologues. The *Zfx* and *Ube1* Y-counterparts are testis-specific and it makes sense that in the absence of functionally equivalent Y homologues, the genes on the X chromosome are subject to X inactivation. The *Rps4* gene does not have a Y homologue so again, X inactivation occurs to maintain dosage compensation with males (reviewed in Disteche 1995). The fewer number of genes expressed from the Xi in mouse may explain the phenotypic differences between mice and humans that lack a second X chromosome. X0 mice are fertile females and may have slight growth delay whereas human X0

females are significantly affected. The majority of 45X fetuses do not survive to birth and the surviving women with Turner's syndrome have short stature, lymphedema, and premature ovarian failure (Ranke and Saenger 2001). The abnormal phenotype in humans may be due to the haploinsufficiency of genes that are expressed from both X chromosomes and the milder phenotype in mice may reflect less haploinsufficiency because very few genes escape X inactivation (Disteche 1997).

It is unknown how genes escape X inactivation but comparing them to genes that are subject to inactivation within and between species may provide clues to the underlying mechanisms. To determine if there are particular elements allowing a gene to escape from X inactivation, the 5' regions of the inactivated *Zfx* and the escaping *ZFX* were compared. The two promoters have a similar structure and no obvious mark protecting *ZFX* from X inactivation was observed (Luoh et al. 1995). There are two closely related genes differing in X inactivation status; the ANT3 gene is a pseudoautosomal gene whereas ANT2 is located on the long arm and is subject to X inactivation (Schiebel et al. 1993), suggesting that the surrounding chromatin environment, not primary promoter sequences, determines the inactivation status of X-linked genes. In mouse, a gene escaping X inactivation, *Smcx*, was compared to flanking genes subject to X inactivation. Again, the comparisons did not disclose unique promoter elements that allow *Smcx* to be expressed from the Xi (Tsuchiya and Willard 2000).

Genes expressed from the Xi lack the epigenetic features associated with the Xi, which supports the idea that these features are involved in the silencing of the second X chromosome. The promoters of genes that escape X inactivation are not methylated on the Xi. For example, the pseudoautosomal gene *MIC2* CpG island is unmethylated on both the active and inactive X chromosome as well as on the Y chromosome

(Goodfellow et al. 1988). The correlation between promoter methylation and inactivation is so strong that the lack of methylation on both Xa and Xi has been used as evidence that a gene escapes X inactivation (Carrel et al. 1996). The Xi generally replicates later in the cell cycle, leading to replication asynchrony between the two homologues. The replication patterns of the *RPS4X*, *ZFX*, and *ANT3* genes, which all escape inactivation, were analysed by FISH and replication synchrony was observed, implying that the homologues on the Xi are replicating earlier. Although the *STS* gene was generally synchronous, there was some discordance, which may reflect the lower expression from the Xi (Boggs and Chinault 1994). The majority of the Xi does not stain with antibodies to acetylated histone H4, indicating hypoacetylation. However, there are three regions that do have some immunolabel, suggesting residual acetylation of histone H4. Two of these bands correspond to the pseudoautosomal regions and Xp11.2-Xp11.3, all of which contain genes that escape X inactivation (Jeppesen and Turner 1993). Only the pseudoautosomal region is associated with the hyperacetylation of histone H3 (Belyaev et al. 1996a). At the individual gene level, the promoter of *ZFX* was associated with acetylated histone H4 on both the Xa and Xi (Gilbert and Sharp 1999). These results strengthen the link between hyperacetylation and potentially active chromatin. The strong correlation between the absence of the above epigenetic features and expression from the Xi implies that the epigenetic changes determine Xi expression status but they may be secondary consequences of gene activation and the local chromatin structural domains may be the primary control.

The genes expressed from the Xi are clustered in particular regions of the Xi, suggesting X inactivation patterns are based on chromosomal domains. It is possible that the different regions correspond to chromatin loops of different transcriptional activity. The 30 nm chromatin fiber is proposed to fold into chromosomal loops, which

can provide a structural compartment for gene organization. The genes are replicated under one origin and therefore controlled as a unit with respect to coiling (torsion) and replication (reviewed in Gartler and Goldman, 1994). The loop domains have boundaries that prevent the spread of one transcriptional state into neighbouring regions, defined by attachment to the nuclear scaffold or matrix (Gasser and Laemmli 1987). Interestingly, the methyl-CpG-binding protein MeCP2 binds to MARs and, through interaction with mSin3A, recruits a corepressor complex containing histone deacetylases, which could create a silenced chromatin structure within one loop (reviewed by Stratling and Yu 1999).

It is unclear whether X inactivation skips these sections at the start of X inactivation or if the silencing is not maintained and the genes reactivate during development. Regions that escape X inactivation in humans have fewer LINE-1 elements, the proposed way stations involved in the spread of the X inactivation signal along the chromosome, suggesting that control is at initiation. However, the *Smcx* gene is first subject to X inactivation but then escapes X inactivation in adult mice, so it must reactivate during development (Lingenfelter et al. 1998). Expression from the Xi may result from a combination of these possibilities. X inactivation may not fully spread into areas with fewer way stations and then this weak silencing may not be properly maintained.

1.2.2 Reactivation of X-linked genes

Reactivation was first demonstrated for the entire X chromosome during oogenesis. The primordial germ cells in humans have Barr bodies indicating one X is inactivated. However, both X chromosomes are active in the egg so there must be a normal inactivation/reactivation cycle in the female germline (reviewed in Gartler and Goldman, 1994). The inactivation may not be as stable as somatic cell inactivation as there is no

hypermethylation of promoters in human fetal female germ cells in 6-24 weeks. The reactivation may be related to a lack of *Xist*, as *Xist* RNA is no longer expressed in oocytes (reviewed in Gartler and Goldman 1994).

The first evidence of reactivation in somatic cells was the change of coat colour in aging mice. These mice carried an X-autosome translocation that was inactivated in all cells. As the mice aged, a pigmentation gene on the translocated autosomal portion reactivated (Cattanach 1974). Reactivation is not limited to inactivated autosomal genes, as the endogenous X-linked gene, *Otc* also reactivates with age. Histochemical staining of liver sections in mice heterozygous for a null mutation showed that older mice had a larger portion of cells with Otc activity (Wareham et al. 1987). The *Otc* gene is 44 cM from the Xic so may be more prone to reactivation but the mottled coat colour gene *Mo*, only 4cM from the Xic, also reactivated with age (Brown and Rastan 1988). To determine if the human *HPRT* gene reactivates with age, fibroblasts from women heterozygous for *HPRT* deficiency at various ages were examined. There was no significant increase of *HPRT*+ cells in older cells, suggesting that age-related reactivation is not common to all X-linked loci and may have species, tissue, or locus-specific controls (Migeon et al. 1988a). *HPRT* is a housekeeping gene with a strong CpG island but the reactivated genes discussed above are tissue-specific without GC-rich promoters. Therefore, it may be easier to disrupt methylation and associated epigenetic controls so the inactivation may not be as well maintained.

Reactivation of X-linked genes is enhanced in interspecies hybrids. The *HPRT* gene is occasionally reactivated in mouse-human hybrids in ~1 in 10^6 cells, but reactivation did not correlate with particular autosomes or the presence of the Xa (Kahan and DeMars 1975). Maintenance of X inactivation may be less stable in these hybrids because slight changes in regulatory proteins could alter epigenetic controls. For

example, the human *XIST* RNA does not localize to the human Xi in rodent-human somatic cell hybrids (Clemson et al. 1996), suggesting that a regulatory protein is not present in mouse. Human X inactivation is maintained in the absence of *XIST* RNA (Brown and Willard 1994) but the lack of localization may indicate a disruption of other epigenetic controls and a predisposition of genes to reactivation. Additionally, the ability to reactivate may depend on the rodent parent because *HPRT* reactivants are observed in some hybrids (Hellkuhl and Grzeschik 1978; Kahan and DeMars 1975) but not in others (Hellkuhl and Grzeschik 1978; Migeon et al. 1978).

Reactivation of X-linked genes has been induced by chemically disrupting the epigenetic features associated with the Xi. Demethylating 5-aza-C analogs can reactivate the expression of inactivated genes (Mohandas et al. 1981; Shapiro and Mohandas 1983), advance the replication time and enhance DNaseI sensitivity of the entire Xi (Jablonka et al. 1985). If more than one chemical is used, the reactivation is higher. Combining inhibitors of histone deacetylase with demethylation treatment leads to a 2 to 5 fold increase in *FMR1* reactivants, indicating a synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of *FMR1* (Chiurazzi et al. 1999).

The reactivation of X-linked genes correlates with the disruption of epigenetic control, and often more than one feature is affected. The demethylating agent 5-aza-C is the most effective chemical treatment to induce expression from the Xi and it interferes with several of the epigenetic features before reactivation is observed. Additionally, X inactivation is maintained in ICF patients who have a lack of promoter methylation, but once replication timing is advanced, genes can be reactivated (Hansen et al. 2000). Therefore, it appears that the epigenetic features work in concert to maintain X inactivation.

1.2.3 Variable X inactivation

Even though the differences between Xi gene expression are well established between species, the inactivation status of an X-linked gene has been believed to be the same among females within a species. However, only *HPRT* and *G6PD* have been examined in adequate numbers of individuals to show consistent X inactivation status (reviewed in Carrel and Willard 1999). For example, the *TIMP1* gene was initially shown to be subject to X inactivation and silent in hybrids retaining an Xi (Brown et al. 1990), but when the analysis was extended, the gene was expressed in a subset of Xi hybrids. In this survey of X-linked gene expression in 9 Xi hybrids, the *ALD* gene was also variably inactivated (Brown et al. 1997). These genes were expressed from the Xi in different hybrids so expression from the Xi was not a lack of maintenance within particular hybrids. A more extensive analysis of the inactivation status of 224 X-linked genes in nine hybrids found 13 genes with heterogeneous inactivation, including *TIMP1* and *ALD* (Carrel et al. 1999).

This variable X inactivation is not limited to the hybrid system as the *REP1* gene was both subject to and escaped X inactivation in some human primary fibroblast lines. In the nine cell lines heterozygous for a transcribed *REP1* polymorphism, three cell lines were subject to X inactivation, with monoallelic expression but the other six cell lines expressed both alleles, indicating escape from X inactivation. The Xi levels ranged from 5 - 42% of Xa levels, which may reflect a mixed cell population with escape from X inactivation in only a subset of cells. Expression of both alleles was detected in the majority of cells using single cell RT-PCR, indicating that all cells express *REP1* from the Xi but at reduced levels. Expression of *REP1* was examined in 10 somatic cell hybrids retaining an Xi and was expressed in seven hybrids but silent in the other three, demonstrating heterogeneous expression as in the human cells (Carrel and Willard 1999). Therefore, the hybrid system would have correctly identified *REP1* as a gene

with variable X inactivation in humans. Consequently, the other genes with heterogeneous X inactivation status in the Xi hybrids may also be variably inactivated in humans.

1.3 Human *TIMP1* gene

1.3.1 Function

The development of multicellular organisms depends on the extracellular matrix (ECM), which maintains aggregates of cells to form tissues and then provides strength and integrity to these tissues. The ECM also has informational cues to direct a cell's behaviour and regulates fundamental cellular processes, including growth, differentiation, motility, signal transduction, and changing cell shape (reviewed in Vu 2001). The remodelling of the ECM is a highly regulated process coordinated by the matrix metalloproteinases (MMP) family. These zinc-containing, calcium-dependent neutral proteases can collectively degrade all the structural proteins of the ECM (reviewed in Vincenti 2001) except in the presence of chelating agents that sequester Zn^{+2} (Borden and Heller 1997). The enzymatic activity of MMP family members is also inhibited by the family of tissue inhibitors of metalloproteinases (TIMPs) whose primary function is to limit the degradative actions of the MMPs during tissue remodelling (Nagase and Woessner 1999).

Humans have four known TIMPs, numbered TIMP1 through TIMP4, based on the order of discovery. In general, TIMPs are secreted proteins that form tight 1:1 complexes with the active forms of MMPs with low selectivity. An exception is the inability of TIMP1 to associate with the MMP-14 (or MT-MMP 1) (Strongin et al. 1993). The four TIMP family members share several structural features: 12 cysteine residues in conserved regions that form six disulfide bonds; the NH2-terminal domain is required to inhibit MMPs; and a 29 amino acid leader sequences is cleaved off to produce the

mature protein (Gomez et al. 1997). There are no human diseases shown to be caused by mutations in the *TIMP1*, *TIMP2*, or *TIMP4* genes but mutations in *TIMP3* cause Sorsby's Fundus Dystrophy (Weber et al. 1994), an autosomal macular disorder with progressive degeneration of the central retina [OMIM 136900] (reviewed in Edwards et al. 2001). An ECM layer between photoreceptors and their blood supply becomes lipid-rich, presumably due to an altered balance between metalloproteinase activity and inhibition (Capon et al. 1989). Because only eyes are affected, other TIMPs may be able to compensate for the lack of TIMP3 elsewhere in the body. Despite the close similarities of the four human TIMPs, each one has distinct properties (Table 1.1) and may have a favoured set of tasks *in vivo*.

	TIMP1	**TIMP2**	**TIMP3**	**TIMP4**
Chromosome Location	Xp11.23	17q2.3-2.5	22q12.1-13.2	3p25
Expression	inducible	constitutive only	inducible	constitutive only
Protein size	28 kDa	21 kDa	24 kDa	25 kDa
Glycosylation	yes	no	yes	no
Soluble	yes	yes	no	yes
Protein Location	diffusible	diffusible	ECM bound	diffusible
MMP Inhibition	all	all	all	1,2,3,7,9
MT-MMP Inhibition	no	yes	yes	yes
Erythroid Potentiation	yes	slight	?	?

Table 1.1. Properties of the Tissue Inhibitors of Metalloproteinases.

The TIMP family members regulated differently and the resulting active proteins differ in size, modifications, position and action. (Edwards et al. 2001 and Woessner and Nagase, 2000 and references within)

The protein-coding information is found within 5 exons in humans and the exon-intron boundaries are conserved between the 4 TIMP proteins, suggesting that the genes arose from a single ancestor (Apte et al. 1995). A single *TIMP* gene was identified in *Drosophila* and sequence alignment with the four human *TIMP*s demonstrated a conserved exon-intron structure with 35% of the amino acids in *Drosophila* identical to one in the human TIMPs (Pohar et al. 1999). By anchoring the phylogenetic tree with the *Drosophila TIMP* gene, Brew et al. (2000) proposed a sequence for the origin of the four TIMPs in mammals. *TIMP1* has the lowest rate of evolutionary change and appears to be the closest to the original gene. The first duplication led to two genes, the *TIMP1* ancestor and the antecedent for the other three *TIMP*s. A further duplication led to *TIMP3* and the ancestor for *TIMP2*/*TIMP4*, which were created by the last duplication (Brew et al. 2000). The *Drosophila TIMP* was discovered within intron 7 of the *SYN* gene, which corresponds to intron 5 of human *SYN1* where *TIMP1* resides. Human *TIMP3* is found in the fifth intron of *SYN3* and initial results suggest that *TIMP2* is near *SYN4* (Pohar et al. 1999). Therefore, the nested organization of *TIMP*s and *SYN*s appears to have been conserved during evolution despite no reported functional relationship between the two genes. This supports the idea of a fourfold amplification of large portions of the genome during evolution from a common invertebrate/ vertebrate ancestor through mammalian radiation.

Most of the literature describing TIMP1 function concentrates on the MMP inhibitory function but TIMP1 has many other functions, shown in Table 1.2. For example, TIMP1 was originally identified as a protein that stimulates the proliferation of erythroid precursors and called EPA for erythroid potentiating activity (Gewert et al. 1987). TIMP1 may also regulate germ cell development, as it is part of the protein complex secreted from Sertoli cells that stimulates steroidogenesis in Leydig and ovarian granulosa cells

(Boujrad et al. 1995). Additionally, secreted TIMP1 appears to block apoptosis because Burkitt's lymphoma cell lines with *TIMP1* expression are resistant to apoptosis, which is reversed by the presence of antibodies to TIMP1 (Guedez et al. 1998).

Functions of TIMP1	Reference
Inhibits active MMPs	(Gomez et al. 1997)
Promote gonadal steroidogenesis	(Boujrad et al. 1995)
Inhibits tumour progression	(Alvarez et al. 1990)
Promote tumour progression	(Lindsay et al. 1997)
Growth factor activity, EPA	(Gewert et al. 1987)
Inhibit apoptosis	(Guedez et al. 1998)

Table 1.2. Reported functions of TIMP1.

Reviewed in (Gomez et al. 1997) and (Denhardt et al. 1993)

1.3.2 Transcriptional control

Generally, the TIMPs are co-ordinately regulated with the MMPs (Denhardt et al. 1993) and where examined, the control of *TIMP1* expression is at the level of transcription (Borden and Heller 1997). *TIMP1* has a unique first exon missing in the other *TIMP* genes that is transcribed but not translated (Dean et al. 2000). Multiple start sites were observed with RPA but the most 5' start gives a 48 bp exon (Clark et al. 1997).

The sequences controlling *TIMP1* expression are concentrated around the 5' end of the gene because a 1.3 kb murine transgene containing 5' flanking sequence, exon1 and most of intron 1 was sufficient to reproduce the spatial and temporal expression of the *Timp1* gene in developing mouse embryos (Flenniken and Williams 1990). Transient transfections of human TIMP1 promoter-CAT reporter constructs have been used to identify promoter elements affecting transcription (Fig 1.2). Deleting -102 to -80 nearly

abolishes CAT activity, indicating that this region is required for transcription (Clark et al. 1997). There is a 38 bp region at -105 to -63 that is strongly conserved (37/38 bp identical) between human and mouse. This level of conservation suggests the sequence is of functional importance. This region contains a consensus AP-1 site and an Ets-binding site (Uchijima et al. 1994). AP1 is a heterodimer of Jun and Fos proteins and Ets proteins are serum-inducible proteins that activate transcription as monomers or within a complex. The AP-1 and Ets sites are often juxtaposed so there may be interaction between the binding proteins. In the *TIMP1* promoter, expression is considerably lower when the 5' AP-1 site is mutated, suggesting that it is required for transcription. There is a non-consensus AP-1 site in the first intron that can also increase transcription. By analysing the amount of expression in various promoter deletion transfections, Logan et al. (1996) have proposed a model of transcriptional activation for the *TIMP1* gene, with Ets activation dependent on previous AP1 binding. When AP1 is not present, there is no transcriptional activation, regardless of the amount of Ets-related proteins. When there is only a low amount of AP1, it binds only to the first AP-1 site and activates transcription. If Ets proteins are also present, they can interact with the binding site and the bound heterodimer, leading to a stable complex that synergistically increases transcription. If AP1 concentration is high, both AP-1 sites are occupied, which leads to the highest rate of transcription and the binding of Ets proteins does not significantly alter the transcription rate (Logan et al. 1996).

Transfection assays have identified other regions that regulate *TIMP1* expression. The +21 to +58 region may control cell-specific transcription because it was required for expression in human skin fibroblasts but not hepatic stellate cells (Clark et al. 1997). Using the CAT reporter system, two regions flanking the promoter region were shown to repress transcription: -1718 to -1458 and +684 to +748 (Dean et al. 2000).

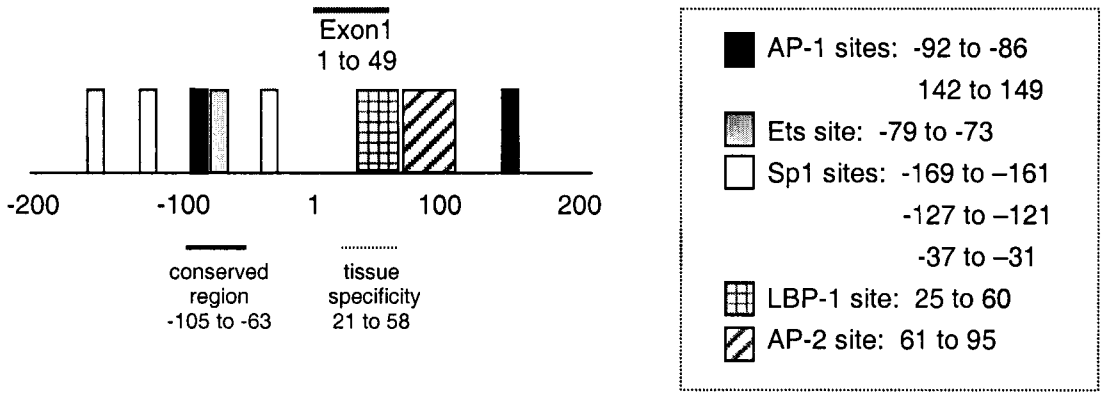

Figure 1.2. Binding sites at the *TIMP1* promoter.

Transient transfection assays have identified a number of transcription factor binding sites at the *TIMP1* promoter. The sequence between –105 to -63 is very conserved between human and mouse, with 37/38 bp identical.

The expression of the human *TIMP1* gene is regulated by a variety of cytokines and growth factors. *TIMP1* is an induced by many factors including retinoic acid, transforming growth factor-β, interleukin 6 and 11, and oncostatin M (Dean et al. 2000). Most of the examination of the induction process has analyzed the action of tetradecanoyl phorbal acetate (TPA), which dramatically increases the production of MMPs and TIMP1. It is unknown what natural signals the TPA is imitating, but its activation of the protein kinase C pathway mimics the action of both growth factors and cytokines (Borden and Heller 1997). The increase in TIMP1 protein is not simply due to a faster transcription rate. In monocytes undergoing TPA induction, RNA levels initially rise due to a longer half-life of the RNA from 3.5 hours to 9 hours and then transcription rate increases (Doyle et al. 1997). The amount of TIMP1 protein is further amplified in mouse because the first exon is not transcribed after induction, leading to increased translation efficiency (Edwards et al. 1987).

1.4 Thesis investigation

I will study variable TIMP1 inactivation in humans, both the potential clinical implications and possible epigenetic mechanisms. The data will be presented in three chapters as follows:

- I determine whether *TIMP1* is variably inactivated in human cells to establish if *TIMP1* expression from the Xi is a reactivation event limited to the hybrid system. (Anderson and Brown (1999) American Journal of Human Genetics)
- I examine possible differences in *TIMP1* RNA levels resulting from polymorphic inactivation because increasing amounts of TIMP1 may explain some phenotypic variations and disease susceptibilities.
  (Anderson and Brown (in press) Human Genetics)
- I compare the epigenetic features associated with the inactive X when TIMP1 expressed or inactivated on the inactive X to determine a hierarchy to heterochromatic organization.
  (Anderson and Brown, manuscript in preparation)

2 MATERIALS AND METHODS

2.1 Cell Culture

2.1.3 Human Cell Lines

The lymphoblast cell lines were acquired from Coriell Cell Repository and grown in RPMI media 1640 (Stem Cell Technologies) supplemented with 15% fetal calf serum (Cansera), L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen). Cells were harvested 12-26 hours after addition of fresh media by centrifugation. The fibroblast cell lines were also received from the Coriell Cell Repository and were grown in minimal essential media (Invitrogen) supplemented with 15% fetal calf serum, L-glutamine, penicillin, streptomycin, and non-essential amino acids (Invitrogen) to sub-confluence before harvesting with trypsin-EDTA (0.25%) (Invitrogen). The passages were noted: GM01813 were passages 16 to 22 and GM02859a went through passages 25 to 33. K. Siminovitch (University of Toronto) supplied the lymphoblast cell lines derived from Wiskott-Aldrich carriers.

2.1.4 Somatic Cell Hybrids

The human/rodent somatic cell hybrids were provided by H. F. Willard (Case Western Reserve University) or S. Hansen (University of Washington) or were generated by S. Baldry using somatic cell fusion as described elsewhere (Hansen et al. 1988; Brown and Willard 1989; Willard et al. 1993). The hybrids were grown in minimal essential media (Invitrogen) supplemented with 7.5% fetal calf serum, L-glutamine, penicillin, streptomycin, and non-essential amino acids to sub-confluence before harvesting with trypsin-EDTA (0.25%). To generate single cell clones, the hybrid cultures were plated to a final concentration of 3-17 cells/60mm plate. After 5 – 10 days in culture, well-separated colonies were isolated by trypsinization in cloning cylinders and transferred to new plates.

2.1.5 Demethylation treatments

Carolyn Brown demethylated an Xi hybrid (t11-4Aaz-5) with 5-aza-cytidine as previously described (Tinker and Brown 1998). The cells were grown in HAT media to select for *HPRT* reactivants and then single cell cloned to test for *TIMP1*-positive cultures. The HAT media consists of minimal essential media supplemented with 7.5% fetal calf serum, L-glutamine, penicillin, streptomycin, non-essential media and 1x HAT. To remove any confounding effects of *HPRT* selection, the cells were transferred back to alpha media once they were growing well.

