

LETTERS

Genomic Organization of the Human Tissue Inhibitor of Metalloproteinases-3 (TIMP3)

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The tissue inhibitors of metalloproteinases (TIMPs) play a crucial role in the physiological turnover of the extracellular matrix (ECM) by tightly regulating matrix metalloproteinase (MMP) activities. Disturbances in the TIMP/MMP system have been implicated in many disease processes where loss of ECM integrity is a principal feature. More recently, we have shown that mutations in TIMP3 cause the autosomal dominant disorder Sorsby's fundus dystrophy (SFD). This is a macular degeneration disorder with characteristic ECM irregularities in Bruch's membrane. To further facilitate mutational analysis and to provide a basis for functional studies, we now report the genomic organization of the human TIMP3 gene.

Cell-cell contacts in multicellular organisms are mediated through the extracellular matrix (ECM), which consists of a highly complex aggregate of secreted proteins and carbohydrates. In response to normal developmental changes and tissue turnover, the components of the ECM are continuously subjected to degradation and resynthesis. Disturbances in these dynamic processes may lead to loss of ECM integrity, which is thought to play an important role in many pathological conditions (Docherty et al. 1992).

The coordinated remodeling of the ECM requires the tightly controlled activity of a number of proteinases, including the family of matrix metalloproteinases (MMPs). These MMPs are able to degrade all components of the ECM and the basement membranes (Matrisian 1990). The regulation of the MMPs occurs at many levels and includes the tissue inhibitors of metalloproteinases (TIMPs). To date, three members of the TIMP gene family have been identified: TIMP1 (Docherty et al. 1985), TIMP2 (Boone et al. 1990), and TIMP3 (Apte et al. 1994a; Silbiger et al. 1994).

The implication of TIMP3 in the pathogenesis of Sorsby's fundus dystrophy (SFD) (Weber et al. 1994) has emphasized further the importance of the inhibitors in ECM homeostasis. SFD is an adult onset macular degeneration characterized by complications arising from ECM disturbances in Bruch's membrane (Capon et al. 1989). How-

ever, the exact molecular mechanisms by which these mutations cause SFD are still unknown. To facilitate the mutational and functional analysis of TIMP3, we determined the genomic structure, identified the exon/intron sequences of the gene, and analyzed the putative promotor region by an interspecies comparison with the mouse *Timp3* promotor.

RESULTS AND DISCUSSION

Genomic Organization of the Human TIMP3 Gene

Overlapping retinal TIMP3 cDNA probes were used to screen two flow-sorted chromosome 22-specific cosmid libraries. Five independent genomic cosmid clones (I-224, P-118, B11B11, A12G11, and D8H6) were isolated and assembled into a contig spanning ~100 kb of genomic DNA (Fig. 1). To identify fragments containing exonic sequences, cDNA probes were hybridized to *EcoRI*- or *PstI*-restricted cosmid DNAs. Three positive *EcoRI* fragments (6.5 kb derived from B11B11; 1.4 kb and 2.5 kb, both derived from D8H6), as well as a 0.3- and a 5.0-kb *PstI* fragment (both derived from I-224) were subcloned and partially sequenced. Subsequently, alignment of the genomic sequences to the published TIMP3 cDNAs revealed the exon/intron organization of the human TIMP3 gene that is encoded by 5 exons (Fig. 1) with the most likely assignments of donor and acceptor splice junctions following the 5'-GT-AG-3' rule (Breathnach et al. 1978).

Exon 1 contains the translation initiation start codon ATG as well as 280 bp of upstream

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STÖHR ET AL.