2.1.6 TPA Induction

The hybrid cell lines were grown in three separate t25 flasks to 50-70% confluence, the alpha media was removed, the cells washed with 1x PBS, and 10 ml alpha media with reduced fetal calf serum (2% FCS) was added to each flask. After 6 hours, this media was removed and 10 ml of new alpha media (2% FCS) with 100 ng/µl TPA (tetradecanoylphorbol-13-acetate) was added to two flasks and left to grow for 6 hours. One flask received alpha media (2% FCS) without TPA as a negative control. RNA was isolated, and then analyzed with RNase Protection, duplex PCR or nested PCR. A change in *TIMP1* expression was calculated as the ratio of RNA levels from the +TPA compared to the flask without TPA.

2.2 DNA extraction

Depending on the number of cells, cell pellets were resuspended in 1 to 10 ml 1M Tris-EDTA buffer (pH 8.0). The following protocol describes 10 ml preps but the amounts can be scaled down accordingly. To cells in 10 ml TE, 500 µl 20% SDS and 4 µl of 10 mg/ml proteinase K was added and this tube was left on the lab bench overnight for at least 12 hours and up to 2 weeks. For tissue culture pellets, a salting out

procedure was used but the phenol/chloroform extraction was performed to isolate DNA from patient blood and tissues.

2.2.1 Salting out genomic DNA

The next day, 800 µl of 5M NaCl was added to the 10 ml of solution in the tube and then incubated at 37C for two hours or until in solution. Once the salt was fully dissolved, a further 3.3 ml of 5M NaCl was added. The tube was shaken vigorously for 30 seconds and then centrifuged for 15 minutes at 2500 rpm at room temperature. The supernatant was transferred to a new 15 ml tube and 0.3 ml 20% SDS and 3.3 ml 5M NaCl were added. Again, the tube was agitated thoroughly for 30 seconds and then centrifuged at 2500 rpm for 15 minutes at room temperature. The supernatant was transferred to a 50 ml tube, twice as much ethanol (~30 ml) was added, and the tube was rocked gently to precipitate the DNA. A Pasteur pipette melted to have a small hook at the end was used to scoop out the DNA and the DNA was placed in a screw cap tube with 1 ml TE. The DNA was left for at least 12 hours to dissolve into the TE and then quantified with spectrophotometry.

2.2.2 Phenol/Chloroform Extraction

This procedure was used to isolate DNA from human samples to minimize the risk from contagious viruses and bacteria and was also used to "clean up" DNA after digestion (see RNA preparation and methylation analysis). Generally, the volumes were smaller with this technique, approximately 1 ml preps. After cell lysis on the lab bench, an equal volume of Tris-buffered phenol (pH 8.6) was added and the tube was vortexed for at least one minute then centrifuged at 13200 rpm for 7 minutes at room temperature. The supernatant was moved to a new tube and an equal volume of 1:1 phenol:chloroform was added. After another complete mix and centrifugation, the supernatant was moved to one more new tube. Now, an equal volume of chloroform

was added, mixed and centrifuged. The supernatant was transferred to a new tube, 1/10 of this volume of 3M KOAc pH 6.0 added along with two times of ethanol. This was then placed at –20C for a minimum of 16 hours. The tubes were then centrifuged at 13200 rpm for 10 minutes at 4C. The ethanol was removed and then the DNA pellet was resuspended in 10 to 100 µl TE according to the size of the pellet. The DNA was left for a minimum of eight hours to resuspend and then quantified with spectrophotometry.

2.3 RNA extraction

RNA was prepared by means of a standard acid-guanidinium extraction protocol (Chomczynski and Sacchi 1987). This protocol can also be scaled depending on the size of the pellet. For a standard fibroblast culture in a t25 flask or 60 ml plate, the following amounts were used. To the cell pellet, 0.6 ml Solution D (guanidinium thiocyanate with β-mercaptoethanol) was added and vortexed at room temp until the cells were evenly distributed without chunks of debris. Then 0.6 ml DEPC-treated water-saturated phenol and 60 µl 2M NaOAc pH 3.5 were added. After the addition of 0.24 ml chloroform, the tube was vortexed for at least one minute after the mixture turned "white". The tube was left on ice for 10 minutes then centrifuged at 13200 rpm for 10 minutes at 4C. The supernatant was transferred to a new 1.5 ml tube and an equal volume of isopropanol was added before the tube was put at –20C for at least 12 hours. The next day (or later), the tube was centrifuged at 13200 for 15 minutes at 4C. The ethanol was removed, then the pellet was washed with 100 µl of 70% ethanol and centrifuged at 13200 for 10 minutes at 4C. The pellet was dried, and then resuspended in 25 to 100 µl DEPC-ddH_20.

To remove DNA contamination, the sample was incubated with 1/10 the total volume of RNase-free DNase (Epicentre) at 37C for 1 hour and then phenol extracted

(as above) to remove excess proteins and single nucleotides. RNA concentration was determined spectrophotometrically prior to analysis of the RNA by RT-PCR or RPA.

2.4 Polymerase Chain Reaction (PCR)

In general, PCR was performed with 1μM primer, 1.5 mM $MgCl_2$, 20 μM of each dNTP, 5 units Taq (Invitrogen) for 30 to 35 cycles of denaturing temperature for 1 minute, annealing for 1 minute and then extending for 2 minutes. The 25 μl reactions were performed in either a Techne Genius or a Thermolyne thermocycler. The primers were pre-mixed into one tube and when first tried, the cycles were as follows: 94C – 1 minute, 54C – 1 minute, and 72C – 2 minutes. The annealing temperature and $MgCl_2$ concentrations were altered to optimize the reactions. For example, if the background was too high, the annealing temperature was raised or the $MgCl_2$ concentration was dropped. For CpG-rich templates, the denaturing temperature was raised to 95C and an extra denaturation step of 95C for 3 minutes was added before the first PCR cycle. Ten μl of the PCR reactions were electrophoresed on a 2% agarose gel and then stained with ethidium bromide to visualize the PCR products.

Primers were designed for a target sequence, trying to avoid repeat elements and if possible, spanning an intron to allow the cDNA and DNA sequences to be discriminated because the PCR products would be different sizes. The primers were usually 20 nucleotides long and spanned 200 to 600 bp, with approximately equal CG and AT content. After designing new primers, the proposed oligonucleotides were run through the BLASTN program (NCBI) to check that they were specific to that particular gene. If the primers matched sequence in mouse or hamster, they would not be useful in the somatic cell hybrids and new primers were designed. If the primers matched multiple sequences at >75% similarity, they were probably within repeat sequences and new primers were designed.

GENE	Name	Sequences	Assay
TIMP1	5'	A: cccttgggttctgcactga	methylation
		B: ccaagctgagtagacaggc	
	C1	A: agatccagcgcccagagaga	expression
		B: ccctgatgacgaggtcggaa	
	3'	A: caaggctctgaaaaggctt	expression
		B: tgctgggtggtaactcttta	
	CA	1: gggttccaagccttagggga	polymorphism
		2: aggctgttccagggagccgc	
		1: gggttccaagccttagggga	PASA – C allele
		C: ctgttccagggagccacg	
		1: gggttccaagccttagggga	PASA – T allele
		T: ctgttccagggagccaca	
	N	5: accttataccagcgttatgagat	nested around CA
		3: cagtgtaggtcttggtgaagc	
ARAF1	M1:4	M1: tgccaaagccctaaggtca	methylation (better)
		M4: cgctgtcgacgatggtct	
	M1:2	M1: tgccaaagccctaaggtca	original methylation
		M2: tgtcaaggcaagaccaatcg	
	B:3	B: tcagcaaaatctccagcaac	expression
		3: tggagatggaggagctccca	
ELK1	5'	A: gcacagctctgtagggaa	methylation
		B: agctcacctgtgtgtagcg	
	m	1: aaacgcaggggcggcttcta	expression (better)
		2: gttaaagagggatgcggagga	
	A:B	A: ggacctagagcttccactca	expression
		B: agagcatggatggagtgacc	
ZNF41	A:B	A: ttttagaagaactgtggcaag	expression
		B: ctcaggatgaattttctgatg	
ZNF157	A:B	A: gcctctcactgaagacttc	expression
		B: agcaagacatgaacagccc	
MIC2	5'	A: agaggtgcgtccgattctt	methylation
		B: cgccgcagatggacaattt	
	m	1: cagagccagctgttcagcgt	expression
		2: cacagcccatgggaaaccgc	
8037	A:B	A: gaggcaagacatccattcc	non-coding
		B: tgactttgagcgagcaggt	
STA	A:B	A: cacctgtgtgtcatctatac	non-coding
		B: ccagtattggtcttccagtt	
XIST	3':5'	3': gaagtctcaaggcttgagttagaag	DNA
		5': ttgggtcctctatccatctaggtag	
	exp	C94: agctcctcggacagctgtaa	expression
		C7B2 (rev): tccagatagctggcaacc	
*	Ac	A5: tatgctctctccgccctca	acetylation
		29r: atcagcaggtatccgatacc	
ZFX*		1: gtgctgtgttaaaggatagt	acetylation
		2: aggagcccaattgggtatgg	
PGK*	A:B	A: acgcggctgctctgggc	acetylation
		B: ttaggggcggagcaggaag	

Table 2.1. The PCR primers used throughout this thesis.

I designed the majority of the primers from GenBank sequences except for the shaded primers (Carolyn Brown) and primers with a * (Gilbert and Sharp, 1999).

2.5 RT-PCR

2.5.1 Reverse Transcription (RT)

The PCR requires a DNA template so cDNA was created from mRNA to analyze expression. To reverse transcribe the RNA, two to five μg of RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), by random hexamer priming (Brown et al. 1990) in a final volume of 20 ul. First, 1X first strand buffer, 0.2 mM DTT, 2.5 mM dNTPs, 1 μM random hexamers were put into each tube, then 9.5 μl of RNA +/- DEPC-ddH_20 was added. Once 12 units RNase Inhibitor (Pharmacia) and 200 units of M-MLV reverse transcriptase (Invitrogen) were added, the reagents were gently mixed and the tube was left at room temperature for 10 minutes. The reaction was then incubated at 42C for 2 hours, followed by incubation at 95C for 5 minutes to kill the reverse transcriptase enzyme activity. One μl of the RT reaction was subsequently used in the PCR reaction.

2.5.2 Duplex PCR

To determine the amount of increased expression in the TPA-induced hybrids, duplex PCR was used for the *TIMP1*+ hybrids. One μl of RT reaction was added to a PCR reaction that had two sets of primers; one for *MIC2* expression as an internal control for the amount of RNA and the efficiency of the RT reaction and one for *TIMP1* to examine the amount of *TIMP1* expression. The duplex PCR was done with two sets of *TIMP1* primers – the TIMP C1A and C1B primers, which are found at the 5' end of the gene (exon 1 and 2) and the TIMP-N primers that are further 3' (exons 4 to 6). The photos of the PCR products electrophoresed on agarose gels were scanned and the images analyzed with NIH image to ascertain the intensity of the bands. The amount of *TIMP1* expression was the intensity of the *TIMP1* band/*MIC2* band intensity. The

increase of *TIMP1* expression after TPA induction was calculated as the level with TPA amount divided by the amount of RNA from cells without exposure to TPA.

2.5.3 Nested PCR

Nested PCR was used to examine very low expression levels in the hybrids. To help detect low level *TIMP1* expression twelve μg of RNA was added to the reverse transcription reaction. One μl of RT reaction was used in the first PCR with TIMP-N primers that flank the CA1: CA2 primer sites. Then one μl of that PCR reaction was used as template for the internal TIMP-CA PCR. Each of the PCR reactions only required 20 cycles due to the increased amount of initial template and two rounds of amplification. Because there was more cDNA template, 20 cycles were enough to amplify a MIC2 product in linear range. Therefore, the MIC2 primers were added to the first TIMP-N reaction tube to control for the amount of input cDNA. The MIC2 and TIMP-NCA PCR products were run side by side on a 2% acrylamide gel and the images analyzed with the NIH image program as above.

2.6 Methylation Analysis

Genomic DNA was isolated from cultured cells with a standard salt extraction protocol. DNA was pre-digested with *EcoRI* at 37C overnight, followed by incubation with 2 μl of RNase at 37°C for 15 minutes. After phenol extraction and ethanol precipitation, the DNA was quantified by spectrophotometry. 2 μg of pre-digested DNA was then incubated overnight at 37°C in a total volume of 20 μl with 20 units of one of the following: mock enzyme (uncut), *HpaII*, or *HhaI*. 1 μl (100 ng) was then used as a template in the PCR reactions (as above). All the primers were human specific and did not flank *EcoRI* sites.

2.7 Northern Analysis

Ten μg of total RNA was run on a 1% formaldehyde gel at low currents in the 4C cold cabinet to prevent melting. I usually made a 40 ml gel, which consists of 36 ml DEPC-ddH_2O, 2 ml 20X MOPS buffer (400 mM MOPS, 100 mM NaOAc, 10 mM EDTA), 2 ml formaldehyde, and 0.4 g agarose. The water and agarose were heated first and immediately before pouring the gel, the MOPS and formaldehyde were added. Two μl of 5x RNA loading dye (60 μl 10x MOPS, 210 μl formaldehyde, 600 μl formamide, 200 μl glycerol) were added to 8 μl of RNA sample, heated at 65C for 15 minutes, and then 0.5 μl of 1 mg/ml ethidium bromide was added just before loading the samples. The gels were run in 1X MOPS buffer.

The gel was transferred to a nitrocellulose membrane by capillary blot overnight. To prepare the gel for transfer, I placed it in an RNase-free dish and rinsed it in DEPC-ddH_20 for 2x 10 minutes each. During these washes, I started to prepare the wick layers by filling the bottom of the transfer apparatus with 10x SSC and putting a layer of Whatman paper that spanned the top plate and reached into the SSC on both sides. Then I placed two pieces of Whatman paper cut slightly larger than the gel area onto the top plate of the transfer surface and completely wet them with more 10x SSC. The gel was then placed on the Whatman and covered by the nitrocellulose membrane, again cut slightly larger than the gel area. Air bubbles were removed by rolling a glass pipette over the layers. A further three sheets of Whatman were placed on the filter and then covered with strips of paper towel. To prevent the layers above and below the gel from touching and wicking the liquid without going through the gel, a sheet of plastic wrap was added to each side of the transfer apparatus. A weight was added to the top of these layers to hold everything in place overnight.

The next day, the paper towels and Whatman filter papers were removed and the nitrocellulose membrane was recovered with the flattened gel. The positions of the wells were marked on the membrane in pencil with a mark or cut to identify both front-back and up-down orientations. The membrane was rinsed in 5x SSC and then placed on a new sheet of Whatman paper to dry. The blots were then baked at 80C for 1 hour.

The DNA probes were labelled by random priming to incorporate ^{32}P-CTP (3000 Ci/mmol, NEN-US). One μg of probe template in 8 μL was added to 2 μl of hexanucleotide primers and heated at 95C for 5 minutes, then immediately placed on ice. The other reagents were added (1x random primed buffer, 5 units Klenow DNA polymerase (Invitrogen) and 4 μl of ^{32}P-UTP) and the reaction was incubated at 37C for a minimum of 45 minutes. To remove unincorporated nucleotides, the resulting probe was purified through a Sephadex column. The nitrocellulose filters underwent a pre-hybridization in hybridization buffer at 42C for 1 hour. The purified probe was added directly to this buffer and incubated overnight at 42C.

The next day, the nitrocellulose membrane was washed to remove unwanted background. Each of the five washes was 20 minutes in duration. The membrane was first washed twice in 2x SSC, 0.3% SDS at room temperature. Then, it was placed in 1x SSC, 0.5% SDS at 65C. Finally, it was washed twice with 0.3x SSC, 1.0% SDS at 65C for each wash. The remaining hybridized probe was visualized with autoradiography. The strength of *TIMP1* probes were compared to the intensity of mouse Actin hybridization to control for the amount of input RNA.

2.8 RNase Protection Analysis

2.8.1 Generation of probes from PCR fragments

Probes were generated by inserting PCR products into the pKRX vector, which has T3 and T7 polymerase sites flanking the multiple cloning site. The pKRX vector is very

similar to the pCR-Script Amp SK(+) but has an extra 15 base pairs in the multiple cloning site. It was linearlized with *XcmI* and is ampicillin resistant.

The insert is a PCR fragment and needs an A/T overhang to clone into pKRX. I used the Deep Vent Taq polymerase with proof-reading ability (NEB) to amplify the fragment so an A/T overhang had to be added. After a 25 μl PCR reaction (as above) with the proof-reading Taq polymerase, the PCR reaction is placed on ice. With as little oil as possible, 20 μl was moved to a new tube and 0.3 μL of the "normal" Taq (Invitrogen) was added and incubated at 72C for 8 minutes. To precipitate the DNA, 2 μl of 3M NaOAc and 40 μl of ethanol were added. The tube was centrifuged at room temp for 5 minutes at 13000 rpm, rinsed with 50 μl 70% ethanol and centrifuged again. The dry pellet was resuspended in 20 μl TE.

This PCR insert was then ligated to the pKRX plasmid at 16C overnight. For 150 to 400 bp inserts, I added either 0.5 μl or 1.5 μl PCR product to 50 ng of linearized plasmid, with 1 μl ligation buffer (NEB) and 1 μl T4 DNA ligase (NEB).

The next day the cells were transformed into the DH5α competent bacteria (Invitrogen). An aliquot of 5 to 10 μl of the ligation mix was added to 50 μl of competent cells. This tube was kept on ice for 45 minutes and I flicked the tube every 5 minutes. While waiting, I added 40 μl of 20 mg/ml X-gal and 4 μl of 200 mg/ml IPTG to LB+Ampicillin plates. After 45 minutes, the cells were heated at 42C for 2 minutes and then placed back on ice for 30 minutes. To help the cells start growing again, 500 μl of LB broth was added and the cells incubated at 37C for 35 minutes. The tube was briefly centrifuged, the supernatant removed to approximately 110 μl, and then 10 or 100 μl was plated onto the antibiotic resistant plates.

White (positive) colonies were picked and grown in a liquid culture of 2 ml LB borth and 4 μL 10 mg/ml ampicillin for 10-16 hours. To check for the PCR insert, 500 μl of liquid culture was removed, centrifuged at 13200 rpm for 1 minute at room temperature, and the supernatant removed. The pellet was resuspended in 100 μl ddH_20, heated at 95C for 5 minutes and 1 μl was used as a PCR template. Once positives were observed, they were grown overnight in liquid culture again and then mini-preps were done to isolate the DNA. From a 2 ml overnight culture, 1.7 ml is transferred to a new tube and centrifuged at room temperature for 1 minute at 13200 to pellet cells. The supernatant was aspirated, 100 μl of lysosyme buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) was added and vortexed vigorously until the pellet was fully resuspended. Then 200 μl of fresh alkaline 1% SDS (0.2N NaOH, 1% SDS) added and inverted to mix, followed by 150 μl of 3M KOAc, pH 6.0 and again inverted several times. The tubes were centrifuged for 5 minutes at room temperature and the supernatant was moved to a new tube. To the supernatant, I added ice cold 95% ethanol to the top of tube and mixed it completely. After this sat at –20C for a minimum of 15 minutes, it was centrifuged at 13200 for 5 minutes at room temperature. The ethanol supernatant was removed and the pellet resuspended in 100 μl TE. Another 200 μl ice-cold 95% ethanol was added, mixed well, and sat on bench for 5 minutes. After another 5 minute centrifuge at room temperature, the ethanol supernatant was removed completely, both by aspiration and air-drying, and the pellet was resuspended in 50 μl ddH_20.

The PCRs were repeated with the original primer pair to check for the correct insert. To determine the orientation of the insert, single primers were paired with primers for the flanking T3 and T7 sites. If an insert was sense to T3, amplification products were seen with the T3 primer and the 3' original primer and with the T7 primer and the original 5' primer.

2.8.2 RNase Protection Assay II (RPA)

RPA analysis was performed with Ambion's RPA II kit, following the manufacturer's directions. Plasmids were purified with the Qiagen mini-prep kit, digested with Gibo-BRL restriction enzymes: BamHI (*TIMP1*) or XhoI (*MIC2*) for a linear template. 1 μg was used for *in vitro* transcription with either T7 (TIMP1) or T3 (MIC2) polymerase (Gibco-BRL) with ^{32}P-UTP. To remove template DNA that may interfere with RNA-RNA hybridization, the labelled probe was denatured (95° - 5 minutes, ice – 5 minutes) and then incubated with 2 units DNase (Epicenter) at 37° for 15 minutes and the enzyme heat-killed at 95° for 5 minutes. To ensure the probes were in excess, an initial RPA was performed with 10 μg of RNA and varying amounts of probe. The probe was not limiting when the intensity of the fragments did not increase by adding more of the probes and therefore the amount of target RNA determined the amount of the protected fragment. After solution hybridization overnight of excess antisense radiolabelled probe to 10 μg of total RNA, any remaining unhybridized probe and sample RNA were removed by RNase digestion. The hybridized product was separated on a native 5% polyacrylamide gel and visualized by autoradiography. To quantify the *TIMP1* fragment, the intensity of the protected *TIMP1* fragment was quantified by phosphoimager (BioRad FX) and compared to the intensity of the band detected for *MIC2* used to control for the amount of input RNA. *MIC2* is expressed from both the active and inactive X chromosomes at comparable levels (Goodfellow et al. 1984) and therefore controls for the number of X chromosomes because the hybrids may have more than one X chromosome (*TIMP1* level = *TIMP1*/*MIC2*). Humans have two expressing copies of *MIC2* for every active copy of *TIMP1* so the *MIC2* band should be twice as intense and therefore, the *TIMP1* strength was determined with (*TIMP1* intensity/0.5**MIC2* intensity). All RPA results were normalized to one stock RNA to decrease variability between gels.

2.9 Nuclease sensitivity

The protocol for nuclease sensitivity was adapted primarily from Gregory and Feil (1999). The hybrids were grown to 75-80% confluent on 60 mm plates before harvesting with trypsin-EDTA (0.25%). The cell pellets were resuspended in 300 µl ice-cold DNase buffer (0.3M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, and 15 mM Tris-HCl pH 7.5). The suspensions were then split into 6 tubes of 50 µl each and another 50 µl of ice-cold DNase buffer with 0.4% Nonidet was added. The tubes were mixed gently and placed on ice for approximately 4 minutes, no longer than 5 minutes. Then 100 µl of freshly diluted DNAse was added: 2.5 U, 1 U, 0.5 U, 0.25 U, 0.1 U and no enzyme and then incubated at 25C for 5 minutes, followed by a 95C heat kill for 15 minutes. To start isolating the DNA, 4 µl of 20% SDS, 8 µl of 0.5M EDTA, and 4 µl of 10 mg/mL proteinase K was added and this was left on the lab bench overnight and a phenol/chloroform extraction was performed the next day. After the DNA was quantified with spectrophotometry, it was diluted to 60 ng/µl, 12 ng/µl and 4 ng/µl and 1 µl was used for PCR templates.

2.10 Chromatin Immunoprecipitation Assay

The hybrid fibroblast cells were harvested with 5 drops of 0.25% trypsin for 2 minutes and then washed in 1x PBS. One ml of 0.37% formaldehyde in alpha media was added to the pellet within a 1.5 ml tube and incubated at 37C for ten minutes. From this point onwards, the cells were kept on ice as much as possible to prevent denaturation of the proteins. The cells were washed 2x with 1/100 proteinase inhibitor cocktail (Sigma) diluted in 1x PBS and then centrifuged at 2500 rpm for 4 minutes at 4C. The pellet was resuspended in 200 µl SDS lysis buffer with 1/100 proteinase inhibitor cocktail and placed on ice for 10 minutes. To help with break up the cells, the suspension was repeatedly drawn up a 25 gauge needle with varying expulsion force. It

was first expelled quickly, then briefly centrifuged to decrease foam. Then, it was expelled slowly and then again, expelled quickly before a brief centrifugation. This was repeated with a slow expulsion and then a forceful release before a final brief centrifugation. The cells were then sonicated for 7 pulses of 10 seconds each at 50% power. There was a one minute wait between pulses to keep the temperature low and to decrease the foam.

The remainder of the protocol follows a kit from Upstate Biochemicals and most of the reagents were supplied ready to use. The suspension was then centrifuged at 13000 for 10 minutes at 4C. The supernatant (~200 µl) was removed to a new tube and 1.8 ml ChIP dilution buffer and 20 µl proteinase inhibitor cocktail were added. One percent of the solution (20 µl) was removed to check the amount of input DNA by spectrophotometry. To the supernatant and buffer, 80 µl of salmon sperm DNA/Agarose slurry was added and mixed on a rocker for 30 minutes at 4C on setting 6. The tube was briefly centrifuged (1000 rpm for 10 seconds) and the supernatant split into two tubes. One tube was the no antibody control but the other tube had 10 µg of the anti-acetylated histone H3 antibody added. Both tubes were kept at 4C overnight with slow mixing to allow hybridization.

The next morning, 60 µl of salmon sperm/agarose slurry was added and then the tubes were mixed gently for one hour at 4C. The elution buffer was prepared at this point (50 µl 20% SDS, 100 µl 1M $NaHCO_3$, 850 µl ddH_20). The following washes were performed, removing the supernatant after each spin:

a. 1 ml Low Salt Immune Complex, 4 minutes rocking at 4C

b. 1 ml High Salt Immune Complex, 4 minutes rocking at 4C

c. 1 ml LiCl Immune Complex, 4 minutes rocking at 4C

d. 1 ml 1x TE buffer, 4 minutes rocking at 4C

e. 1 ml 1x TE buffer, 4 minutes rocking at 4C

After the last wash, 250 µl of elution buffer was added and the tube vortexed briefly and then mixed slowly on the rocker for 15 minutes at room temp, setting 7. The tube was then briefly centrifuged, the supernatant removed, and centrifuged again briefly with any remaining supernatant moved to the same tube. Adding 20 µl 5M NaCl and incubating at 65C for 4 hours then reversed the protein crosslinks. Then 10 µl 0.5M EDTA, 20 µL Tris-HCl, and 2 µl of 10 mg/ml proteinase K were added and the sample was incubated at 45C for one hour. A phenol/chloroform extraction was performed to isolate the DNA, adding 20 µg of glycogen as a carrier to help precipitate the DNA. The pellet was resuspended in 25 µl TE and quantified by spectrophotometry. The DNA was diluted to 20 ng/µl for PCR reactions in the linear phase.

3 VARIABLE X INACTIVATION OF THE HUMAN *TIMP1* GENE

3.1 Introduction

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). Although the majority of X-linked genes are believed to be subject to the dosage-compensation mechanism of X inactivation, over 15% of X-linked genes escape this silencing and are expressed from both the active and Xi chromosomes (Carrel et al. 1999). 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 Willard et al. 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 Xi 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 (Fisher et al. 1990; Migeon 1972; Schneider-Gadicke et al. 1989). Surprisingly, some genes are expressed in some but not all of the Xi-containing hybrids (Brown et al. 1997). One of the genes showing this variable expression is the human tissue inhibitor of metalloproteinases-1 (*TIMP1* [MIM 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 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 Xi chromosome behave more like the active chromosome, replicating earlier and retaining more acetylation of histones than does the remainder of the inactive X chromosome (Jeppesen and Turner 1993; Schempp and Meer 1983). Human *TIMP1* is in a gene-rich region containing both *ARAF1* and *ELK1* within 100 kb (Brandau et al. 1998; Coleman et al. 1994; Derry et al. 1995; Knight et al. 1994). I therefore assessed whether this entire region was expressed from the Xi 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 (Ellis et al. 1987; Kahan and DeMars 1975), 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 Xi 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 female cells heterozygous with respect to the *TIMP1* gene previously shown to have extreme skewing of X inactivation.

3.2 Results

3.2.3 Expression of TIMP1 and Flanking Genes in Hybrid Cells

Expression of human *TIMP1* was examined in six active X- and eight Xi-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 3.1). To determine if the expression from the inactive X chromosome extended beyond *TIMP1,* I analyzed the expression of genes surrounding *TIMP1,* within the gene-rich region of Xp11.23 (Fig. 3.1A) (Brandau et al. 1998; Coleman et al. 1994; Derry et al. 1995; Knight et al. 1994). RT-PCR was performed with primers for *ARAF-1* (a raf-related gene located 20 kb distal to *TIMP1*) and *ELK1* (a transcription factor and proto-oncogene 50 kb proximal to *TIMP1*). The two zinc-finger genes (*ZNF41* and *ZNF157*) were thought to be located <100 kb from *TIMP1* but subsequent sequencing results have placed these genes 100 kb (*ZNF41*) and 170 kb (*ZNF157*) further away (contig NT_011584). Although *TIMP1* is found within intron 5 of *SYN1* (Derry and Barnard 1992), *SYN1* expression was not examined, as 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 3.1B and as summarized in Table 3.1, *ARAF1, ELK1, ZNF41,* and *ZNF157* were expressed only in hybrids that retained a human active X chromosome. No expression of these genes were observed in the Xi-containing hybrids, including those in which *TIMP1* expression had been detected.

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

When *TIMP1* cDNA sequences in the GenBank DNA-sequence repository were compared, a single base pair polymorphism (SNP) was found, eliminating the need for the traditional labour-intensive methods of mutation detection and sequencing. A T → C

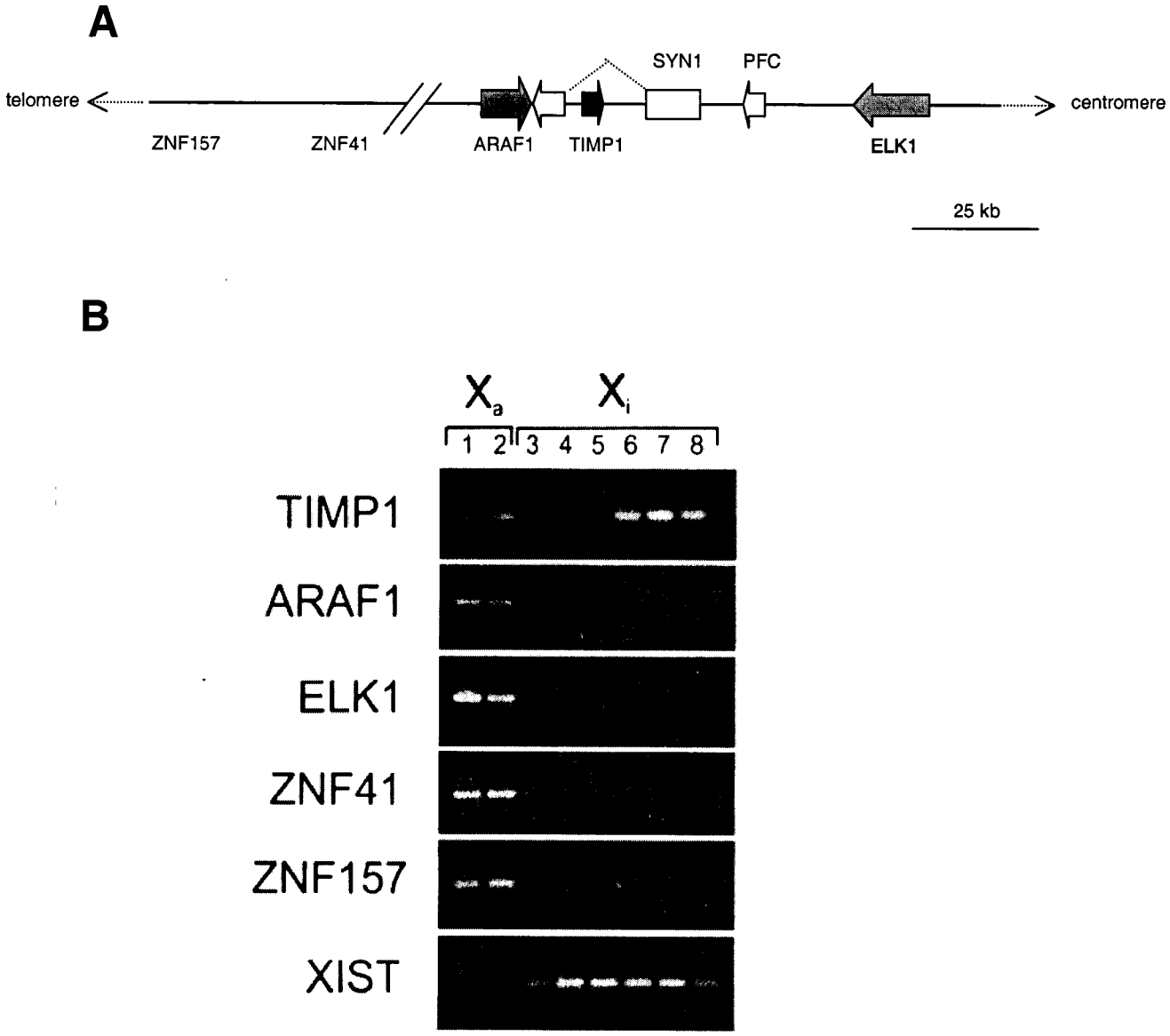

Figure 3.1. Expression analysis of genes in Xp11.23.

A, Schematic map of genes flanking *TIMP1*. The *ZNF41* and *ZNF157* genes were originally thought to be closer to the *TIMP1* gene but sequencing efforts have places them >100kb away. The *SYN1* and *PFC* genes are not expressed in fibroblasts and therefore could not be examined in the hybrid system. **B**, Products from amplification of cDNA derived from the hybrids, tested with human-specific primers for the genes shown. Hybrids retaining the active X chromosome (Xa) or inactive X chromosome (Xi) are denoted as follows (numbers represent 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-O7.

Hybrid Cell Line	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	+	-	-	-	-	+
t75-1maz-34-1a	+	-	-	-	-	+
CHO-01416-O7[a]	+	-	-	-	-	+

Table 3.1. Expression of the five genes in various somatic cell hybrids.

[a] Hybrid with a hamster parental line.
[b] A plus sign (+) denotes presence of expression and a minus (-) sign represents no expression.

base-pair change in exon 5 was observed in 4 of 15 compared sequences. Although this polymorphism is part of the coding region, the transition is silent. This polymorphism has been independently identified (Hardcastle et al. 1997). I initially determined that this base pair change discovered *in silico* was a common variant in the human population by analyzing human cell lines with a PCR-digestion assay. TIMP-CA primers were designed to flank the polymorphic base and because there was no natural restriction enzyme site near the SNP, the primers incorporated a mismatch base to artificially create a BstU1 recognition site dependent on the polymorphism. To distinguish the C and T alleles, PCR products were digested with BstU1, which would only cleave when the C variant was present (Fig. 3.2A). When 5 human cell lines were examined, three heterozygous females and two males with either the C or T allele were observed (Fig. 3.2B) confirming that the base pair is polymorphic in the human population. The TIMP-CA primers amplify cDNA and they could have been used to analyze expression in heterozygous females but the BstU1 enzyme does not cut when a heteroduplex is present at its recognition site. If the C and T allelic sequences annealed in a heterozygous female, no digestion would occur, inflating the presence of the upper (T) allele, which may obscure low level expression of the C allele. To eliminate this technical concern, I designed a PCR allele-specific amplification (PASA) assay to analyze *TIMP1* expression in human females.

The PASA assay (Fig. 3.3) consisted of a constant primer and two allele-specific primers and 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 the DNA from homozygous females in different ratios (Fig. 3.3B), and an allele present in ≥10% of the total DNA was consistently detected (Fig.

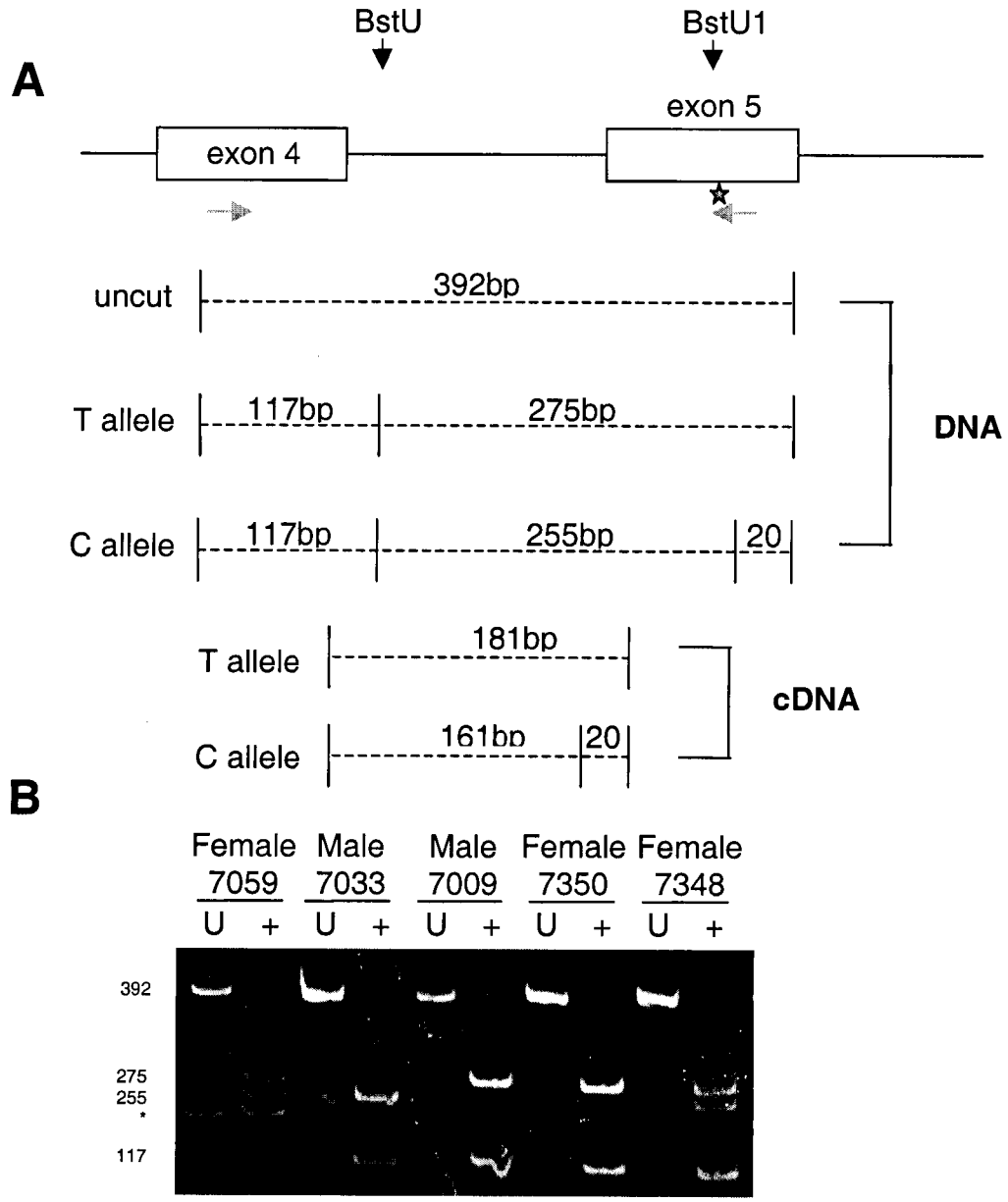

Figure 3.2. *TIMP1* PCR-digestion assay and results.

A, Schematic outline of the PCR-digestion assay. Oligonucleotide primers were designed with a mismatch to create a restriction enzyme site when the C allele was present at the polymorphism (star). PCR amplification yielded a 392 bp fragment that was then digested with BstU1. There was a second BstU1 site within the intron, providing a control for complete digestion. If the C allele was present, the lower (255 bp) band was observed while the upper band indicated the presence of only the T allele. A heterozygous female would have both the upper and lower bands. To check expression, the primers amplify cDNA, leading to smaller fragments because the primers flank an intron. **B**, TIMP1 alleles in the DNA of 5 lymphoblast cell lines. Both the 7059 and 7348 females were heterozygous as the upper and lower bands are seen after digestion. The 7350 female was homozygous for the lower (C) allele and the 7033 and 7009 hemizygous males had the C and T allele respectively. There was an artefact band from PCR amplification (*) in several lanes but it was a different size and did not interfere with typing the alleles.

3.3B). The different alleles had almost equal frequencies (136 C and 141 T in 277 X chromosomes tested) in a sample of unrelated human females, males, cell lines, and somatic-cell hybrids (Table 3.2 and Appendix A).

Female cell lines with previously demonstrated extreme skewing of X inactivation were used to assess expression of *TIMP1* from the inactive X chromosome. 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 (Plenge et al. 1997; Rupert et al. 1995); or lymphoblasts derived from normal females (in GM07059 and GM07348), which may have become clonal either during derivation or in culture (Migeon et al. 1988b). 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. 1995)) or methylation differences near a polymorphic site (androgen receptor (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.

To determine if *TIMP1* was expressed from the Xi in human females, I analyzed these females with non-random X inactivation patterns with the PASA assay. As shown schematically in Figure 3.3, 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 3.3C: panels 1 and 2 show the results of analysis of homozygous females, demonstrating the specificity

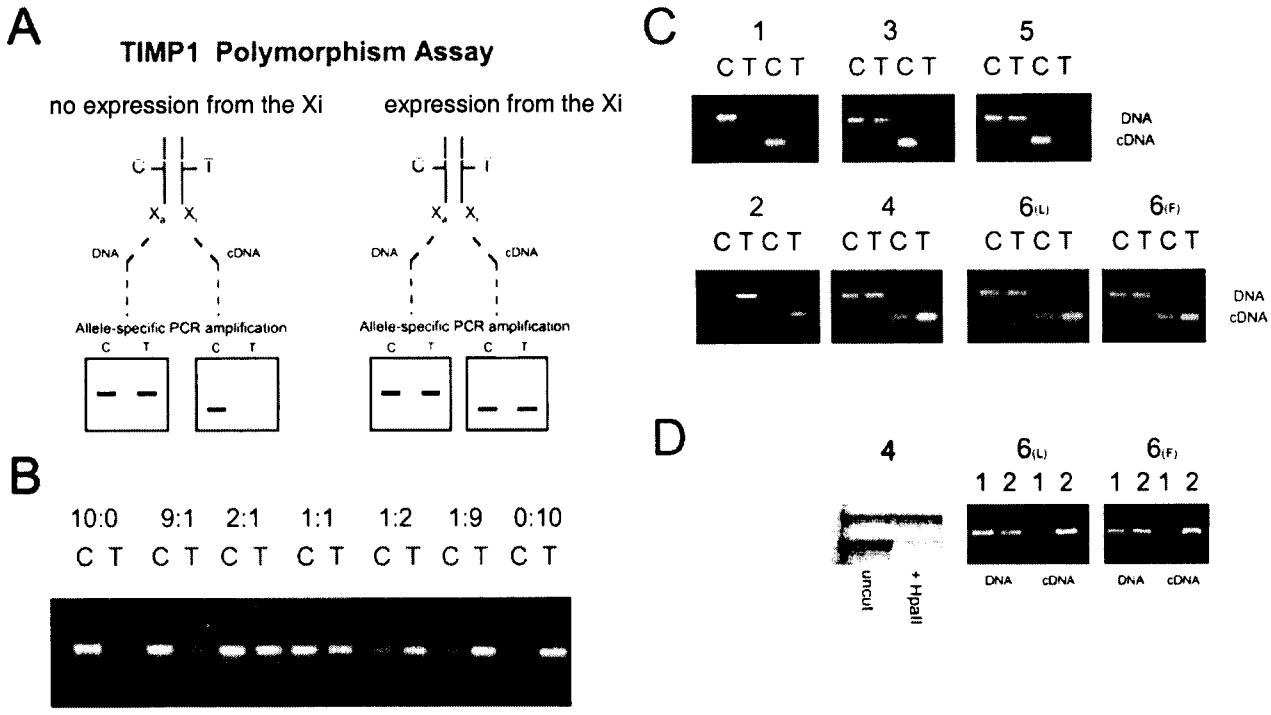

Figure 3.3. *TIMP1* polymorphism assay and results.

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.

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 3.3D. For sample 4, 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 3.3D, the female was heterozygous for *XIST,* as has been described elsewhere (Plenge et al. 1997; Rupert et al. 1995). In cDNA, only one allele was amplified, establishing that the same X chromosome was active in all cells. In the initial analysis, *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 3.3).

	Count	Frequency
C allele	136	49%
T allele	141	51%
expected female heterozygosity		50%
observed female heterozygosity	89	48%

Table 3.2. The allele frequencies of the *TIMP1* polymorphism.

Two hundred and seventy-seven X chromosomes were typed with the TIMP1 PASA assay. The genotypes for all individuals examined are listed in Appendix A.

Individual	Cell Type	*TIMP1* Alleles	
		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

Table 3.3. Expression of *TIMP1* in human female cells (published).

The [symbol indicates that these two cell lines were derived from differing tissues in the same individual.

Female cell lines with extreme skewing of X inactivation continued to be tested for *TIMP1* expression from the inactive X chromosome (Table 3.4). Wiskott-Aldrich syndrome (WAS [MIM 301000]), is an X-linked recessive (Xp11.23-p11.22) immunodeficiency characterized by recurrent infections, thrombocytopenia and eczema that is caused by mutations in the WAS protein (*WASP*) gene (Lemahieu et al. 1999). Female carriers have extreme skewing of X inactivation in all peripheral blood cells, presumably due to early selection against cells that express the mutant *WASP* (Puck et al. 1990; Wengler et al. 1995). Twelve lymphoblast cell lines derived from WAS carrier females, which included 4 pairs of first-degree relatives were analyzed. None of these individuals expressed *TIMP1* from the Xi, dropping the frequency to 19% (3/16).

Individual	Cell Type	*TIMP1* Alleles	
		DNA	cDNA
4023]	Cultured lymphoblasts	C + T	C
2443]	Cultured lymphoblasts	C + T	C
JS4190]	Cultured lymphoblasts	C + T	T
JS4194]	Cultured lymphoblasts	C + T	T
3411]	Cultured lymphoblasts	C + T	C
3554]	Cultured lymphoblasts	C + T	C
3096]	Cultured lymphoblasts	C + T	T
3098]	Cultured lymphoblasts	C + T	T
3149	Cultured lymphoblasts	C + T	C
2659	Cultured lymphoblasts	C + T	T
3251	Cultured lymphoblasts	C + T	T
3757	Cultured lymphoblasts	C + T	T

Table 3.4. Expression of *TIMP1* in additional female cell lines from WAS carriers.

The] symbol indicates that these cell lines were derived from the blood of first-degree relatives.

3.3 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 human-hamster hybrids (Table 3.1). 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. I postulated that the *TIMP1* expression from the inactive chromosome might be restricted to the hybrid system, because reactivation (sporadic expression from the inactive X chromosome) of other genes is observed at an elevated frequency in hybrids (Gartler and Goldman 1994; Kahan and DeMars 1975). *TIMP1* expression from the inactive X chromosome was therefore investigated by examination of heterozygous 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 initially observed in three of the eight informative human females and cell lines analyzed (Table 3.3). This frequency is similar to that observed in the hybrids, and, in the two cases in which human cell lines and the Xi-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, because variable inactivation has been described in females with choroideremia (Carrel and Willard 1999). 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.

After the initial frequency of *TIMP1* expression from the Xi was published, 12 more female lymphoblast cell lines were analyzed and no additional individuals with expression from the Xi were observed, dropping the frequency to 19% (Table 3.4). The additional cell lines were all derived from Wiskott-Aldrich syndrome carriers but mutant *WASP* does not prevent *TIMP1* expression from the Xi because patient 07 in the published results was also a WAS carrier. In comparison to the initial set of cell lines examined, the new lines were all lymphoblasts so there could be a reduced frequency of variable X inactivation in cultured lymphoblasts. However, *TIMP1* expression from the Xi was concordant between lymphoblasts and fibroblasts from one individual, suggesting that cultured lymphoblasts are not strongly protected from variable X inactivation. Although it is possible that expression from the Xi does occur more often in the hybrid system, more Xi-containing hybrids have to be examined because the hybrid frequency could also drop. It may have been fortuitous that *TIMP1* expression from the Xi was observed more than once in both the initial eight hybrids and eight human female cell lines. The small number of samples will have to be increased to determine an accurate frequency of expression from the inactive chromosome.

The mechanism leading to *TIMP1* expression from the Xi is unknown. 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, suggesting 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 (Giannoukakis et al. 1996; Jinno et al. 1995). 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, as 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 should 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 15% - 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 I 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 (Brown and Rastan 1988; Cattanach 1974; Wareham et al. 1987), but long-term culture of Xi-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. 1988a), but *HPRT* may be strongly silenced due to the presence of a dense CpG island and therefore 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 (Kaslow and Migeon 1987; Migeon et al. 1985), 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. There are no predicted CpG islands (CpG island finder, EMB) near the *ALD* and *REP1* genes also shown to be variably inactivated (Carrel et al. 1999), suggesting that they do not have strong CpG islands. 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. In subsequent chapters, I will discuss the analysis of epigenetic factors normally associated with the inactive X chromosome, such as methylation (chapter 4), chromatin structure and acetylation of histone H3 (chapter 5).

Whatever the mechanism allowing *TIMP1* expression from the inactive X chromosome, I anticipate that expression levels will vary between individuals, because *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 (Carrel et al. 1996; Migeon et al. 1982; Sheardown et al. 1996). Since TIMP1 and its target metalloproteinases are widely expressed proteins involved in a variety of processes, elevated TIMP1 may be clinically significant. This is the subject of study in chapter 4.