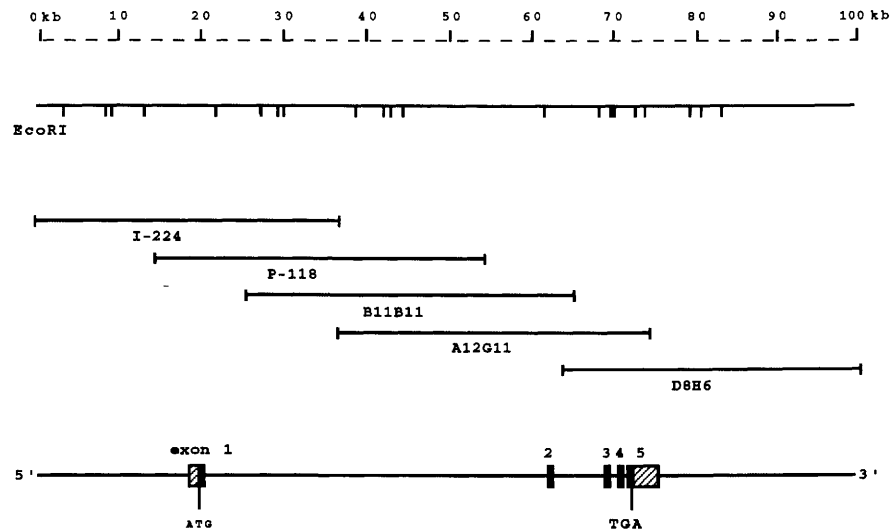


Figure 1 Structure of the human TIMP-3 gene. The genomic organization was deduced from five overlapping genomic clones (I-224, P-118, B11B11, A12G11, and D8H6) indicated by solid lines. *EcoRI* restriction sites within the genomic clones are shown as solid bars and the size of the contig is represented as a broken line above the restriction map. The relative positions of the 5 exons are based on their location in the respective *EcoRI* fragment and are shown below the cosmids. Coding exons are indicated by solid boxes, 5'- and 3'-flanking regions by hatched boxes, and introns by solid lines.

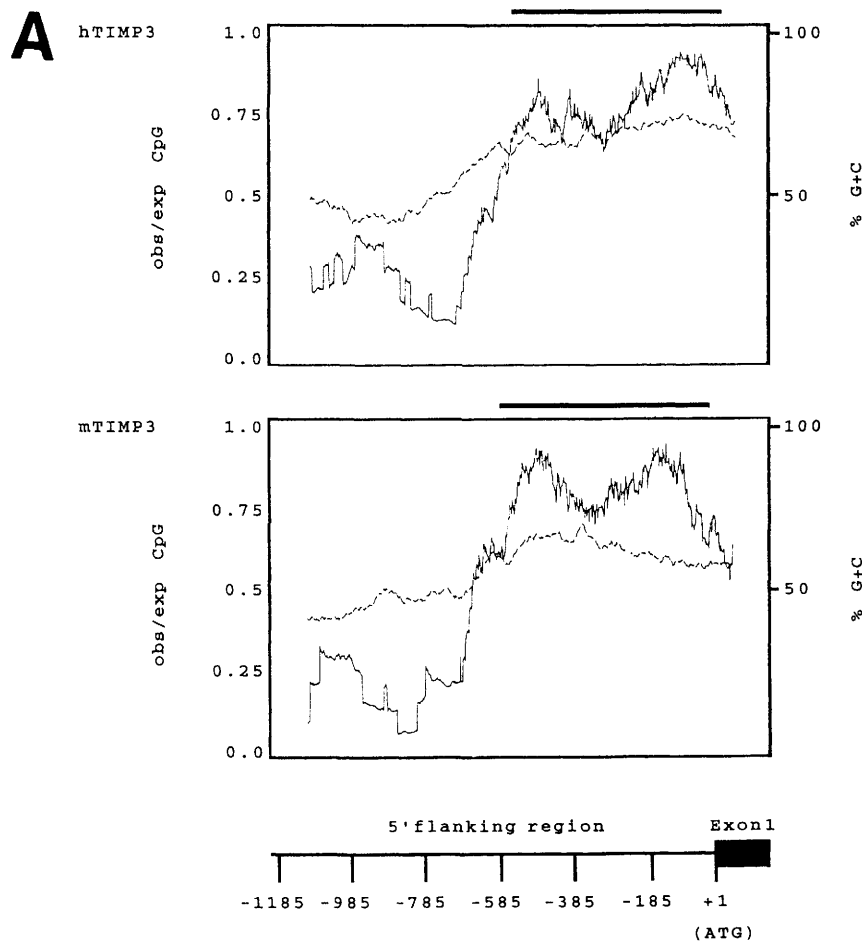
sequence corresponding to the most 5'-extending cDNA isolated (Silbiger et al. 1994). This suggests that the 5'-flanking region of the TIMP3 gene is not interrupted further by intervening sequences. Exons 2–4 are 84, 112, and 122 bp, respectively. The 2.5-kb *EcoRI* fragment from cosmid clone D8H6 contains exon 5 with the translation stop codon TGA and an additional 1057 bp of 3'-untranslated region. An *EcoRI* restriction site 1057 bp downstream of the stop codon is also present in published TIMP3 cDNAs (Byrne et al. 1995).