4 EFFECT OF VARIABLE X INACTIVATION ON *TIMP1* RNA LEVELS AND ROLE OF DNA METHYLATION

4.1 Introduction

The majority of the genes on one of the two X chromosomes is transcriptionally silenced, however, a substantial number of genes escape X inactivation and are expressed from both the Xa and the Xi (Carrel et al. 1999). In addition, some genes have been shown to have variable X inactivation, being expressed from both X chromosomes in some females but subject to X inactivation in others (Anderson and Brown 1999; Carrel and Willard 1999). Of the genes that escape X inactivation, some have homologues on the Y chromosome, and thus continue to maintain dosage equivalence between males and females, but for those genes without a Y homologue, the expression from the Xi may generate dosage differences between the sexes (reviewed in Disteche 1999). For a gene that is variable in its inactivation status, expression levels may be different amongst females as well as between males and females.

The human *TIMP1* gene is variably expressed from the Xi, as demonstrated in the previous chapter with an allele-specific PCR assay to examine expression in heterozygous women with extreme skewing of X inactivation (Anderson and Brown 1999). Because variations in the MMP-TIMP balance are observed in pathological conditions, it is surprising to see discrepancies among women in their inactivation of *TIMP1*. However, it can not be assumed that women expressing *TIMP1* from both X chromosomes will have twice as much *TIMP1* RNA, as the expression levels from genes on the Xi are often lower than those on the Xa (*e.g.* (Carrel et al. 1996; Migeon et al. 1982), perhaps due to the surrounding facultative heterochromatin state. The Xi acquires many features of heterochromatin, including hypermethylation, nuclease

insensitivity, delayed replication timing and hypoacetylation of histones (reviewed in Avner and Heard 2001). These features are believed to maintain the stable silencing of the chromosome. Genes escaping X inactivation are often located in clusters along the X chromosome, suggesting that some of these factors are acting regionally (Disteche 1999; Tsuchiya and Willard 2000). These regions that escape inactivation behave more like the active chromosome, lacking promoter methylation (Goodfellow et al. 1988; Yen et al. 1984), replicating earlier in the cell cycle (Schempp and Meer 1983) and retaining histone acetylation (Jeppesen and Turner 1993) compared to the rest of the Xi chromosome. However, the variable expression from the Xi in the *TIMP1* region was limited to *TIMP1* alone, as two flanking genes, *ARAF1* and *ELK1*, were expressed only from the Xa even when *TIMP1* expression was observed from the Xi (Anderson and Brown 1999).

To determine whether women expressing two copies of *TIMP1* have increased *TIMP1* mRNA, I analyzed expression levels in male and female cell lines. Surprisingly, there was considerable variation in *TIMP1* expression from cells with monoallelic expression. The diverse expression levels precluded analyzing the contribution from the Xi to total *TIMP1* RNA in females, so I examined expression in rodent/human somatic cell hybrids. The human X chromosome retains its original X-inactivation status in such hybrids (Migeon 1972), and because the Xa and Xi can be studied independently, hybrids have been a valuable tool to assess the inactivation status of X-linked genes (*e.g.* Brown et al., 1997). *TIMP1* expression levels varied more widely in hybrids retaining the Xi than in those with an Xa, perhaps reflecting residual features associated with X inactivation. The gene-specific nature of *TIMP1* expression was suggestive of promoter-related features, so I have studied whether methylation, a feature of inactive chromatin that is correlated with transcriptional repression (Jones and Wolffe 1999),

might be altered when *TIMP1* is expressed from the Xi. Understanding what allows some genes to be expressed from an otherwise inactive X chromosome will provide clues about the hierarchy of epigenetic factors that normally result in the stable inactivation of an X chromosome. Furthermore, variable expression of genes from the Xi in some females may alter expression levels sufficiently to result in different susceptibilities to disease.

4.2 Results

4.2.5 Analysis of TIMP1 expression levels

To quantify the amount of *TIMP1* RNA, Northern analysis was initially performed. The results were concordant with previous RT-PCR analysis and there appeared to be expression level differences in the various cell lines relative to mActin, which was used to control for the amount of input RNA (Figure 4.1). However, technical problems prevented consistent results so further analysis was performed using the more quantitative RNase Protection Assay (RPA).

RPA analysis was performed with Ambion's RPA II kit, following the manufacturer's directions. After overnight solution hybridization of excess antisense radiolabelled probe to 10 μg of total RNA, any remaining unhybridized probe and sample RNA are removed by RNase digestion. The hybridized product was separated on a native 5% polyacrylamide gel and visualized by autoradiography (Fig. 4.2A). Inserting PCR products into the pKRX vector generated *TIMP1* and *MIC2* probes. To ensure that the probes were in excess, an initial RPA was performed with 10 μg of RNA and 8-80 ng (TIMP1) or 11-290 ng (MIC2) of probe. No change in the intensity of the fragments was observed above 10 ng (TIMP1) and 25 ng (MIC2) of probe (Fig. 4.2B) and therefore the amount of target RNA would determine the amount of the protected fragment.

The intensity of the protected *TIMP1* fragment was quantified by phosphoimager and compared to the intensity of the band detected for *MIC2* used to control for the amount of input RNA. *MIC2* is expressed from both the active and inactive X chromosomes at comparable levels (Goodfellow et al. 1984) and therefore controls for the number of X chromosomes (*TIMP1* level = *TIMP1*/*MIC2*). Because humans have two expressing copies of *MIC2* for every active copy of *TIMP1*, the *MIC2* band should be twice as intense and therefore, the *TIMP1* strength was determined with (*TIMP1*

intensity/0.5*_MIC2_ intensity). The RPA was repeated using mouse Actin as a control for levels of RNA and similar results were observed (Fig. 4.3), confirming that the variability observed was due to different amounts of _TIMP1_ RNA, not _MIC2_. All RPA results were normalized to one stock RNA to decrease variability between gels. While the variability between replicates of the same RNA preparation was 7.4%, different RNA preparations from the same cell line showed a larger variability (average of 14%), which I attribute to cell cycle differences, as the cells used were not synchronised.

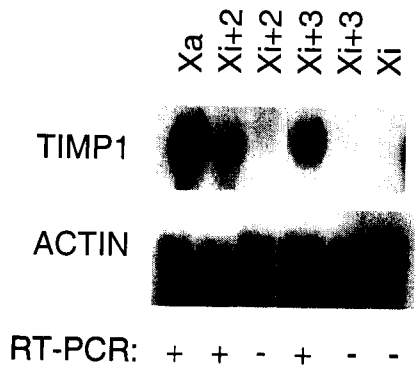

Figure 4.1. Northern Analysis in hybrid cell lines.

The bands observed after hybridization with a _TIMP1_ cDNA probe are shown in the top panel and the amount of input RNA is controlled by the Actin probe. The expression status corresponds to the _TIMP1_ expression determined by RT-PCR, shown along the bottom. The hybrid cell lines are as follows: Xa = AHA-11aB1, Xt2 = t86-B1maz1b-3a with _TIMP1_+ and _TIMP1_- subclones, Xt3 = t81az1D with _TIMP1_+ and _TIMP1_- subclones, and Xi – t48-1a-1Daz4a.

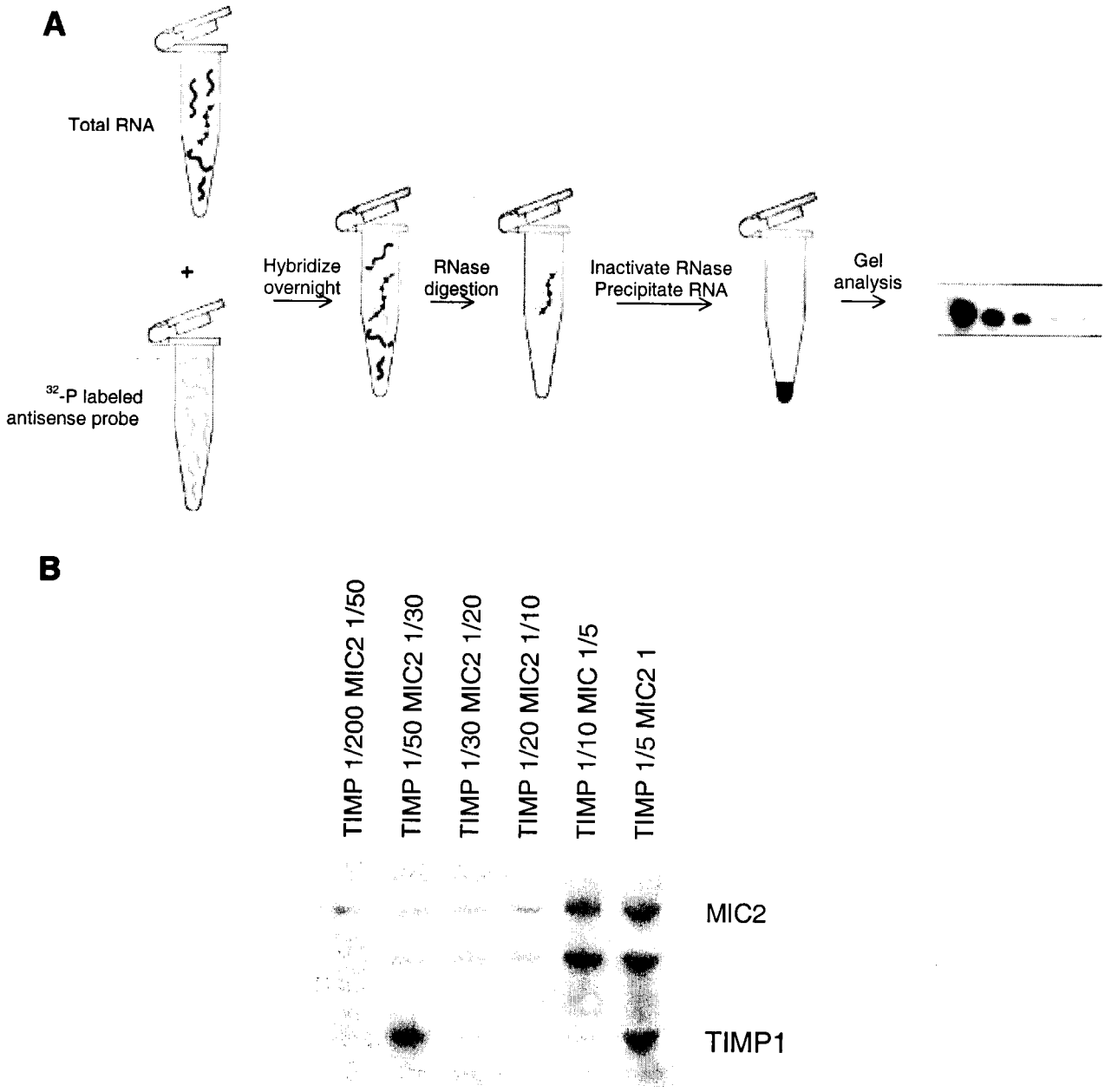

Figure 4.2. RNase Protection Assay.

A Schematic drawing of the technique adapted from the Ambion website (www.ambion.com/basics/rpa/101_index.html). **B** Increasing amounts of probe added to 10 μg of AHA-11aB1RNA. The protected *TIMP1* fragment is the bottom band and the top band is the *MIC2* control for equal amounts of input RNA. The middle band is an artefact that consistently appears with the *TIMP1* probe. *MIC2* bands did not increase in intensity after the 1/50 dilution although more background appeared. The *TIMP1* bands did not increase significantly above the 1/50 dilution. The strong intensity of the *TIMP1* band in the last lane (1/5 dilution) may be due to increased background and/or ethanol present in the sample, which can create misshapen bands.

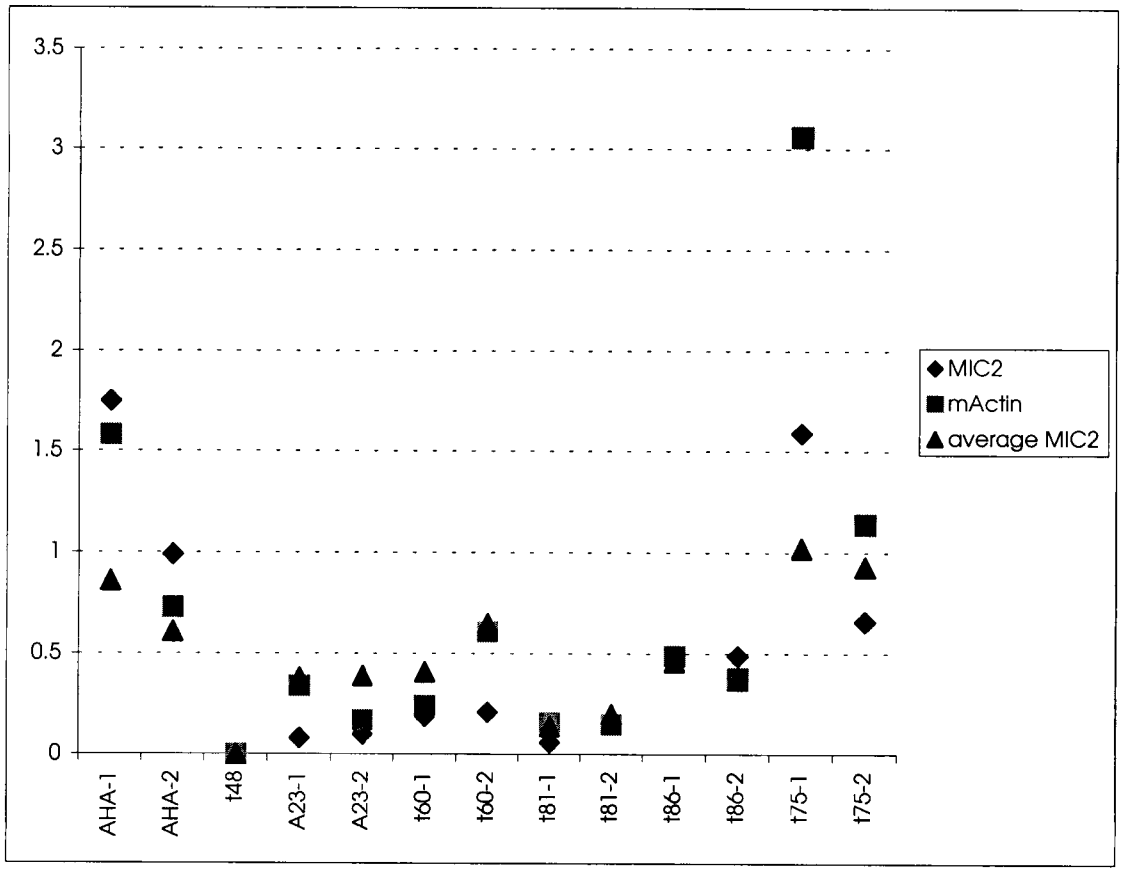

Figure 4.3. Comparison of normalized *TIMP1* levels with two different controls.

TIMP1 expression levels in hybrid cell lines were calculated after comparing to different controls for the amount of input RNA. Each cell line expressing *TIMP1* was analyzed twice, shown by the 1 or 2 following the cell line name. The t48 cell line did not express *TIMP1* by RT-PCR so RPA was only performed once. Xa hybrids are: AHA, A23, and t60 and the Xi+ cell lines are: t81, t86, and t75. The *MIC2* and mActin RPA were done on the same day and these represent a single gel. The average *MIC2* is the average value for each RNA prep when normalized to *MIC2* input amounts for all gels analyzed. The relative trends between the points were similar for all three classes of controls, indicating that different intensities were due to different amount of *TIMP1* RNA.

4.2.6 *Cell lines show a range of TIMP1 expression levels*

I established the levels of *TIMP1* RNA from the Xa chromosome by examining expression in either male cells or female cells previously demonstrated to express *TIMP1* from only the Xa (Anderson and Brown 1999). In these cell lines, there was a 1.7 fold range of expression (Fig. 4.4 and Table 4.1). This variability was appreciably greater than the differences between different RNA preparations from the same cell line, and made it difficult to determine if *TIMP1* RNA levels were elevated in females expressing two copies of *TIMP1*. While the RNA level was increased in HSC593, a cell line previously demonstrated to express *TIMP1* from the Xi, this difference was not significant ($p=0.2$). Because the allele-specific PCR assay used to identify cells expressing *TIMP1* from the Xi requires a heterozygous female sample with non-random X inactivation, it is difficult to obtain informative female samples. Therefore, I also examined RNA levels in female cell lines whose X inactivation status could not be established with our PCR assay because they were homozygous for the *TIMP1* polymorphism (Anderson and Brown 1999). One cell line (GM07005) showed significantly higher expression ($p=0.004$) and it is tempting to suggest that this reflects biallelic expression of *TIMP1*. However, the other two cell lines of unknown X inactivation status (GM07350 and GM01416D) had expression levels within the upper range of Xa levels and may or may not express *TIMP1* from the Xi. Therefore, the variability possible in RNA levels within a cell line as well as between cell lines monoallelically expressing *TIMP1* precludes identification of cell lines with biallelic *TIMP1* expression based on RNA levels.

Diverse expression levels from the active X chromosomes could reflect sequence differences at the *TIMP1* promoter that alter transcription factor binding sites. For instance, a single nucleotide polymorphism in the MMP-1 promoter creates an Ets-

binding site and enhances expression (Rutter et al. 1998). The *TIMP1* minimal promoter contains both AP-1 and Ets sites and changes in these binding sites could modify transcription, but the promoter sequence (-122 to +130 of Clark et al. 1997) was the same between the active hybrids with the highest and lowest expression levels (Appendix B).

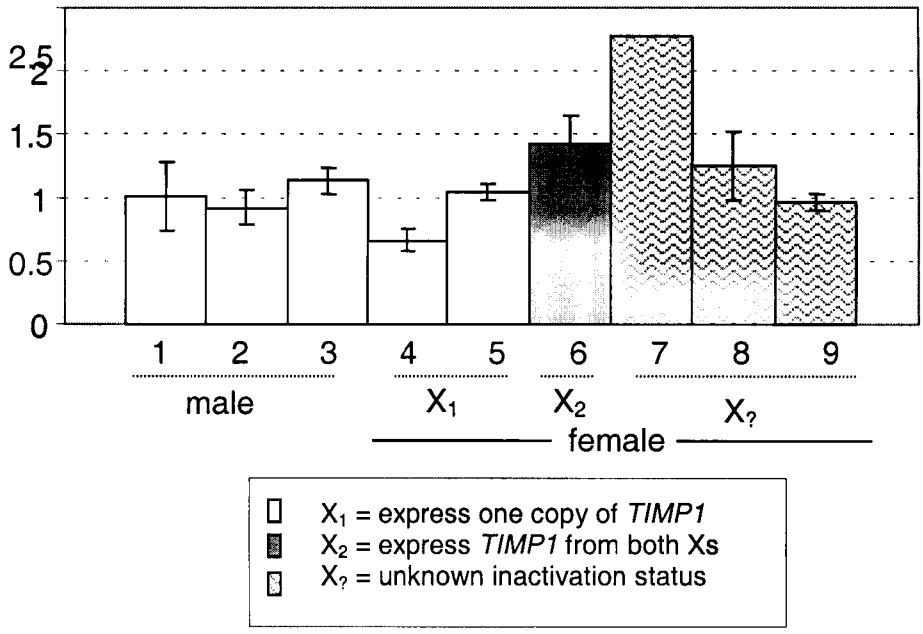

Figure 4.4. *TIMP1* expression levels in human lymphoblast cell lines.

The bars represent the average normalized value (TIMP/0.5*MIC2) of multiple analyses.

Cell Line	*TIMP1* expression[A]	Expressed allele	Level of *TIMP1*
GM07009	X_aY (1)	C	1.01 +/- 0.27
GM07033	X_aY (1)	T	0.92 +/- 0.13
GM11200	X_aY (1)	T	1.13 +/- 0.10
GM07059	$X_aX_i^-$ (1)	C	0.66 +/- 0.09
GM07348	$X_aX_i^-$ (1)	T	1.04 +/- 0.07
HSC593	$X_aX_i^+$(2)	C, T	1.43 +/- 0.22
GM07005	$X_aX_i^?$ (?)	T *	2.27 +/- 0.00
GM07350	$X_aX_i^?$ (?)	C *	1.25 +/- 0.27
GM01416D	$X_aX_i^?$ (?)	T *	0.96 +/- 0.06

Table 4.1. Normalized *TIMP1* expression levels in human lymphoblast cell lines.

[A]The number of alleles expressing *TIMP1* is shown in brackets; some of the females have previously been demonstrated to have mono-allelic (Xi^-) or biallelic (Xi^+) expression, while for females who are homozygous (marked by asterisk), it is unknown if there is expression from the Xi ($Xi^?$)

Diverse *TIMP1* RNA levels prevented assessing the contribution of the Xi to total *TIMP1* levels so I analyzed expression in rodent/human somatic cell hybrids retaining either the human Xa or Xi. The expression levels of *TIMP1* in Xa-containing hybrids were similar to the lymphoblast cell lines expressing one copy of *TIMP1* with a comparable range of expression (Fig. 4.5 and Table 4.2). Combining the data from the Xa-containing hybrid cell lines and lymphoblast cell lines expressing *TIMP1* only from the Xa, we found a range of normalized *TIMP1* expression from 0.58 to 1.13, with an average expression of 0.89, and a standard deviation of 0.20.

To determine the contribution of *TIMP1* expression from the Xi, levels of *TIMP1* RNA were determined by RPA for Xi-containing hybrids previously shown to express *TIMP1* by RT-PCR (Table 4.2). For t81-az1D (Xi+3), no expression was detected above the resolution limit for the RPA (0.25). RT-PCR can detect as little as 1/100 of the *TIMP1* expression from the Xa (Fig. 4.6) (Brown et al. 1990), thus the RNA level for t81-az1D can be estimated to be between 0.01 and 0.25 of Xa levels. Because the Xi+1 cell line

showed expression at the upper end of the Xa levels, the span of expression levels from the Xi was greater than that observed for the Xa cell lines. I hypothesised that this might reflect a residual epigenetic feature of the Xi, and thus examined methylation of the promoter region.

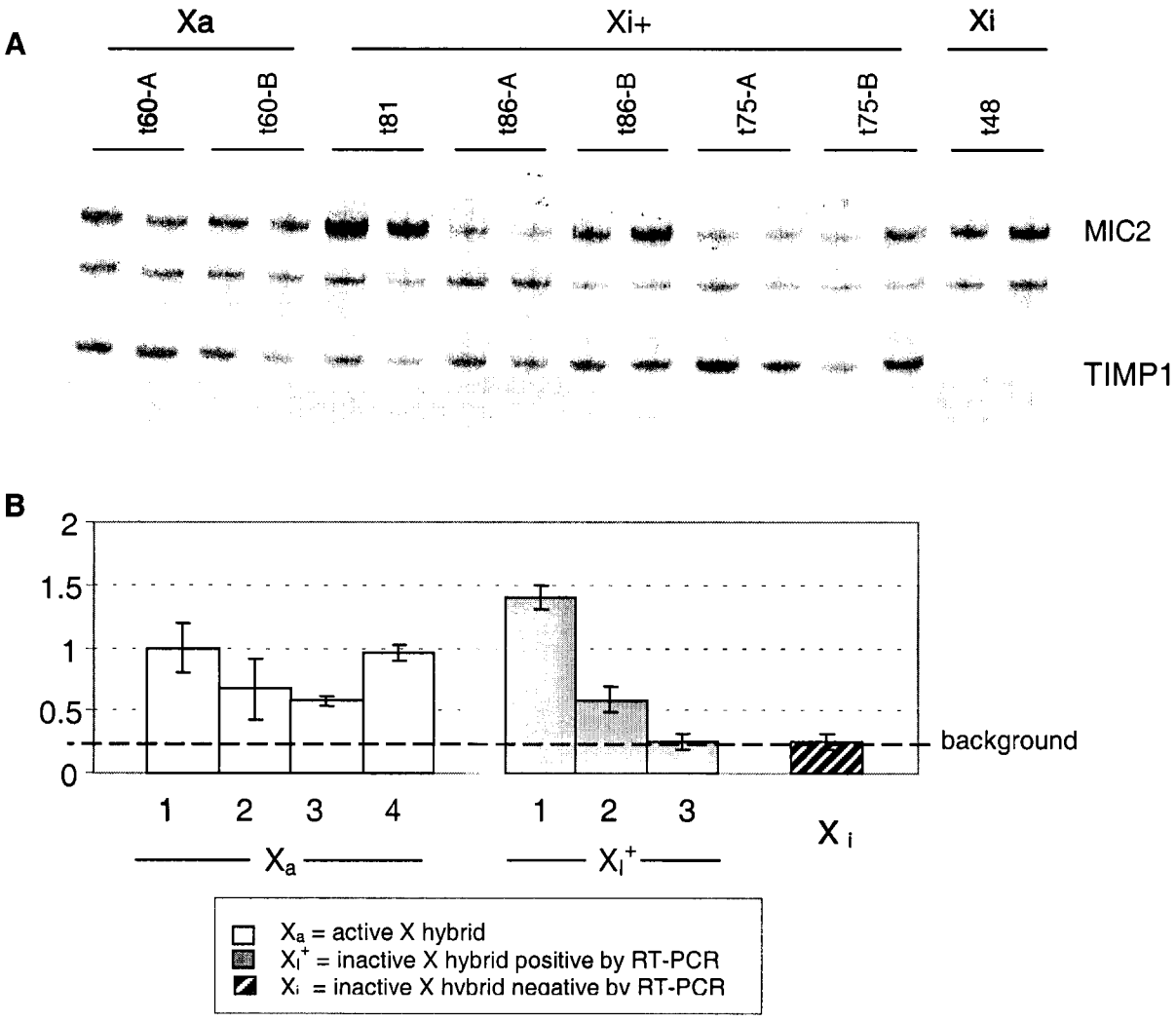

Figure 4.5. *TIMP1* expression levels in somatic cell hybrid lines.

A Representative RPA for the hybrid cell lines. Each RNA is done twice on the same gel and two different RNA preparations from the same hybrid are labelled A and B. The t81 cell line has *TIMP1* bands but the level is low compared to the strong upper *MIC2* band and is not significant above the background seen for Xi hybrids. **B** The average values obtained for each cell line. The t81 (Xi+3) cell line does have obvious *TIMP1* bands but the level of *TIMP1* is at the level of background. The dotted line represents the level of background since Xi hybrids shown to be negative by RT-PCR give this positive value.

Cell Line	X content and *TIMP1* expression	Expressed allele	Level of *TIMP1*
AHA-11aB1	X_a	T	1.00 +/- 0.20
t60-12	X_a	T	0.67 +/- 0.24
A23-1aC15	X_a	C	0.58 +/- 0.04
tHM-34-2A41B	X_a	T	0.96 +/- 0.06
t75-2maz34-1a	X_i^+	C	1.40 +/- 0.10
t86-B1maz1b-3a	X_i^+	C	0.59 +/- 0.10
t81-az1D	X_i^+	T	NS
t11-4Aaz5	X_i^-	-	NS

Table 4.2. Normalized *TIMP1* expression levels in the hybrid cell lines.

The human/rodent somatic cell lines contain either an active (X_a) or inactive (X_i) chromosome. The X_i^+ cell lines have been shown to express *TIMP1* from the X_i by RT-PCR. NS = not significant above background.

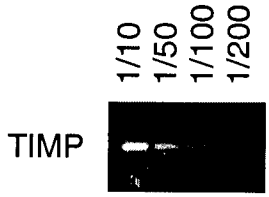

Figure 4.6. Sensitivity of the TIMP C1A:C1B primers in RT-PCR.

Five μg of RNA from AHA-11aB1 was used in a standard reverse transcription reaction. The resulting cDNA was serially diluted and the TIMP C1A:C1B primers were used to amplify this template. A band was consistently observed at the 1/100 dilution.

4.2.7 *Variable loss of methylation in X_i-containing hybrids*

The methylation status of the 5' region of *TIMP1* was determined by PCR after methylation-sensitive digestion with *HpaII* or *HhaI*, which assesses the methylation status of 4 of 11 CpG sites in the *TIMP1* minimal promoter (Fig. 4.7). If the DNA was methylated, the enzymes would not be able to cut and there would be a fragment amplified by PCR. No band after *HpaII* or *HhaI* digestion indicated that at least one of the enzyme recognition sites was unmethylated. The XIST 3':5' primers have no enzyme recognition sites and consequently control for the presence of DNA after digestion whereas amplification of the *MIC2* promoter region was used to demonstrate complete digestion.

No PCR amplification was observed after either *HpaII* or *HhaI* digestion of the Xa-containing hybrids, demonstrating a lack of methylation. PCR amplification was observed for the X_i-containing hybrids that failed to express *TIMP1*, indicating that the enzyme sites were methylated (Fig. 4.7C). However, when *TIMP1* was expressed from the inactive chromosome, various methylation states were observed. The t75-2maz34-1a hybrid (Xi+1) was unmethylated at the *TIMP1* promoter, while methylation was present in the t86-B1maz1b-3a (Xi+2) and t81-az1D (Xi+3) hybrids. *ARAF1* and *ELK1* flank *TIMP1* and were silent in all X_i hybrids studied (Anderson and Brown 1999). *ARAF1* is 5' to *TIMP1* with ~11kb of intervening DNA, while *ELK1* is ~35 kb downstream. After methylation-sensitive digestion, PCR products were observed for both genes in all X_i-containing hybrid cell lines but none of the Xa-containing hybrids (Fig. 4.7C), indicating that both the *ARAF1* and *ELK1* promoters were methylated when silent and unmethylated when expressed. *TIMP1* appears to be unique in having methylation even when expressed, however the two methylated, *TIMP1*-expressing cell lines (Xi+2 and Xi+3) showed lower levels of *TIMP1* expression. Thus we hypothesised that the

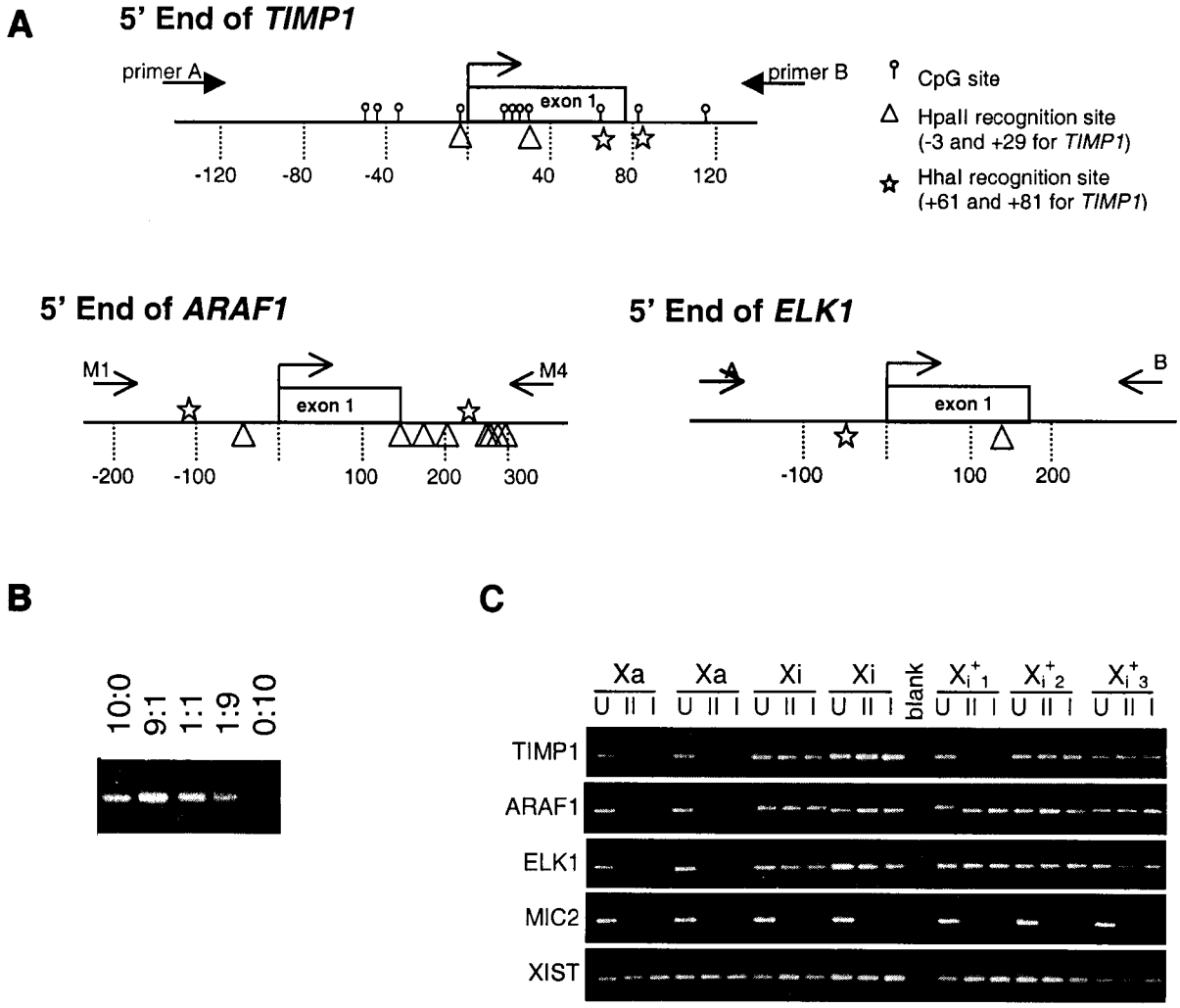

Figure 4.7. Methylation analysis in the hybrid cell lines.

A Schematic map of the 5' end of *TIMP1*. The primers flank the minimal promoter from –102 to +60 (Clark et al. 1997). The AP-1 and Ets sites required for basal transcription are found in a 38 base pair region with 37 bp 100% conserved between human, mouse, and rat. The methylation of CpG sites within *HpaII* (triangle) and *HhaI* (star) recognition sites were examined. **B** The *TIMP1* 5' primers can detect 10% of methylated template mixed with unmethylated template. The first lane is methylated template and the last lane is unmethylated temple with the mixes in the middle lanes. When these DNA samples were mixed in the various proportions as listed, 10% methylated DNA could be detected in the presence of 90% unmethylated (1:9 lane. **C** Products from amplification of DNA without digestion (U), after *HpaII* digestion (II), or after *HhaI* digestion (I). The presence of a band after digestion (II or I lanes) indicates that those enzyme sites were methylated and resistant to digestion. *ARAF1* and *ELK1* flank *TIMP1* in Xp11.23. *MIC2* primers were used to control for complete digestion while *XIST* was used to confirm the presence of DNA. The analyzed cell lines listed left to right: active X hybrids (Xa) AHA-11aB1 and t60-12; inactive X hybrids (X_i) t48-1a-1Daz4a and t11-4Aaz5; inactive X hybrids that express *TIMP1* (X_i^+) t75-2maz34-1a, t86-B1maz1b-3a, and t81-az1D.

methylated and expressing cell populations might reflect mixed cell populations with some unmethylated expressing cells and some methylated silent cells as amplification with the *TIMP1* primers can readily detect 10% of the total DNA as uncut/methylated (Figure 4.7B).

Clones of the three Xi+ hybrids were examined for methylation and expression (Table 4.3). The Xi+1 hybrid was originally unmethylated with high level *TIMP1* expression and all 13 subclones were *TIMP1*$^+$ and unmethylated. Clones from the other methylated *TIMP1*$^+$ hybrids (Xi+2 and Xi+3) included several clones that failed to express *TIMP1*, supporting the idea that low level expression was due to the presence of a mixed population of silent and expressing cells. These *TIMP1*$^-$ clones were all methylated, and in fact no clone has ever been observed that is *TIMP1*$^-$ but unmethylated. However, more than half of the *TIMP1*$^+$ subclones remained methylated (69% Xi+2 and 67% Xi+3), perhaps reflecting ongoing instability of expression or inactivation. To examine the stability of *TIMP1* inactivation, I generated further single cell clones from individual *TIMP1*$^+$ and *TIMP1*$^-$ clones (Table 4.4). *TIMP1*$^-$ cells gave rise to *TIMP1*$^+$ clones at a low frequency (1/33 for both lines) whereas methylated expressing clones continued to give rise to a mixed population of subclones.

	Original culture		
Class of subclone	Xi+1 *TIMP1*$^+$ unmethylated	Xi+2 *TIMP1*$^+$ methylated	Xi+3 *TIMP1*$^+$ methylated
TIMP1$^-$ methylated	0	5	3
TIMP1$^+$ methylated	0	11	6
TIMP1$^+$ unmethylated	13	0	0

Table 4.3. Subclones of the inactive X hybrids that express *TIMP1*.

The hybrids were single cell cloned and the resulting clones are grouped according to expression and methylation categories. *TIMP1*- unmethylated cultures were never observed so this class was not included in the tables.

Class of subclone	Xi+2 *TIMP1*$^-$ methylated	Xi+2 *TIMP1*$^+$ methylated	Xi+3 *TIMP1*$^-$ methylated	Xi+3 *TIMP1*$^+$ methylated
TIMP1$^-$ methylated	20	7	12	0
TIMP1$^+$ methylated	0	5	1	10
TIMP1$^+$ unmethylated	0	1	0	0

Table 4.4. Expression and methylation groups of the next generation of clones.

The clones were generated from an individual culture of the indicated class. Expression and methylation were determined with the PCR assays.

4.2.8 Methylation correlates with Expression Levels

To determine whether the low level expression was a characteristic of the cell lines or due to the presence of methylation at the promoter I examined expression levels of subclones of two sister clones that expressed *TIMP1* but differed in their methylation states. These clones arose during the second subcloning of Xi+2 from a methylated *TIMP1*$^+$ clone. The only difference between these sibling subclones should be epigenetic characteristics given that they arose from the same clone. When the clone was unmethylated at the promoter, 16/16 subclones expressed *TIMP1*, and expression was in the range seen for an Xa (Fig. 4.8). The methylated *TIMP1*$^+$ culture continued to show instability, with only 13/31 (42%) of its clones remaining *TIMP1*$^+$, and those that were positive showed low expression levels (Fig. 4.8).

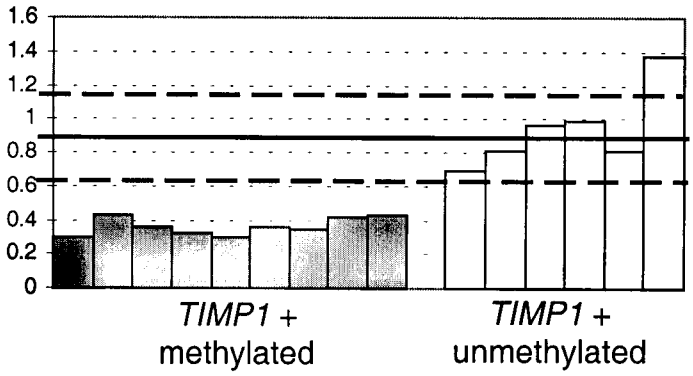

Figure 4.8. Lower level expression correlates with presence of methylation.

Expression levels were measured with RPA in clones shown to express *TIMP1* by RT-PCR. The RNA was isolated from single cell clones derived from two sibling cultures, differing only in their methylation status. There is a consistent increase in RNA level when methylation is absent. The solid line represents the average Xa expression level (0.89) and the dotted lines depict the standard deviation (+/- 0.20).

A summary for three generations of clones is shown in Figure 4.9. When the promoter was unmethylated, the cells stably expressed *TIMP1* because all subclones expressed *TIMP1* at Xa levels. Expression in the presence of methylation continued to be unstable, with only 46% of the subclones continuing to express *TIMP1*. Methylation was lost in one of the 100 subclones from a methylated *TIMP1*$^{+}$ and that culture led to a stable *TIMP1*-expressing clone. The subclones that no longer expressed *TIMP1* were generally stably silenced because they stayed negative in culture and the majority of subclones were non-expressing. However, one of 33 subclones expressed *TIMP1*, suggesting that these cultures were prone to reactivation.

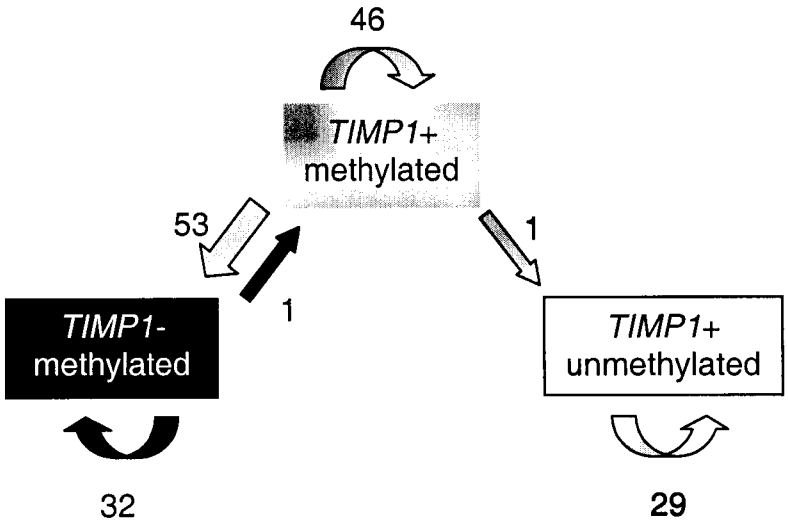

Figure 4.9. Summary of subclones.

The observed expression and methylation status for the 162 clones analyzed over three generations of subclones. The clones with unmethylated promoters have stable expression as all following subclones express TIMP1. Expression in the presence of methylation continued to be unstable, with only 47/99 continued to express *TIMP1*. The silent clones were fairly stable because only one subclone reactivated and began to express *TIMP1* again. There is a slight trend toward expressing *TIMP1* in a stable manner.

4.2.9 Methylation in human cell lines

I analyzed the methylation status of the *TIMP1* promoter in human cell lines to determine if expression from the X_i is likely to significantly alter total *TIMP1* RNA levels. The male lymphoblast cell lines examined have only the one Xa and were subsequently unmethylated at the *TIMP1* promoter (Fig. 4.10) and female cell lines that inactivated *TIMP1* (X_i^-) showed methylation. Females are generally a mosaic of cells with different X chromosomes active, and thus methylation would be expected if either X chromosome were subject to inactivation. However the three females previously shown to have expression from the X_i had extremely skewed X inactivation (>90% inactivation of one X), and thus could be considered a monoclonal population with expression from both X chromosomes. DNA from these females (derived from blood, a fibroblast culture and a lymphoblast culture) showed amplification after the *HpaII* and *HhaI* digestion, indicating that the *TIMP1* promoter on the *TIMP1*-expressing X_i remained methylated. The female cell line shown to have increased *TIMP1* RNA (GM07005) does not have extremely skewed X inactivation (~75%) and is not informative because the methylation of either X will be detected.

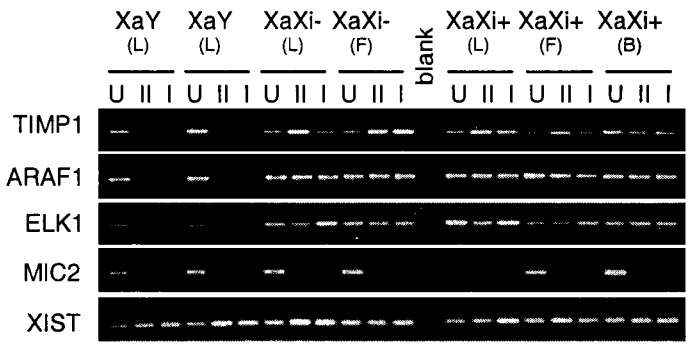

Figure 4.10. Methylation analysis of the human cell lines.

PCR products from: uncut DNA (U); after *HpaII* digestion (II); or after *HhaI* digestion (I). The presence of a band after digestion (II or I lanes) indicates the presence of methylation. The DNA was isolated from (left to right): male GM07009 and GM07033; $X_aX_i^-$ = GM07059; X_aX^{i-} = GM02859; $X_aX_i^+$ = HSC593; $X_aX_i^+$ = GM01813; $X_aX_i^+$ = 07. L = lymphoblast cell line. F = fibroblast cell line. B = blood.

4.3 Discussion

X inactivation is an extraordinary example of co-ordinate gene control, silencing most of the ~2000 genes on one of the two X chromosomes of females to achieve dosage compensation with hemizygous males. However, >10% of X-linked genes escape this inactivation and are expressed from both X chromosomes (Carrel et al. 1999). Dosage compensation is maintained when these genes have functional Y homologues (Disteche 1995) but when there is no functional Y homologue, females may have higher amounts of that gene product. For some genes, overexpression in females may be necessary and play a role in normal female development; while for other genes, it may have no impact. Dosage is, however, clearly important for a subset of X-linked genes. For example, abnormal duplication of *DAX-1* in males results in sex reversal whereas deletions lead to X-linked congenital adrenal hypoplasia (Goodfellow and Camerino 2001). The dosage of the *PLP* gene is also strictly regulated because both duplication and deletion of the gene in males cause Pelizaeus-Merzbacher disease, an X-linked demyelination disorder (Sistermans et al. 1998). However, expression from the X_i does not necessarily cause a significant increase in gene product because gene expression from the X_i is often lower than that of the active chromosome, as observed for the *STS* gene (Migeon et al. 1982). As TIMP1 is involved in a delicate biological balance with MMPs, any alterations may disrupt normal physiology so *TIMP1* RNA levels were examined to determine the contribution of expression from the X_i chromosome.

When determining the baseline level of *TIMP1* expression, I discovered considerable variation in *TIMP1* RNA levels. Cell cycle variability and/or culture conditions likely account for much of the differences between RNA preparations of the same cell line because the *TIMP1* gene responds to a variety of growth factors

(Denhardt et al. 1993). Surprisingly, however, beyond the variation within a cell line, there was an almost two fold range of *TIMP1* RNA between cell lines expressing only one copy of the gene. Diverse expression levels from the active X chromosomes could reflect sequence differences at the *TIMP1* promoter but the *TIMP1* promoter sequence was the same between the two Xa hybrids with the most divergent expression levels. The intrachromosomal variability was also not correlated with the expressed polymorphism (Tables 4.1 and 4.2)(Anderson and Brown 1999). However, other sequence differences beyond the minimal promoter and exon 5 polymorphism may influence expression levels. Although it is possible that translational mechanisms may regulate the divergent RNA levels to provide a consistent amount of protein across cells, *TIMP1* is known to be regulated at the transcriptional level, suggesting that RNA levels are a major determinant of protein levels (Doyle et al. 1997). Because the TIMP1 protein is an inhibitor of MMPs, the balance of TIMPs to its target proteins, not absolute amounts, is important in determining proteinase activity. Therefore, variable *TIMP1* levels between cell lines may reflect the amount of MMPs present within cell lines.

Expression levels from the X_i chromosome showed greater variability than levels from the X_a, due to very low level expression in some X_i^+ hybrids. The low level expression observed in X_i^+ hybrids could be characteristic of these cell lines or due to residual silencing by epigenetic features associated with X inactivation such as methylation. By analyzing sibling cultures differing in methylation, I determined that low level expression was not inherent to the cell line but associated with the presence of methylation at the *TIMP1* promoter. The observation of methylation and expression in clones was unexpected because methylation generally correlates with silencing of X-linked genes. It is possible that there are critical sites required to silence *TIMP1* that I did not examine. For example, it has recently been demonstrated that there are 3

crucial sites determining expression status for the X-linked *HPRT* gene (Chen et al. 2001a). Alternatively, there may be ongoing instability of expression and/or inactivation. In the time it takes a single cell to grow into a culture for analysis, different cell types might arise leading to a mixture of silent, methylated cells and expressing, unmethylated cells. This could also explain lower level expression in the presence of methylation, presumably due to the lack of contribution from the methylated cells. However, both the unmethylated *TIMP1*$^{+}$ state and the methylated silent clones were fairly stable, indicating that the premise of a simple mixture of unstable cells is unlikely. The persistence of methylated *TIMP1*$^{+}$ cultures after single cell cloning suggests that *TIMP1* promoter methylation and gene expression are not mutually exclusive. *REP1* is another gene with variable X inactivation and expression from the Xi is 5 to 42% of the Xa level. Both *REP1* alleles were detected using RT-PCR on single cells, demonstrating that *REP1* is expression from the Xi in all cells but at lower levels than the Xa allele (Carrel et al. 1999). *TIMP1* expression from the X_i when the promoter was methylated was at or below the RPA assay background level of 0.25. This small amount of *TIMP1* RNA from the X_i is unlikely to be biologically significant given the variability of *TIMP1* expression from the active chromosome.

TIMP1 is not always expressed from the X_i. Comparing cell lines with and without this X_i expression may reveal which epigenetic factors are required to maintain the inactive state. The X_i is associated with the hypermethylation of promoters, nuclease insensitivity, delayed replication timing and the hypoacetylation of histones. These epigenetic features are most likely redundant and I propose that one of these features can be lost without disrupting dosage compensation. For example, cell lines from patients with the ICF immunodeficiency syndrome have a hypomethylated X_i chromosome but a gene was expressed from the X_i only when replication timing was

advanced similar to the X_a homologue (Hansen et al. 2000). In the X_i^+ cells, one of the epigenetic controls may be absent at *TIMP1*, allowing low level expression from the X_i before methylation is disrupted. However, once methylation is lost, *TIMP1* stably expresses at a high level, which could result in the loss of dosage equivalence.

Cells from the three females with expression from the X_i showed methylation at the *TIMP1* promoter. In the hybrid cell lines, *TIMP1* promoter methylation was associated with low level expression and ongoing instability whereas unmethylated *TIMP1* promoters correlated with stable expression from the X_i at levels comparable to the active chromosome. Therefore, as women age, they may accumulate cells that stably express *TIMP1* from the X_i, leading to escalating *TIMP1* RNA levels. If TIMP1 rises above the typical range of expression, it may lead to aberrant regulation of metalloproteinases activity. Expression from the X_i chromosome with the eventual rise of a gene product above a normal range may help explain observed sex differences in the susceptibility to some late-onset disorders such as arthritis and multiple sclerosis.

5 ANALYSIS OF VARIABLE X INACTIVATION TO ESTABLISH A HIERARCHY OF EPIGENETIC FEATURES

5.1 Introduction

The variable X inactivation of the human *TIMP1* gene provides a unique system to analyze the epigenetic features associated with the Xi, an extraordinary example of stable gene silencing. Genes subject to X inactivation are associated with hypermethylation of promoters, nuclease insensitivity, delayed replication timing and hypoacetylation of histones in addition to the localization of *XIST* RNA and macroH2A to the Xi (reviewed in Avner and Heard, 2001). Genes escaping X inactivation have the epigenetic characteristics of the Xa: hypomethylated promoters, hyperacetylation of histones, sensitivity to nucleases and replication timing similar to the Xa. Therefore, comparing cell lines with and without *TIMP1* expression from the Xi may reveal which epigenetic factors are required to maintain the inactive state.

The presence of methylation at the *TIMP1* promoter was not sufficient to fully inactivate *TIMP1* (chapter 4). However, methylation did have a suppressive effect as methylation correlates with unstable, low level *TIMP1* expression and an unmethylated promoter expressed *TIMP1* at levels similar to the Xa. The correlation between lack of promoter methylation and high *TIMP1* levels was clear but the initial event was unknown.

Lack of promoter methylation may precede stable expression or increased expression could drive the demethylation of the promoter. A strong transcriptional activator can transcribe a gene in the presence of a repressive chromatin structure, altering the epigenetic modifications to a more permissive state. The *TIMP1* gene is inducible and expression significantly increases in the presence of several growth factors and signals (Brown et al. 1990; Clark et al. 1997; Edwards et al. 1987). To

determine whether a transient increase in *TIMP1* expression led to demethylation of the promoter and long-term stable expression, I induced *TIMP1* expression with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA).

Alternatively, the methylation at the *TIMP1* promoter may have to be removed before full transcription occurs. Methylated transgenes are repressed whereas unmethylated constructs are not (Kass et al. 1997), suggesting that methylation has a decisive role in silencing. I analyzed expression of *TIMP1* after demethylation treatments to determine whether removing methylation leads to *TIMP1* expression from the Xi. *TIMP1* was expressed in a subset of cells and subsequent subcloning led to various classes of clones with expression patterns similar to the Xi+ hybrids.

There may be several layers of repression set up by the epigenetic features associated with silencing and more than one feature may need to be disrupted before a gene is reactivated. This is supported by the observation that the demethylating agent 5-aza-cytidine appears to interfere with several epigenetic features before X-linked genes are reactivated. After treatments with 5-aza-cytidine, three genes within the 100 kb *G6pd* region (*G6pd, P3,* and *Gdx*), were reactivated together, suggesting that a regional feature was disrupted in addition to the loss of methylation (Toniolo et al. 1988). For the *HPRT* gene, replication timing advances after demethylation treatments (Hors-Cayla et al. 1983; Schmidt and Migeon 1990). Further detailed molecular analysis of *HPRT* reactivation determined that the promoter becomes nuclease sensitive before transcription factors bind (Chen et al. 2001a), indicating that both promoter demethylation and relaxation of chromatin structure are required before transcription can begin. Furthermore, combining inhibitors of histone deacetylase with demethylation treatment leads to a 2 to 5 fold increase in reactivation of the *FMR1* gene (Chiurazzi et al. 1999), suggesting that histone hyperacetylation and DNA demethylation work

synergistically to silence genes. Additionally, patients with ICF syndrome have a significant lack of DNA methylation due to mutations in the *DMNT3b* methyltransferase, yet X inactivation is generally maintained and when genes do reactivate, it correlates with an advance in replication timing (Hansen et al. 2000), supporting the idea that more than one epigenetic feature must be disturbed due to the redundant nature of the silencing process.

In the *TIMP1*- clones derived from Xi+ hybrids that had expressed *TIMP1*, inactivation was fairly stable as the clones stayed negative in culture and the majority of clones remained inactive. However, one of 33 clones expressed *TIMP1*, implying that these cell lines are prone to reactivate. It is possible that one or more epigenetic feature may already be lost in the Xi+ cell lines, allowing unstable, low level *TIMP1* expression which is fully reactivated once methylation is lost at the *TIMP1* promoter. To test this hypothesis, I examined nuclease sensitivity and the acetylation of histone H3 in the various classes of hybrids.

To help understand why *TIMP1* is expressed from some Xi but not others, I examined inheritance patterns. To do this, I analyzed *TIMP1* expression from the Xi in first-degree relatives carrying a mutation in the *WASP* gene located at Xp11.23 –11.22. Wiskott-Aldrich syndrome (WAS [MIM 301000]) is a severe X-linked recessive disease characterized by thrombocytopenia, recurrent infections, and eczema. Female carriers have non-random X inactivation in all peripheral blood cells, due to selection against cells that express the mutant *WASP* (Puck et al. 1990; Wengler et al. 1995). Because of the close proximity to *TIMP1* at Xp11.23, almost no recombination is expected between the two genes and carrier females should inherit the same *TIMP1* allele and inactivate it along with the mutated *WASP* gene. Therefore, these first-degree relatives will have the same *TIMP1* allele on the Xi so patterns of inheritance can be established.

5.2 Results

5.2.10 TPA induction

Expression levels in hybrid cultures were measured after six hours in media with low FCS plus 10 ng/μL of TPA, a chemical that induces *TIMP1* expression (Brown et al. 1990). The change in expression was determined by comparing the expression level in cultures exposed to TPA to those cultures grown only in alpha media with 2% FCS (Table 5.1). Expression levels were measured with PCR and RPA techniques and the results were consistent across assays. *TIMP1* expression was quantified by comparing the intensity of a *TIMP1* band to the intensity of the *MIC2* band. *MIC2* is expressed from both the active and inactive X chromosomes at comparable levels (Goodfellow et al. 1984), controlling for both the amount of input RNA and also for the number of X chromosomes, which can be variable in the hybrids. In cultures that originally expressed *TIMP1* at detectable levels, both RPA and duplex PCR were used. The duplex PCR used *MIC2* primers and *TIMP1* primers in the same PCR reaction. The results were similar between two sets of *TIMP1* primers: C1A:C1B within exons 1 and 2 and the downstream CA1:CA2 in exons 4 and 6 (Figure 5.2). To examine possible induction in *TIMP1*- hybrids, nested PCR was performed with TIMP-N primers that flank the TIMP1 CA1:CA2 primers. In all hybrids, irrespective of the technique used, less than a two fold increase was observed (Table 5.1). This slight increase was not maintained after 48 hours in culture without TPA, indicating that induction did not alter *TIMP1* expression permanently. However, *TIMP1* expression did not appear to be fully induced because the RNA levels at most doubled in contrast to the 2 – 5 fold increase expected (Brown et al. 1990; Edwards et al. 1987).

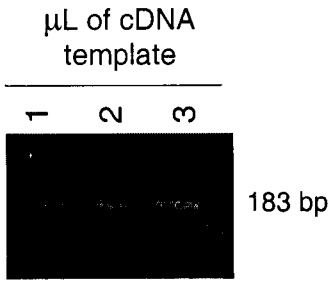

Figure 5.1. Linearity of the *TIMP1 C1A:C1B* PCR.

Increasing amounts of the same cDNA template was used to show that the PCR amplification was occurring in linear phase. When the above photo was scanned and quantified using the NIH image program, the intensity of the bands increased relative to the amount of template (1.0, 1.9x, 2.9x). RNA from the AHA-11aB1 cell line was reverse transcribed with random hexamers to create the cDNA template.

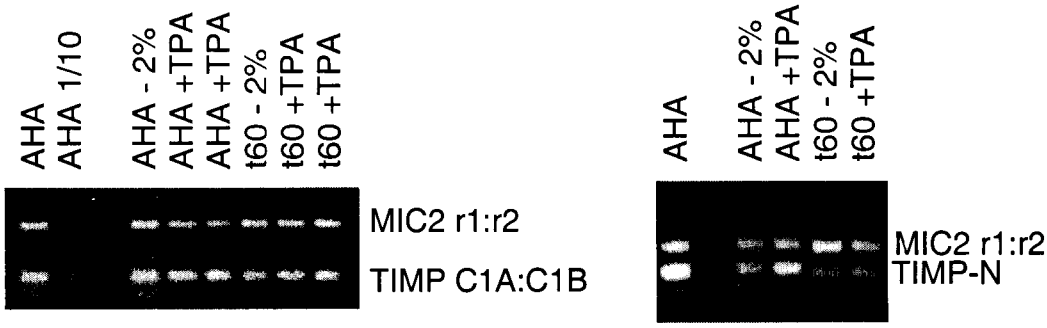

Figure 5.2. Duplex PCR reactions.

Both *TIMP1* and *MIC2* primers were added to the same PCR reaction to reduce variability between reactions. *MIC2* was used to control for the amount of amplifiable cDNA, determined by the amount of input RNA and the efficacy of the RT reaction. The gel images were scanned and analyzed by NIH image. The intensity of the *TIMP1* band was divided by the intensity of the *MIC2* band to provide *TIMP1* expression levels. Comparing the expression level after TPA induction to the *TIMP1* expression without induction gave the increase in expression after TPA induction (Table 5.1). Two sets of *TIMP1* PCR primers were used in the duplex PCR reactions. The TIMP C1A:C1B primers are 5' within exon 1 and 2 whereas TIMP-N flank the polymorphism primers from exon 4 to 6.

Hybrid + TPA [A]	Increase determined by *TIMP1 C1A:C1B* duplex PCR	Increase determined by *TIMP1-N* duplex PCR	Increase determined by RPA	Increase remaining after 48 hrs [C]
Xa = *TIMP1*+, unmethylated				
AHA-a	1.8x	1.7x	1.9x	1.1x
AHA-b	1.4x	1.9x	1.9x	0.9x
t60-a	1.2x	1.5x	1.0x	
t60-b	1.3x	1.4x	1.2x	
Xi+1 = *TIMP1*+, unmethylated				
t75-a	1.3x	1.6x	1.3x	1.0x
t75-b	1.7x	1.3x	0.7x	
Xi+2 = *TIMP1*+, methylated				
t86-6K-a	1x	1.7x	1.4x	
t86-6K-b	1x	1.7x	1.3x	
Xi+2 = *TIMP1*-, methylated [B]				
t86-6P-a	1.3x (nested)	1.5x (nested)		
t86-6P-b	1.8x (nested)	1.9x (nested)		
Xi = *TIMP1*-, methylated [B]				
t11-a	1.6x (nested)	1.8x (nested)		
t11-b	1.4x (nested)	2.1x (nested)		

Table 5.1. Summary of *TIMP1* RNA increase after TPA induction.

The increase columns (⇑) are the ratio for the RNA levels after TPA induction compared to the same cell line grown in 2% FCS alpha media. [A] The "a,b" label designates two separate flasks induced by TPA, cultured and analyzed at the same time. Each PCR assay result listed is the average of two separate PCR reactions on the same cDNA template. The RPA assay was done once for each RNA. The results are similar for the PCR and RPA assays. [B] Nested PCR was used to detect low level expression. To confirm observed increases, a second reverse transcription reaction was performed and the PCR amplficiations repeated. [C] Some cultures were examined after 48 hours in alpha media (7.5% FCS) and the previously induced cultures were compared to the control grown only in media with 2% FCS. There was no long term increase of *TIMP1* expression.

5.2.11 Demethylation treatments

A Xi hybrid with stable X inactivation of *TIMP1* (t11-4Aaz-5) was demethylated by 5-aza-cytidine treatment. Cells were first grown in HAT-supplemented media to select for *HPRT* reactivation. The expression of *TIMP1* and surrounding genes (*ARAF1, ELK1, ZNF157,* and *ZNF41* shown in Figure 5.3) were analyzed in 16 *HPRT*+ clones by RT-PCR with human-specific primers. Nine of the cultures expressed *TIMP1* as well as one or more of the genes in the region. Different flanking genes were expressed in different *TIMP1*+ cultures and there was no obvious pattern of reactivation. All of the *TIMP1*+ clones had methylation at the *TIMP1* promoter, which may reflect remethylation and possible heterogeneous cell populations of methylated, silent cells and expressing, unmethylated cells.

To isolate any fully demethylated cells as well as examine the stability of *TIMP1* reactivation, I generated clones of four cultures that expressed different genes in the *TIMP1* region (Table 5.2). The *TIMP1*- culture remained inactive in all 10 subclones. The *TIMP1*+ clones that also expressed *ELK1* with or without *ZNF157* did not retain expression in the majority of subclones and *TIMP1* expression was lost as clones grew in culture. This suppression of *TIMP1* reactivation may have been due to remethylation of the *TIMP1* promoter because the cells were no longer exposed to the demethylating treatment. In contrast, the *TIMP1*+ culture that also reactivated *ARAF1* and *ZNF157* was fairly stable as 82% of the subclones continued to express *TIMP1*. Of the 14 *TIMP1*+ subclones, nine were also *ARAF1*+, suggesting that these genes are being co-ordinately regulated. Interestingly, *TIMP1* and *ARAF1* are flanked by putative Matrix Attachment Region (MARs) sites, which were placed by analyzing the *TIMP1* region shown in Figure 5.3 with the MARFinder program (www.ncgr.org/MarFinder).

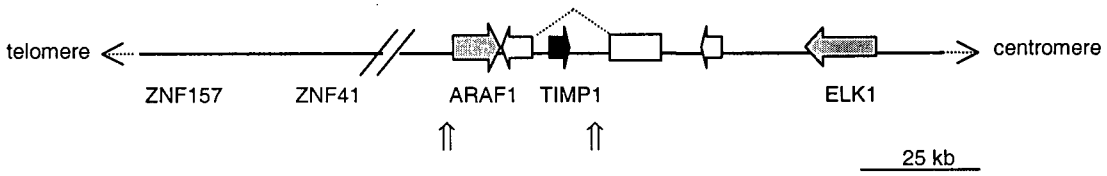

Figure 5.3. Genes in the *TIMP1* region.

Expression of the genes labelled in this figure were examined in the demethylated Xi hybrid clones. The ⇑ symbol shows the location of putative Matrix Attachment Regions when this region is analyzed with MARFinder. These MARs are close to the *TIMP1* and *ARAF1* genes: 635 bp from the 5' end of *ARAF1* and ~18 kb downstream of *TIMP1*.

Subclones	t11-az-4 TIMP1-	t11-az-8 [a] TIMP1+ (ELK1+)	t11-az-9 [a] TIMP1+ (ELK1+, ZNF157+)	t11-az-10 TIMP1+ (ARAF1+, ZNF157+)
Expression				
TIMP1-	10	2 [a]	2 [a]	3
TIMP1+	0	10	15	14 [b]
Methylation				
methylated	10	12	17	16
unmethylated	0	0	0	1

Table 5.2. Clones from HPRT+ clones of the demethylated Xi hybrid.

Clones were generated from four cultures with different genes reactivated. The expression was determined by RT-PCR with human-specific primers. Methylation was examined with PCR after methylation-sensitive enzyme digestion. [a] Expression in these branches was initially weak and became silent after time in culture. *ELK1* expression was initially retained in one of the t11-az-9 *TIMP1*+ clones but was not analyzed after *TIMP1* expression was lost. [b] Nine of the TIMP1+ cultures were also ARAF1+. The expression of *ZNF157* was not examined in the subclones.

The majority of subclones were methylated at the *TIMP1* promoter even when *TIMP1* was expressed. In the one *TIMP1*+ clone without methylation, the *ARAF1* promoter was also unmethylated, providing additional evidence that these two genes are

reactivated co-ordinately. The presence of methylated and unmethylated cultures expressing the same genes with the same history provided the opportunity to further examine the role of methylation in *TIMP1* expression from the Xi. Single cell clones were generated from *TIMP1*+, *ARAF1*+ (t11-az-10) cultures that differ in methylation states and expression and methylation were analyzed in these clones (Table 5.3). All subclones continued to express both *TIMP1* and *ARAF1*, indicating stable expression even in the presence of methylation. The expression levels in the individual subclones were determined by RPA and, as in the Xi+ clones, *TIMP1* expression was significantly lower when the promoter was methylated.

Subclones	*TIMP1*+ and *ARAF1*+	
	unmethylated t11-az-10-10	methylated t11-az-10-7
TIMP1 expression	4/4	8/8
ARAF1 expression	4/4	8/8
TIMP1 methylation	0/4	8/8
ARAF1 methylation	0/4	8/8
Average *TIMP1* expression level	0.99	0.57

Table 5.3. Comparison of two sibling cultures differing in methylation states.

These cultures arose during the subcloning of t11-az-10 from a methylated *TIMP1*$^+$ *ARAF1*$^+$ clone. All subclones continue to express both *TIMP1* and *ARAF1* assayed by RT-PCR. Despite the relative stability of the methylated *TIMP1*+ culture, the *TIMP1* expression level was significantly lower in the methylated cultures (p=0.01).

5.2.12 Nuclease sensitivity correlates with methylation status

The accessibility of the *TIMP1* and *ARAF1* promoter sequences to digestion by exogenous nucleases was examined in various classes of hybrids: +/- *TIMP1* expression with differing methylation states in both the natural (Xi+) and chemically induced (t11-az) *TIMP1* expression from the Xi. If the DNA is more sensitive to digestion, it is presumed to have a more relaxed chromatin structure. The PCR

amplification products obtained after amplification of DNA digested with increasing amounts of DNase1 are seen in Figure 5.4 and depicted graphically in Figure 5.5. Less DNaseI enzyme was required to fully digest the promoter regions in the Xa hybrids compared to the Xi hybrids, indicating a difference in chromatin conformation. There were no significant differences between Xa and Xi hybrids at the non-coding sequences or within the body of *TIMP1*, so subsequent analysis concentrated on the promoter regions. At the *ARAF1* promoter, only the cell lines that express *ARAF1* were sensitive to digestion, requiring ≤ 0.25 units of DNaseI to digest the DNA and lose PCR amplification. The *ARAF1*- hybrids were relatively insensitive as PCR amplification was still seen with 0.5 – 1.0 units of DNaseI. Intriguingly, the accessibility of the *TIMP1* promoter correlated with the methylation state of a hybrid rather than its expression status. When the promoter is unmethylated in the Xi+ hybrids, sensitivity to digestion is similar to that observed in the Xa hybrids. More DNaseI was required to digest the methylated Xi+ hybrids, suggesting that the promoter is less accessible even though the gene is transcribed.

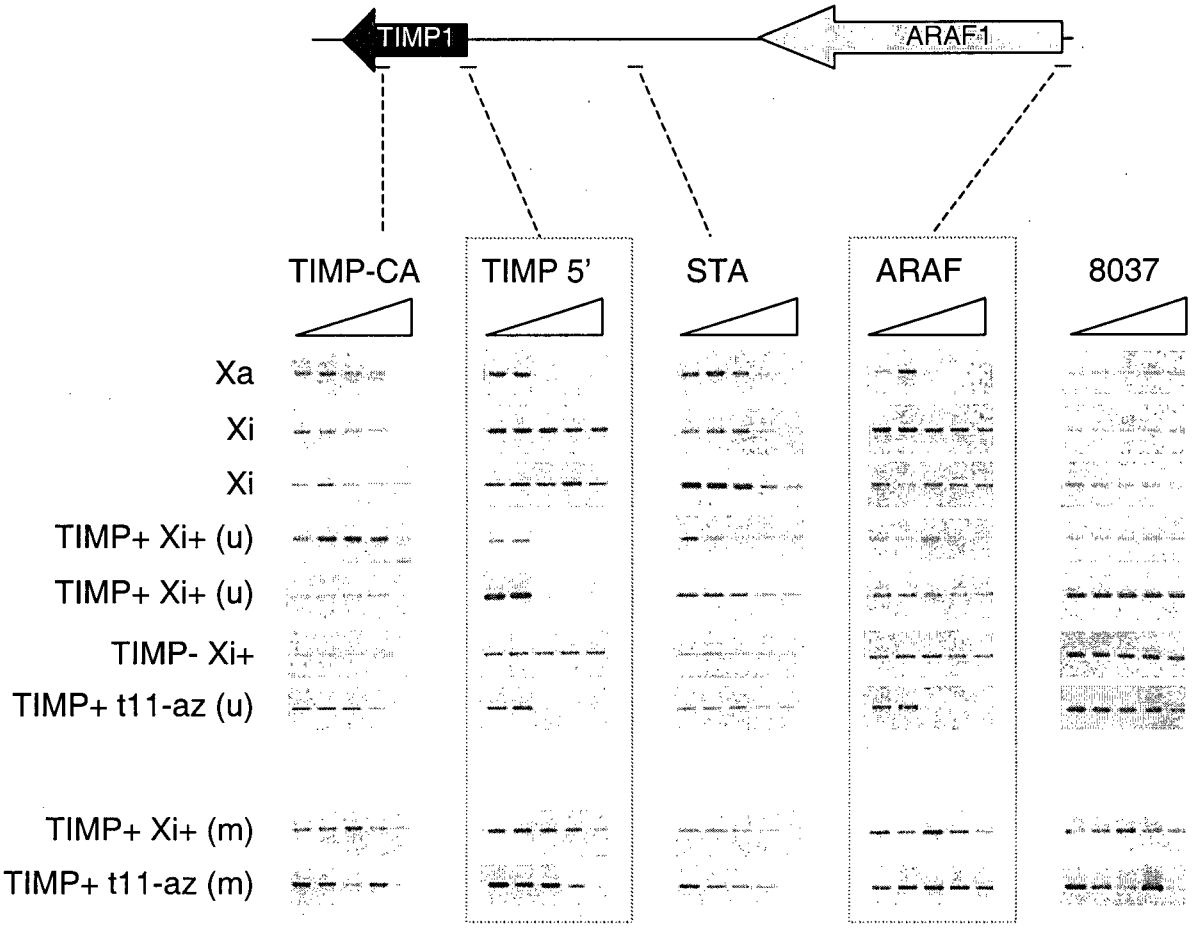

Figure 5.4. Nuclease sensitivity results.

The amount of DNase I (0, 0.1, 0.25, 0.5, 1.0 units) increases from left to right (triangle). The longer a band remains, the less sensitive the region to digestion. The cell lines are listed on the left of the figure. For the Xi+ hybrids, the status of *TIMP1* expression is written first. The "t11-az" designates the Xi hybrid with stable X inactivation of *TIMP1* demethylated by 5-aza-cytidine treatment. The (u) indicates that the *TIMP1* promoter was unmethylated and the presence of methylation is represented by the (m). There were significant differences when the promoter sequences were analyzed (boxed with dotted line). The STA primers flank a non-coding sequence halfway between the *TIMP1* and *ARAF1* gene and this region was examined to determine if the entire region become nuclease sensitive. The 8037 primers flank another non-coding region and were used to check that equal digestion and PCR amplification occurred across all cell lines. The hybrids examined were (top to bottom): t60-12; t48-1a-1Daz4a; t11-4Aaz5; t75-1maz-34-1a; t86-6J, t86-6P; t11-az-10-10; t86-6K; and t11-az-10-7.

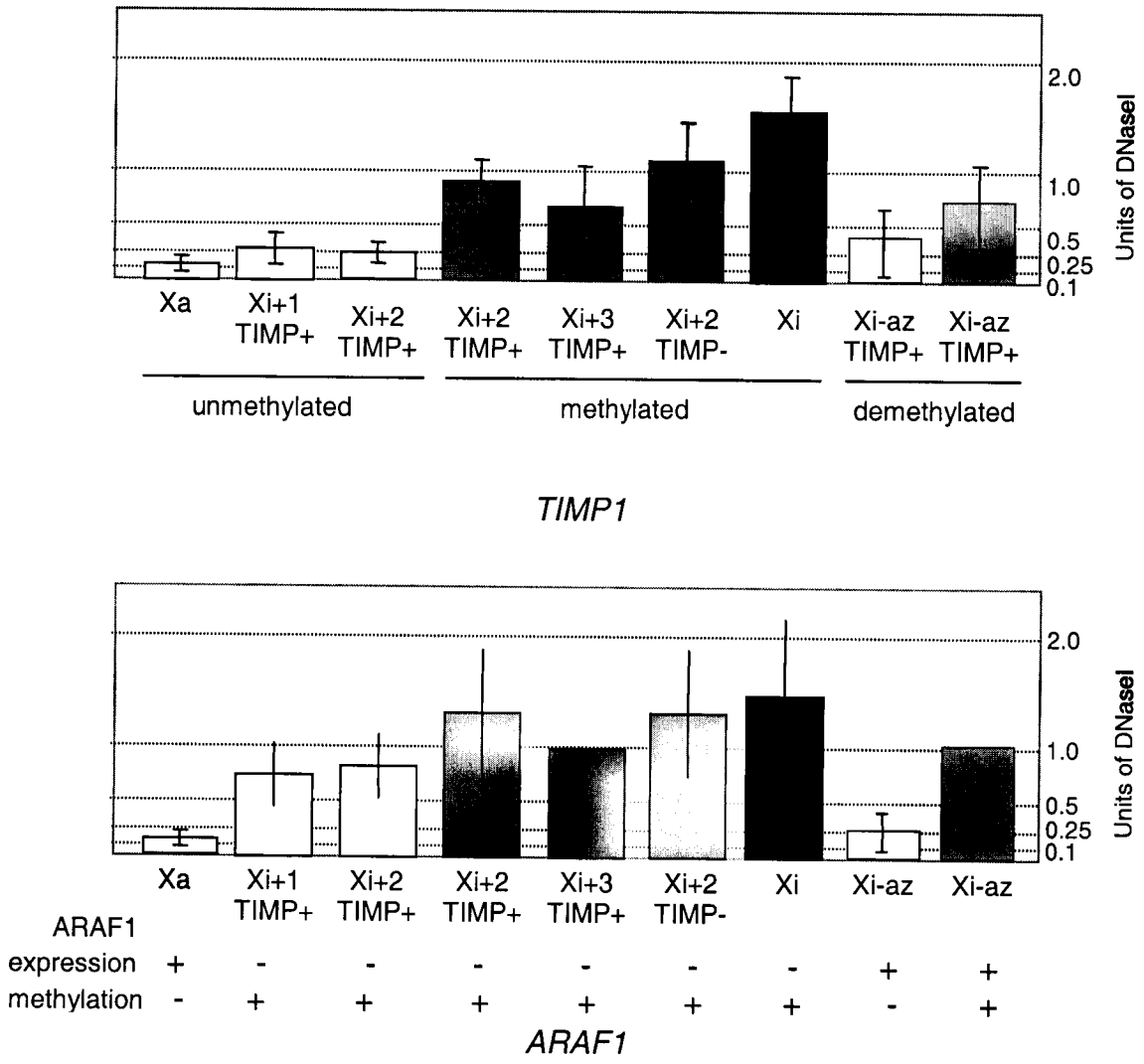

Figure 5.5. Nuclease sensitivity at the *TIMP1* and *ARAF1* promoters.

These graphs represent the amount of DNaseI enzyme per μL required to digest the promoter region and lose PCR amplification. The white bars represent unmethylated cultures that express *TIMP1*, the grey bars are methylated hybrids that express *TIMP1*, and the black bars are Xi hybrids that have never expressed *TIMP1* and are methylated. The cell lines are (from left to right): AHA-11aB1, t75-2maz34-1a, t86-6J, t86-6K, t81-14, t86-6P, t48-1a-1Daz4a, t11-az-10-10, and t11-az-10-7.

5.2.13 Histone acetylation precedes expression from the Xi

The acetylation of histone H3 at promoters was examined in the various classes of hybrids with a chromatin immunoprecipitation assay (ChIP). To determine what DNA sequences are associated with acetylated histones, DNA and associated proteins were cross-linked and then antibodies to acetylated histone H3 were added. When the acetylated histone H3 was immunoprecipitated, the cross-linked DNA and other proteins were also pulled out. The DNA in the bound fraction was isolated and used in PCR reactions. A PCR product indicated that the sequence was associated with acetylated histone H3 (Figure 5.6).

In general, promoters were associated with acetylated histone H3 when a gene was expressed. The *ZFX* gene escapes X inactivation and the promoter is hyperacetylated in all hybrids because *ZFX* is expressed from both Xa and Xi chromosomes. The *ARAF1, ELK1,* and *PGK1* genes are subject to X inactivation and the promoters were associated with acetylated H3 only when expressed (i.e. in the Xa hybrids). When the *ARAF1* gene was reactivated after demethylation treatments in the t11-az cell lines, the promoter sequences were enriched in the immunoprecipitated fraction, indicating histone acetylation. The *XIST* gene is expressed from the Xi and again, the promoter is hyperacetylated when *XIST* is expressed. The opposite expression patterns between *XIST* and the genes subject to X inactivation demonstrate that expression and acetylation are linked, not that the Xa hybrids are generally acetylated.

The *TIMP1* promoter was associated with acetylated histone H3 when expressed in the Xa hybrids but not in the Xi hybrids that had never expressed *TIMP1*. When Xi+ hybrids and derived clones were examined, the *TIMP1* promoter was always hyperacetylated, irrespective of the current expression status. In the cell lines that underwent demethylation treatments, the *TIMP1* promoter was associated with

acetylated histone H3 only when the gene was expressed. In a *TIMP1*- clone derived from a demethylated *TIMP1*+ culture, the promoter was no longer acetylated, suggesting that the retention of acetylation in the Xi+ hybrids was not just a memory of previous *TIMP1* expression. Therefore, acetylation of histone H3 at the *TIMP1* promoter may be a mark allowing expression from the Xi.

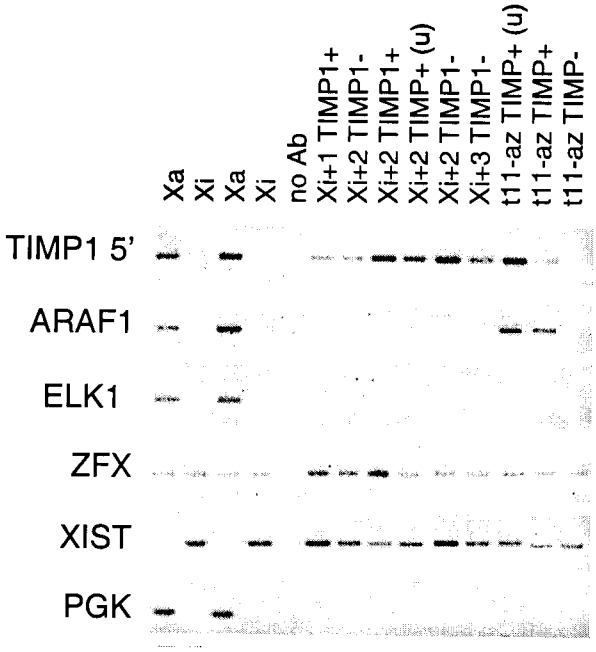

Figure 5.6. Acetylation of histone H3 at various X-linked gene promoters.

A PCR amplification product indicates that the sequence was associated with acetylated histone H3. The promoters of *ARAF1, ELK1, ZFX, XIST,* and *PGK* genes were acetylated only when the genes were expressed. In contrast, the *TIMP1* promoter was associated with acetylated histone H3 in all Xi+ hybrids, regardless of the current expression state. This appears to be specific to the Xi+ hybrids, as the demethylated hybrids were not hyperacetylated when *TIMP1* was no longer expressed. The cell lines are (from left to right): AHA-11aB1; t48-1a-1Daz4a; t60-12; t11-4Aaz5; t75-2maz34-1a; t86-6P; t86-6K; t86-6J; t86-1U; t81-4; t11-az-10-10; t11-az-10-7; and t11-az-9-10.

5.2.14 TIMP1 *expression from the Xi may be inherited*

To assess whether *TIMP1* expression from the Xi is inherited, I analyzed *TIMP1* expression in four pairs of first degree relatives that are likely to have inherited the same *TIMP1* allele. These lymphoblast cell lines were derived from WAS carriers who have nonrandom X inactivation. If *TIMP1* was subject to X inactivation, only the one allele on the Xa would be expressed. Expression of both alleles indicates that *TIMP1* is also being expressed from the Xi. The cell lines were heterozygous for the *TIMP1* polymorphism as both C and T alleles were amplified from DNA. However, only one *TIMP1* allele was observed in cDNA, indicating that *TIMP1* was only expressed from the Xa in these cells (Table 5.4).

The concordant lack of *TIMP1* expression from the Xi in all four pairs is consistent with the hypothesis that determination of *TIMP1* expression from the Xi is inherited. However, this argument would be more convincing if there was a pair that had expression from the Xi. Only 19% of females examined have expression from the Xi (chapter 3) so the agreement in four pairs of women is not statistically significant ($p=0.43$). Although the initial analysis is promising, more pairs of first-degree relatives will have to be examined to confirm that there is an inherited predisposition of *TIMP1* expression from the Xi.

		TIMP1 Alleles	
Individual	Family	DNA	cDNA
4023]	WAS1	C + T	C
2443]	WAS1	C + T	C
3096]	WAS6	C + T	T
3098]	WAS6	C + T	T
JS4190]	WAS8	C + T	T
JS4194]	WAS8	C + T	T
3411]	WAS13	C + T	C
3554]	WAS13	C + T	C

Table 5.4. Concordant *TIMP1* expression from the Xi in first-degree relatives.

Pairs of WAS carriers were examined for *TIMP1* expression from the Xi using the PASA assay (chapter 3). The] symbol indicates that these cell lines were derived from the blood of first-degree relatives. Only one allele was observed in all cDNAs, indicating that all eight women did not express *TIMP1* from the Xi.

5.3 Discussion

I analyzed the epigenetic features associated with inactivated X-linked genes in hybrid cell lines that have various states of *TIMP1* expression. If there are differences between the epigenetic modifications when *TIMP1* is expressed from the Xi, it may distinguish the epigenetic modifications required for *TIMP1* inactivation and the epigenetic changes secondary to expression status.

Active genes are generally unmethylated at their promoters but *TIMP1* can be expressed from the Xi in the presence of methylation. However, this expression was at low levels and unstable; high levels of stable expression were only observed when the promoter was unmethylated, suggesting that methylation still has a suppressive effect on *TIMP1* expression. It is unknown whether methylation must be lost before full expression occurs or whether strong expression drives the demethylation. To differentiate which is the primary event, I compared *TIMP1* expression after demethylation and TPA induction of the hybrid cell lines.

Inducing *TIMP1* expression in methylated Xi+ hybrids did not lead to stable, high level expression, suggesting that increasing expression does not stimulate demethylation at the *TIMP1* promoter. However, the less than two fold increase in *TIMP1* expression after TPA induction was lower than expected so expression may not have risen high enough to change promoter modifications. The presence of methylation does not explain low induction because all hybrids tested had only a slight increase in *TIMP1* expression, including the unmethylated Xa and Xi+ hybrids. Because the RPA probe and the first set of duplex primers (C1A:C1B) start in exon 1, there was a possibility that they would not detect expression as mouse *Timp1* does not transcribe the first exon after TPA induction (Edwards et al. 1987). When the duplex PCR was

repeated with the downstream CA1: CA2 primers, there was no difference in induction levels, suggesting that the human *TIMP1* gene retains exon 1 after TPA induction.

There are several other explanations for the observed lack of induction. The amount of *TIMP1* RNA was normalized to the expression of *MIC2* but previous investigations used mouse Actin to control for the amount of input RNA (Brown et al. 1990; Edwards et al. 1987) and the decreased induction may reflect differences between these controls. *MIC2* may also be induced in the presence of TPA, although it has not been reported in the literature. An increased baseline of *TIMP1* expression could also account for the lack of induction. All cell were first "starved" for six hours in alpha media containing only 2% FCS and the non-induced cells were kept in this media for another six hours (Brown et al. 1990; Clark et al. 1997; Edwards et al. 1987). The pre-treatment may not have been long enough to drop *TIMP1* expression or there may have been additional growth factors in the media. Given that the expected rise in *TIMP1* expression was not observed, the results are difficult to interpret. Although the TPA induction did not lead to stable expression methylated *TIMP1*+ cells, strong expression could still drive demethylation of the promoter, allowing stable expression to be maintained.

To further examine the role of methylation in repressing *TIMP1*, a Xi hybrid that had never expressed *TIMP1* was demethylated. In 16 *HPRT*+ clones examined, nine expressed *TIMP1*, suggesting that methylation contributes to the inactivation of *TIMP1*. As in the non-treated Xi+ hybrids, methylation of the promoter and *TIMP1* expression were seen in the same subclones. This may have been due to remethylation as the cells were no longer exposed to the demethylating chemical and further clones were generated to determine if *TIMP1* became inactive. In two of the *TIMP1*+ hybrids (t11-az-8 and t11-az-9), the majority of subclones were silenced and the *TIMP1*+ clones became negative in culture, suggesting that remethylation suppressed *TIMP1* expression. The

other *TIMP1*+ demethylated hybrid had stable expression when the promoter was methylated or unmethylated. The stability was significant compared to the methylated Xi+ hybrids where 50% of subclones were *TIMP1*- (p=0.004). This stable demethylated cell line had also reactivated *ARAF1* and *ZNF157*. The reactivation of *ZNF157* probably did not contribute to the stable expression of *TIMP1* because it is 70 kb away and was reactivated in one of the hybrids that did not maintain *TIMP1* expression. The co-ordinate expression of *ARAF1* in stable reactivation suggests that a regional control of silencing was also disrupted. When the *TIMP1* region shown in Figure 5.1 was analyzed by the MARFinder program, putative matrix attachment regions were placed on either side of *TIMP1* and *ARAF1*. MARs delineate chromatin loops and may prevent the spread of one transcriptional state into the neighbouring structural unit. The increased stability in *TIMP1* expression when *ARAF1* is also expressed may represent a change across the entire chromatin loop whereas when only the *TIMP1* gene is expressed, the area may reside in a repressive environment.

Both the regionality of *TIMP1* reactivation and the expression of *TIMP1* in the presence of methylation suggest that another epigenetic modification may differ when *TIMP1* is expressed from the Xi. There are several lines of evidence indicating that epigenetic features work together to maintain X inactivation. As in my demethylated hybrids, stable reactivation of *G6pd* also resulted in the reactivation of two nearby genes, *P3* and *Gdx*, suggesting that regional control was lost in addition to gene-specific methylation. There is increased reactivation of X-linked genes when one feature associated with X inactivation is missing. It is easier to reactivate X-linked genes in somatic cell hybrids (Kahan and DeMars 1975), which may be due to the lack of *XIST* RNA localization to the Xi or a regulatory control missing in mouse. In cells with decreased methylation as found in chorionic villus or in patients with ICF syndrome,

there is enhanced expression from the Xi. In the ICF cells, expression correlated with an advance in replication timing, demonstrating that another layer of repression is removed with stable reactivation. In the Xi+ hybrid cell lines, the unstable *TIMP1* expression from the Xi may result from the lack of another epigenetic control. Therefore, I searched for differences in other epigenetic features associated with X inactivation.

Transcriptionally inactive genes are generally insensitive to nucleases, which may reflect a more compacted chromatin structure. A relaxed chromatin configuration could promote *TIMP1* expression from the Xi because the promoter would be accessible to transcription factors so I assessed the sensitivity of the *TIMP1* and *ARAF1* 5' regions to exogenous DNaseI in the various types of hybrids. Cell lines that stably expressed *TIMP1* at high levels required less enzyme to digest the promoter region than cells without *TIMP1* expression, indicating an open chromatin structure when *TIMP1* was expressed. Surprisingly, the *TIMP1* promoter was relatively insensitive to DNaseI digestion when *TIMP1* was expressed at low levels from the Xi. Because unmethylated promoters were accessible but methylated promoters were not, chromatin packaging appears to be linked to methylation status. The Xi+ cell lines do appear to be more DNaseI sensitive than the Xi hybrid that has never expressed *TIMP1* but the variability of this assay prevents distinguishing small differences in chromatin structure. The digestion at low enzyme concentrations observed at unmethylated promoters may reflect hypersensitive sites free of nucleosomes due to the binding of transcription factors. The entire Xa chromosome was previously reported to be twice as sensitive as the Xi (Lin and Chinault 1988) but my results show similar DNaseI sensitivity between the Xa and Xi chromosomes outside of promoter regions. In the methylated Xi+ hybrids, the promoter may be relaxed enough to allow transcription but factors may only bind occasionally so nucleosomes are not displaced and no hypersensitivity is seen.

Alternatively, the lack of nuclease sensitivity could reflect a mixed population of expressing and silent cells as proposed for the low level *TIMP1* expression in the presence of methylation (chapter 4). However, all of the subclones from the demethylated Xi hybrid retained expression and methylation, suggesting each cell expresses *TIMP1* (and *ARAF1*) at low levels with methylation and a more condensed chromatin structure present. The difference in chromatin structure with the various classes of *TIMP1* expression imply that chromatin packaging is involved in *TIMP1* silencing. However, the *TIMP1* promoter remained nuclease insensitive when *TIMP1* was expressed from the Xi and only became fully accessible as *TIMP1* was stably expressed from an unmethylated promoter, indicating that an open chromatin structure was not the primary event allowing *TIMP1* expression from the Xi.

In contrast, there is acetylation of histone H3 at the *TIMP1* promoter in the Xi+ hybrids even when the gene is no longer expressed. The acetylation does not appear to be a mark remembering earlier expression as it was not retained in a *TIMP1*- hybrid that had previously reactivated *TIMP1*. Because H3 acetylation is present in Xi+ hybrids prone to reactivate *TIMP1*, it may be the mark allowing *TIMP1* to be expressed from the Xi. This acetylation may disturb higher order chromatin packaging as the first step towards transcriptionally competent chromatin. All Xi+ hybrids had acetylated H3, suggesting acetylation is important for *TIMP1* expression from the Xi. If all the epigenetic features were equivalent, loss of any feature should lead to unstable expression resulting in heterogeneous subclones. However, I never observed cells with unstable *TIMP1* expression when the promoter was methylated or DNaseI sensitive, indicating that hyperacetylation is the first change required for *TIMP1* expression from the Xi. Therefore, there is a hierarchy to the epigenetic silencing of the *TIMP1* gene.

I examined the acetylation of histone H3 as a representative member of the nucleosome. It will be interesting to examine the acetylation state of histone H4 to determine if the acetylation extends to other histone tails or is specific to histone H3. There are several lines of evidence that the acetylation of histones H3 and H4 are uncoupled. For example, in the imprinted Prader-Willi and Angelman Syndrome region of chromosome 15, the expressed paternal allele was associated with acetylation of H4, not H3 when there was biallelic methylation. Therefore, acetylated H4 was associated with expression and hypoacetylated H3 was associated with methylation (Gregory et al. 2001). It is also possible that other histone tail modifications are regulating *TIMP1* expression from the Xi. There are several covalent modifications (acetylation, methylation, and phosphorylation) that have been proposed to extend the information carried in the primary DNA sequence by altering transcriptional competence. There is mounting evidence that these modifications are not independent. For example, on the histone H3 tails, phosphorylation and methylation are antagonist whereas phosphorylation and acetylation are synergistic and mark transcriptionally active chromatin (reviewed in Jenuwein and Allis 2001). Therefore, it is possible that the observed acetylation of histone H3 at the *TIMP1* promoter is a reflection of a phosphorylation state required for transcription. Examination of the histone modifications in the various classes of *TIMP1* expression from the Xi should provide clues to decipher the "histone code".

The next obvious question is what determines the differential modification of histone H3 at the *TIMP1* promoter? The co-incidence of acetylation and methylation is unexpected because MeCP2 binds methylated promoters and recruits histone deacetylase (HDAC) activity that removes acetyl groups from the histone tails. This system could be disrupted at several points. MeCP2 may generally fail to recruit HDACs

or the deacetylase activity may be weak but these changes should affect more than the *TIMP1* gene. The *TIMP1* promoter has very few CpG sites (11 CpG within 200 bp) so there may not be sufficient MeCP2/HDAC complexes to remove acetylation. Sequence differences in the *TIMP1* gene may decrease MeCP2 binding further or recruit another protein complex with HAT activity. If sequence differences direct the acetylation of histone H3 at the *TIMP1* promoter, this signal may be inherited and initial analysis of *TIMP1* expression from the Xi in first-degree relatives supports an inherited predisposition to express *TIMP1* from the Xi.

Although differences in epigenetic control may be set up by underlying sequence differences, it is the loss of epigenetic features associated with X inactivation that allows *TIMP1* expression from the Xi. The epigenetic features are most likely redundant and I propose that altering one feature leads to unstable expression from the Xi. Once other features are disrupted, a gene is stably expressed. At the *TIMP1* gene, the epigenetic features are not equal, as acetylation of histone H3 appears to be the primary mark allowing *TIMP1* expression from the Xi. Therefore, there is a hierarchy to the epigenetic silencing of TIMP1, with histone acetylation preceding expression whereas methylation and chromatin structure are concordant with expression.

6 DISCUSSION

The process of X inactivation has captivated researchers since it was first proposed in 1961. During the last four decades, it has been shown that a significant number of genes escape inactivation and more recently, that genes expressed from the inactive chromosome can be different between individuals. To examine what controls the expression of individual genes on the Xi, I used the human *TIMP1* gene as a model. *TIMP1* is subject to X inactivation on some Xi but expressed from others in the somatic cell hybrid system (Brown et al. 1997). My thesis investigated if this variable *TIMP1* expression from the Xi occurs in human female cells and the possible phenotypic consequences of polymorphic inactivation for the *TIMP1* gene. Variable X inactivation suggests a lack or instability of a process required to maintain the inactive state so differences in *TIMP1* expression from the Xi were used to examine the epigenetic features of silencing.

6.1 Variable *TIMP1* expression the Xi also occurs in human female cells

Using an allele-specific PCR assay to examine expression in heterozygous women known to have extreme skewing of X inactivation, I demonstrated that *TIMP1* was variably inactivated in human female cells (chapter 3). Although differential inactivation patterns imply some females express more *TIMP1* than others, total *TIMP1* RNA levels did not clearly identify women with expression from the Xi. Because there was a 1.7x range of *TIMP1* levels in cells that express *TIMP1* only from the Xa, cells with a higher level of *TIMP1* RNA could either express *TIMP1* from both X chromosomes or express high levels of *TIMP1* solely from the Xa. The variability in *TIMP1* RNA levels was initially surprising as the TIMP1 protein is involved in a delicate balance with MMPs to determine proteinase activity However, the differing *TIMP1* levels between cell lines may reflect the amount of MMPs present within cell lines as the balance between proteinases and

inhibitors is likely important, not the absolute amounts of one protein. The initial low level of *TIMP1* expression from the Xi in the presence of methylation probably falls within normal variation and *TIMP1* is still considered inactive on an organism level. However, the balance of MMPs and TIMPs may be disrupted if *TIMP1* was fully reactivated on Xi chromosomes. The MMPs and TIMPs are co-ordinately regulated by similar transcription factors, which presumably prevents large fluctuations in proteinase activity but reactivation of *TIMP1* would provide a second copy of the gene, similar to an inserted transgene. The increased *TIMP1* expression would not be due to an increase in transcription rate so the other TIMPs and MMPs would not be expected to similarly increase expression. Because TIMP1 is a secreted protein, there would no selection for or against individual cells that expressed too much TIMP1 protein.

6.2 Is variability due to reactivation or failure of initiation?

It appears that the *TIMP1* gene was initially subject to X inactivation and has subsequently reactivated. The majority of female cell lines inactivate *TIMP1*, implying that inactivation is the normal course of events. Low level expression from the Xi correlated with features associated with silencing, which suggests that the gene was previously inactivated. Although this unstable expression in the presence of heterochromatic features could reflect spreading of the surrounding environment over the *TIMP1* gene, once the majority of inactivating features were removed, *TIMP1* was stably expressed from the Xi without interference from the flanking heterochromatic states. Furthermore, the trend towards increased *TIMP1* expression in the Xi+ hybrids with the accompanying loss of epigenetic modifications associated with silencing indicates that this expression from the Xi is a reactivation event. Full transcription was observed only when the repressive features of methylation and condensed chromatin packaging were removed, indicating that there had been an initial inactivation event.

Reactivation and escape from inactivation are often distinguished as distinct processes but the labels may just reflect differences in timing. The mouse gene *Smcx* is initially inactivated during development but is then reactivated and biallelically expressed in adult tissues (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. The variable X inactivation of *TIMP1* may be showing "escape in action". *TIMP1* may be initially inactivated along with its surrounding genes but be prone to reactivation.

TIMP1 may not be able to maintain X inactivation as well as other X-linked genes for several reasons. There are fewer proposed way stations in the *TIMP1* region, only one primate-specific LINE element is observed within 100 kb of the *TIMP1* gene, so *XIST* RNA may not bind. There is an enhanced reactivation rate in the hybrid system where the *XIST* RNA does not localize to the Xi (Clemson et al. 1996). Furthermore, there may be less steric hindrance at the promoter to directly interfere with transcription factor binding because there are few CpG sites at the *TIMP1* promoter. More importantly, in light of the increased acetylation of histones, the sparse CpG sites may bind less MeCP2 leading to less histone deacetylase activity at the promoter.

6.3 Epigenetic mechanisms of variable *TIMP1* expression from the Xi

The epigenetic features associated with silencing are most likely redundant and I propose that altering one feature, in this case acetylation of histones, leads to unstable expression from the Xi. This low level of expression does not significantly alter total RNA levels. Once another feature is lost, such as promoter methylation, *TIMP1* is stably expressed from the Xi at levels similar to the Xa. I hypothesize the following model (Fig. 6.1) after combining the analysis of promoter methylation, nuclease sensitivity, and acetylation of histone H3 in hybrids with several levels of *TIMP1* expression:

A. When all the repressive features are present, *TIMP1* is stably inactivated and cell lines do not express *TIMP1* from the Xi despite extended periods of time in tissue culture.

B. The acetylation of histone H3 tails leads to a slight relaxation of the chromatin structure, perhaps by interfering with internucleosomal packaging. A transcription factor occasionally binds with the limited access to the promoter and unstable, low level *TIMP1* expression results. This transcription factor must act on a methylated substrate. One possible player is the Sp1 transcription factor as it binds DNA and activates transcription even when the binding site is methylated (Holler et al. 1988). There are three Sp1 sites near the minimal *TIMP1* promoter. The majority of clones at this stage have unstable expression but 1% of clones begin to express *TIMP1* at levels comparable to the Xa.

C. Once methylation and a higher order of chromatin packaging are removed, the promoter is similar to that on the Xa and the *TIMP1* gene is fully transcribed.

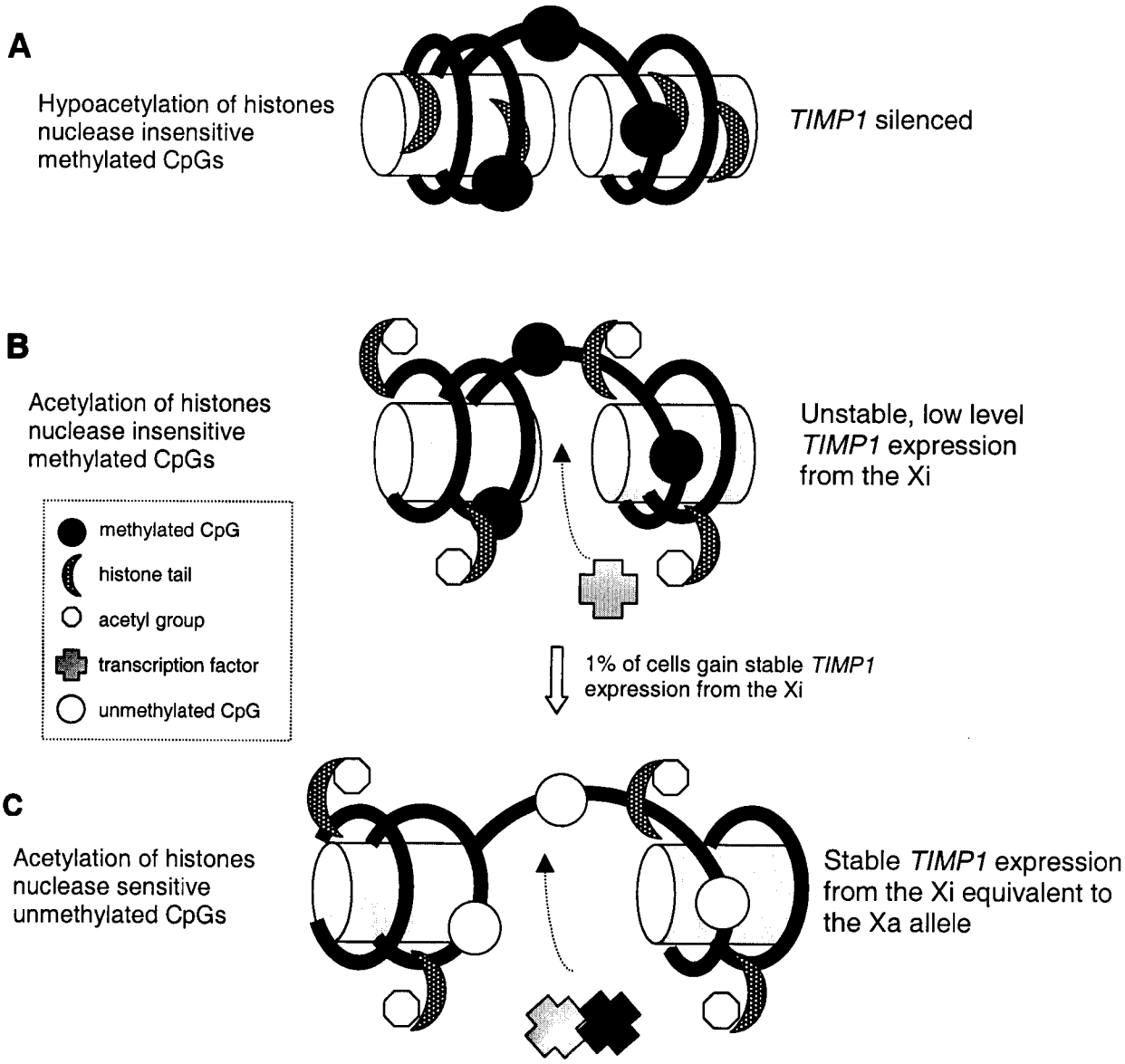

Figure 6.1. Hierarchy of epigenetic features in the inactivation of *TIMP1*.

A. *TIMP1* expression is completely silenced when epigenetic features are similar to other inactivated genes. (Xi hybrids) **B.** There is a slight relaxation of the chromatin structure when histone H3 is acetylated which allows a transcription factor access to the promoter. (Xi+ hybrids) **C.** Full transcriptional activation occurs when the promoter has lost the epigenetic features associated with X inactivation. The two joined transcription factors represent the Jun/Fos heterodimers that act at the AP-1 sites of the *TIMP1* promoter.

6.4 Generality of the hierarchy

I examined *TIMP1* inactivation as a paradigm of gene silencing and I have shown that there are layers of inactivation. Moreover, dosage compensation can be maintained when some suppressive features are still present. For the *TIMP1* gene, the epigenetic features were not equivalent because acetylation preceded expression in all three Xi+ hybrids, suggesting that hyperacetylation promotes *TIMP1* expression from the Xi. Demethylation and nuclease sensitivity were secondary events concordant with stable expression. Different epigenetic features may be the primary determinant of expression status for other genes. Clusters of genes which all escape inactivation as contiguous groups, such as seen in the pseudoautosomal regions, may have additional regional effects that are important, whereas promoter-specific features may be more important to influence the expression of "solo" genes expressed from the Xi surrounded by genes subject to X inactivation. For instance, CpG methylation or histone acetylation at the promoter may affect the *ZFX* gene more than the pseudoautosomal gene *MIC2*. The role of each epigenetic feature associated with silencing is most likely determined by its context and can mean numerous things depending on its current situation. It is only through the type of molecular analysis in this thesis will we begin to understand the complexities of gene regulation.

6.5 Further Investigations

To fully understand the mechanism of *TIMP1* expression from the Xi, additional features of the epigenetic control of *TIMP1* inactivation should be examined. A late replicating chromosome is one of the first distinguishing characteristics of the Xi and it seems to be an integral part of the inactivation process. The replication timing of the *TIMP1* locus could be compared between Xi with and without *TIMP1* expression to

determine where late replication timing fits into the hierarchy established above. It would also be interesting to examine more histone variants to determine if acetylation of histone H3 is the primary modification allowing *TIMP1* expression from the Xi. Additionally, Xi hybrids could be treated with the histone deacetylase inhibitor, TSA, to determine whether histone acetylation alone allows *TIMP1* expression from the Xi. If acetylation of histones is the only requirement, this treatment should create a situation similar to the Xi+ hybrids.

There are 12 other genes currently known to be variably inactivated from the human Xi (Carrel et al. 1999). It will be interesting to analyze the histone acetylation patterns at their promoters to determine whether acetylation of histone H3 is a common mechanism for all the genes or if it is unique to the *TIMP1* gene. Comparing promoter features such as extent of CpG methylation and the number of LINEs (proposed way stations) may also provide clues to polymorphic X inactivation.

The *TIMP1* gene was only expressed from the Xi in a subset of cells so there may be sequence differences underlying the variable X inactivation. The analysis of *TIMP1* expression in families with WAS should determine whether the reactivation is inherited with the *TIMP1* gene. The *TIMP1* sequence could be compared between Xi and Xi+ chromosomes to find any sequence differences that leads to *TIMP1* expression from the Xi. A "predisposing polymorphism" could be used to identify women that express *TIMP1* from the Xi who may be prone to fibrotic disorders due to increased inhibition of metalloproteinase activity.

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APPENDIX A - GENOTYPING THE TIMP1 C/T POLYMORPHISM.

Cell line	Cell type	Family/Group	*TIMP1*	
			Allele1	Allele2
GM7005	lymphoblast	CEPH 1331	C	C
GM7023	lymphoblast	CEPH 1331	C	C
GM7059	lymphoblast	CEPH 1331	C	T
GM7033	lymphoblast	CEPH 1331	C	-
GM7016	lymphoblast	CEPH 1331	C	-
GM7340	lymphoblast	CEPH 1331	C	T
GM7050	lymphoblast	CEPH 1331	C	T
GM6990	lymphoblast	CEPH 1331	C	T
GM6988	lymphoblast	CEPH 1331	C	C
GM6999	lymphoblast	CEPH 1331	C	C
GM11818	lymphoblast	CEPH 1331	C	T
GM7009	lymphoblast	CEPH 1333	T	-
GM7002	lymphoblast	CEPH 1333	T	T
GM7017	lymphoblast	CEPH 1333	T	-
GM7341	lymphoblast	CEPH 1333	C	T
GM6987	lymphoblast	CEPH 1333	C	T
GM7011	lymphoblast	CEPH 1333	C	T
GM11820	lymphoblast	CEPH 1333	T	T
GM7348	lymphoblast	CEPH 1345	C	T
GM7350	lymphoblast	CEPH 1345	C	C
GM7357	lymphoblast	CEPH 1345	C	-
GM7346	lymphoblast	CEPH 1345	C	T
GM7345	lymphoblast	CEPH 1345	C	T
GM7354	lymphoblast	CEPH 1345	C	T
GM01416D	lymphoblast		T	T
HSC593	lymphoblast	mother 59-25	C	T
59-25	lymphoblast		C	C
SA70	lymphoblast		C	T
AG	lymphoblast		T	T
11197	lymphoblast		T	T
11198	lymphoblast		T	T
11199	lymphoblast		C	T
11200	lymphoblast		T	-
11201	lymphoblast		C	T
GM01813	fibroblast		C	T
GM02859	fibroblast		C	T
pt 07	blood		C	T
pt 09	blood		C	T
10-02	blood	family 10	C	T
10-01	blood	family 10	C	C
t75-Xa	mouse hybrid	GM01813 Xa	T	-
t75-Xi	mouse hybrid	GM01813 Xi	C	-
t48-Xi	mouse hybrid	GM02859 Xi	C	-
t11-Xi	mouse hybrid		T	-
t86-Xi	mouse hybrid		C	-

Cell line	Cell type	Family/Group	*TIMP1*	
			Allele1	Allele2
t81-Xi	mouse hybrid		T	-
t60-12	mouse hybrid		T	-
AHA-11aB1	mouse hybrid		T	-
A23-1aC15	mouse hybrid		C	-
tSA70	mouse hybrid	SA70 Xa	C	-
GM06318D	hamster hybrid		C	-
tHM-34-2A-41B	mouse hybrid		T	-
tHM-34-2A-3az1a	mouse hybrid		T	-
tHM-34-2A-3HAT1E	mouse hybrid		C	-
tHM-34-1a-2az1D	mouse hybrid		C	-
2443	lymphoblast	WAS1	C	T
4023	lymphoblast	WAS1	C	T
3239	lymphoblast	WAS2	T	T
3149	lymphoblast	WAS2	C	T
3506	lymphoblast	WAS3	C	T
2500	lymphoblast	WAS3	T	T
3675	lymphoblast	WAS4	C	T
3671	lymphoblast	WAS4	C	C
2036	lymphoblast	WAS5	T	T
1341	lymphoblast	WAS5	C	T
3096	lymphoblast	WAS6	C	T
3098	lymphoblast	WAS6	C	T
2659	lymphoblast	WAS7	C	T
3815	lymphoblast	WAS7	C	C
4140	lymphoblast	WAS8	T	T
4192	lymphoblast	WAS8	T	T
1440	lymphoblast	WAS9	C	T
1443	lymphoblast	WAS9	T	T
1206	lymphoblast	WAS9	T	T
7079	lymphoblast	WAS10	~~C~~	~~C~~
3259	lymphoblast	WAS10	~~C~~	~~C~~
3251	lymphoblast	WAS10	~~C~~	~~T~~
3413	lymphoblast	WAS10	~~C~~	~~C~~
WAS1	lymphoblast	WAS11	C	C
5662	lymphoblast	WAS11	C	C
WAS2	lymphoblast	WAS12	~~C~~	~~T~~
4018	lymphoblast	WAS12	~~C~~	~~T~~
3554	lymphoblast	WAS13	C	T
3411	lymphoblast	WAS13	C	T
3417	lymphoblast	WAS13	T	T
2524	lymphoblast	WAS14	C	C
4241	lymphoblast	WAS14	C	C
3604	lymphoblast	WAS15	C	C
3757	lymphoblast	WAS15	C	T
RSA 1	blood		C	C
RSA 2	blood		C	T
RSA 3	blood		C	T

Cell line	Cell type	Family/Group	*TIMP1*	
			Allele1	Allele2
RSA 4	blood		C	C
RSA 5	blood		T	T
RSA 6	blood		C	T
RSA 7	blood		C	T
RSA 8	blood		C	C
RSA 9	blood		T	T
RSA 10	blood		C	C
RSA 11	blood		C	C
RSA 12	blood		T	T
RSA 13	blood		C	T
RSA 14	blood		C	T
RSA 15	blood		C	T
RSA 16	blood		C	C
RSA 17	blood		C	T
RSA 18	blood		T	T
RSA 21	blood		C	T
RSA 22	blood		C	T
RSA 23	blood		T	T
RSA 24	blood		C	T
RSA 25	blood		C	T
RSA 26	blood		C	C
RSA 27	blood		C	C
RSA 28	blood		C	T
RSA 29	blood		C	C
RSA 30	blood		T	T
RSA 31	blood		C	C
RSA 32	blood		C	T
RSA 33	blood		T	T
RSA 34	blood		C	T
RSA 35	blood		C	T
RSA 36	blood		C	T
RSA 37	blood		C	T
RSA 38	blood		C	T
RSA 40	blood		C	C
RSA 41	blood		C	C
RSA 42	blood		C	C
RSA 43	blood		C	T
RSA 44	blood		C	C
RSA 45	blood		T	T
RSA 46	blood		C	T
RSA 47	blood		T	T
RSA 48	blood		C	T
RSA 49	blood		T	T
RSA 50	blood		T	T
RSA 52	blood		T	T
RSA 53	blood		C	C
RSA 54	blood		C	C

Cell line	Cell type	Family/Group	*TIMP1*	
			Allele1	Allele2
RSA 55	blood		C	C
RSA 56	blood		C	C
RSA 57	blood		C	T
RSA 70	blood		C	C
RSA 78	blood		C	C
RSA 80	blood		C	C
RSA 89	blood		C	T
RSA 108	blood		T	T
RSA 118	blood		C	T
RSA 122	blood		C	T
RSA 141	blood		T	T
RSA 160	blood		C	T
C004	lymphoma		C	C
C172	lymphoma		C	T
C168	lymphoma		C	T
C528	lymphoma		C	T
C107	lymphoma		C	T
C369	lymphoma		T	T
C24	lymphoma		C	T
C311	lymphoma		T	T
C128	lymphoma		C	T
(no name)	lymphoma		C	T
C334	lymphoma		C	C
C441	lymphoma		C	T
C29	lymphoma		T	-
C101	lymphoma		T	-
C401	lymphoma		T	-
C443	lymphoma		C	-
C45	lymphoma		T	-
C121	lymphoma		T	-
C185	lymphoma		C	-
C200	lymphoma		C	-
C283	lymphoma		T	-
01-03	blood from	46 del (X)(q22-q26)	T	T
PA-1	ovarian teratocarcinoma		C	T
NTera-1	embryonal carcinoma		T	T
NTera-2	embryonal carcinoma		T	-
NCCIT	teratocarcinoma		T	-
1304	embryonal carcinoma		C	C
Px5	placenta		T	T
Px6	placenta		C	C
Px7	placenta		T	T
Px8	placenta		T	T
Px9	placenta		C	C
TX26	placenta		T	-
TX22	placenta		C	T
XE45	blood	mother XE51	~~C~~	~~T~~

Cell line	Cell type	Family/Group	*TIMP1*	
			Allele1	Allele2
XE51	placenta		~~C~~	~~T~~
YB10	blood	mother YB12	T	T
YB12	placenta		T	T

The allele frequency is 49% C and 51% T, with heterozygosity of 48%. The alleles listed in grey (C T) were already counted in other individuals because the alleles are identical by decent. The crossed out alleles (~~C T~~) were removed from the calculation of allele frequency because I was unable to determine which allele was shared within a set of samples.

APPENDIX B – SEQUENCE COMPARISONS AT THE 5' END OF THE TIMP1 GENE

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      1            15 16           30 31           45 46           60 61           75 76           90
1 AHA GGTGGGTGGAGAGTA ATGCATCCAGGAAGC CTGGAGGCCTGTGGT TTCCGCACCCGCTGC CACCCCCGCCCCTAG CGTGGACATTTATCC
2 A23 GGTGGGTGGAGAGTA ATGCATCCAGGAAGC CTGGAGGCCTGTGGT TTCCGCACCCGCTGC CACCCCCGCCCCTAG CGTGGACATTTATCC
3 t75 -GGGGGTGGAGAGTA ATGCATCCAGGAAGC CTGGAGGCCTGTGGT TTCCGCACCCGCTGC CACCCCCGCCCCTAG CGTGGACATTTATCC

      91          105 106          120 121          135 136          150 151          165 166          180
1 AHA TCTAGCGCTCAGGCC CTGCCGCCATCGCCG CAGATCCAGCGCCCA GAGAGACACCAGAGG TAAGCAGGGCCCGGG GTGGCCCAGCAGGGA
2 A23 TCTAGCGCTCAGGCC CTGCCGCCATCGCCG CAGATCCAGCGCCCA GAGAGACACCAGAGG TAAGCAGGGCCCGGG GTGGCCCAGCAGGGA
3 t75 TCTAGCGCTCAGGCC CTGCCGCCATCGCCG CAGATCCAGCGCCCA GAGAGACACCAGAGG TAAGCAGGGCCCGGG GTGGCCCAGCAGGGA

      181         195 196         210 211         225 226         240 241         255 256         270
1 AHA CCGGAGCTGGGCTGC AGCTTGGGTGCTGGC CACCAGGCCCGTGCA CTCCCGTGCCAGATG CCTGTCTACTCAGCT TGGCCTGCGGGTACC
2 A23 CCGGAGCTGGGCTGC AGCTTGGGTGCTGGC CACCAGGCCCGTGCA CTCCCGTGCCAGATG CCTGTCTACTCAGCT TGGCCTGCGGGTACC
3 t75 CCGGAGCTGGGCTGC AGCTTGGGTGCTGGC CACCAGGCCCGTGCA CTCCCGTGCCAGATG CCTGTCTACTCAGCT TGGCCTGCGGGTACC

      271         285 286         300 301         315 316         330 331         345 346         360
1 AHA AGGACCCTGGGCTAG TCTAGGGGGAAGAGG GTCGGAGGCTGGAAC TGCTTTCCCAACCCC GAGGCTCCAAACTCC CTAGAACCCCTGACA
2 A23 AGGACCCTGGGCTAG TCTAGGGGGAAGAGG GTCGGAGGCTGGAAC TGCTTTCCCAACCCC GAGGCTCCAAACTCC CTAGAACCCCTGACA
3 t75 AGGACCCTGGGCTAG TCTAGGGGGAAGAGG GTCGGAGGCTGGAAC TGCTTTCCCAACCCC GAGGCTCCAAACTCC CTAGAACCCCTGACA

      361         375 376         390 391         405 406         420 421         435 436         450
1 AHA TCCGCCCCCAATTCC CCCAAACCCATGACC CCCTAATATCACAAA TGCTTTCCAAATCCC CCCGCAAATTCCTTC ATCCCGAAATTTCCC
2 A23 TCCGCCCCCAATTCC CCCAAACCCATGACC CCCTAATATCACAAA TGCTTTCCAAATCCC CCCGCAAATTCCTTC ATCCCGAAATTTCCC
3 t75 TCCGCCCCCAATTCC CCCAAACCCATGACC CCCTAATATCACAAA TGCTTTCCAAATCCC CCCGCAAATTCCTTC ATCCCGAAATTTCCC

      451         465 466         480 481         495 496         510 511         525 526         540
1 AHA TCATCGCCCCCTAAA TACCCACCGCTAACC CCTGCAGCTCCCTAA ACTCCCCCAGCTCCC CAATCCCCAGTTCCC CAGCTCCCAAACTTC
2 A23 TCATCGCCCCCTAAA TACCCACCGCTAACC CCTGCAGCTCCCTAA ACTCCCCCAGCTCCC CAATCCCCAGTTCCC CAGCTCCCAAACTTC
3 t75 TCATCGCCCCCTAAA TACCCACCGCTAACC CCTGCAGCTCCCTAA ACTCCCCCAGCTCCC CAATCCCCAGTTCCC CAGCTCCCAAACTTC

      541         555 556         570 571         585 586         600 601         615 616         630
1 AHA TTTTGCCCTCAAATT CCTCAAGTATCCCCA TTGCCTTAAGCCCCC AAATTCCCCCAACCC CTTCAACCTCCAAAC TCCCCCAGCTCCCTA
2 A23 TTTTGCCCTCAAATT CCTCAAGTATCCCCA TTGCCTTAAGCCCCC AAATTCCCCCAACCC CTTCAACCTCCAAAC TCCCCCAGCTCCCTA
3 t75 TTTTGCCCTCAAATT CCTCAAGTATCCCCA TTGCCTTAAGCCCCC AAATTCCCCCAACCC CTTCAACCTCCAAAC TCCCCCAGCTCCCTA

      631         645 646         660 661         675 676         690 691         705 706         720
1 AHA AACCCCCAACCTCCT CCAAATTCCTCAACC TCCCTAAATTCCCCC AGCCTCTTATATCTC TTAATATACACAACC CCTAAACTCTGCCGT
2 A23 AACCCCCAACCTCCT CCAAATTCCTCAACC TCCCTAAATTCCCCC AGCCTCTTATATCTC TTAATATACACAACC CCTAAACTCTGCCGT
3 t75 AACCCCCAACCTCCT CCAAATTCCTCAACC TCCCTAAATTCCCCC AGCCTCTTATATCTC TTAATATACACAACC CCTAAACTCTGCCGT

      721         735 736         750 751         765 766         780 781         795 796         810
1 AHA CTCCAAACTCCTCCA GCCCCCAATCCCAAA TTCCTCACCCCCAAA CTCCTCAACCTCCAG AATTCCCCAAT-CCC CTCATTCCTCAAACT
2 A23 CTCCAAACTCCTCCA GCCCCCAATCCCAAA TTCCTCACCCCCAAA CTCCTCAACCTCCAG AATTCCCCAAT-CCC CTCATTCCTCAAACT
3 t75 CTCCAAACTCCTCCA GCCCCCAATCCCAAA TTCCTCACCCCCAAA CTNCTNAACCTNCAG AATTNCCCAATTCCC CTNATTCCTCAAACT

      811         825 826         840 841         855
1 AHA TCCTCAGCCCCCTTC TCCCCCAAGTGCCAC AACCCCATAACCCCG
2 A23 TCCTCAGCCCCCTTC TCCCCCAAGTGCCAC AACCCCATAACCCCG
3 t75 TNCTCAGCCCCCTT- TTCCCCAAGNGCCAC AACCCCATAACCCCG
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The sequences at the 5' end of *TIMP1* are identical for the two Xa hybrids with the highest and lowest expression levels (AHA-11aB1 and A23-1aC15 respectively) and one of the hybrids that expressed *TIMP1* from the Xi. The sequences were single read of PCR products and the sequences were aligned using CLUSTAL (BCM Search Launcher).