The overall organization of the human TIMP3 gene seems very similar to that of the recently reported murine homolog (Apte et al. 1995). A comparison between human and murine exon/intron splice junctions reveals that the coding regions in both species are interrupted by intervening sequences at identical positions within the respective triplet codons. In addition, a similar conservation of the relative locations of the exon/intron boundaries has also been reported for the murine *Timp3* and *Timp1* genes (Apte et al. 1995). This suggests that the TIMP gene family might have evolved from a common ancestor and might have arisen by duplication of an ancient precursor TIMP locus.

Comparison of the 5'-flanking Regions of the Human and Mouse TIMP3 Genes

Assuming very similar functions of the highly homologous TIMP3 genes in human and mouse (Silbiger et al. 1994; Leco et al. 1994), the regulation of TIMP3 expression should be subjected to strong evolutionary constraints for preservation of putative regulatory elements. Therefore, we compared ~1200 bp of the 5'-flanking region of

Figure 2 Comparison between the 5' flanking regions of human and murine TIMP3 gene. (A) Schematic representation of the G + C content and frequency of CpG dinucleotides across the coding region of exon 1 and 1184 bp of the 5'-flanking region of the human and murine TIMP3 genes. The G + C composition is indicated by a broken line; the observed versus expected frequency of CpG (obs/exp CpG) dinucleotides by a solid line along the sequence. Human and murine CpG islands that fulfill the criteria of moving averages of >50% G + C and a value of >0.6 for observed versus expected frequency of CpG dinucleotides (Gardiner-Garden and Frommer 1987) are shown by thick bars above the graphs. Below the plot, the relative position of the coding region of exon 1 is indicated as a solid box, indicating the translation start codon. The solid line represents 5'-flanking sequence, and the numbers below correspond to base pairs relative to the start codon ATG, with A being +1. (B) BESTFIT alignment of the nucleotide sequences of the putative promoter regions of human and murine TIMP3. Sequence data for the mouse gene are taken from Sun et al. (1995). The translation codon is marked by a solid box. The asterisks above the human and below the murine sequence denote the beginning of the longest isolated cDNA clones in the corresponding species (Leco et al. 1994; Byrne et al. 1995); the solid circle below the nucleotide adenine at position -316 indicates the major transcription start site of the mouse *Timp3* gene (Sun et al. 1995). The G + C-rich cluster with significant sequence similarity between human and mouse as well as the conserved putative binding sites for SP1 and a possible TATA box are boxed.



B hTIMP3
mTimP3

TTTCCTCTNTNCTGCTCTCTCCAGAGAAACTGGAGGGTAGCAGTT.....AGCATTCCTCCC...GCTGGTTCCACCAAGCACAGTCAAGGTC.TC -706
TTTCCTCTTACTCCCTGCTCTCTTAGGAAAGATAAGAAGGTGAAAATTCTCTYTGAGGCTTCTCTTGACTTTTTCAGGAGCGGTGATCAAGGACTTC -685

TAGGACATGGCCACCCCTCACCTGTGGAAGCGGTCTGCTG...GGTGGTGGGTGTAGTTGGTTCTGGTTGGGTTCAGAGACACCCAGTGGCCCAAG -609
AGGGTGGCGGCCACCTGGGGCCCTTGAAGAGATTATGCAGGAAGGCTGGGTGGCTGGGACTGAATCCTTGTATTAGGTCCAAGGCTCATAGAGAGGC.GG -586

TGGCGTGGGCGCCAGGGCGCAGAGCAGAGAAGGGGCACGAGGGCTCCGCTCCGAGGACCCAGCGGCAAGCACCCGGTCCCGGNGGGCCAGCCACCCACT -509
TGGCGTAGGGC.....GAAGGGCCGTGGGACCGCT.....GTAGCGCACACCGG.CCCAGGACCGCCAGCCACACCCCT -512

CGCGTGGCCACGGCGGCATTAATCCCTATAAAGGATCTGAACGATCCGGGGGCGGC...CCGCCC...GTTACCCCTTG.CCCCCGGCC...CCGCCCTTTTGTGA -410
CGCGTGGCCACAACGATTAATCCCTATAAAGGATCTGAACGATCCGGGGGCGGC...CCGCCC...GTCGCCCCGCGCCCCCGGC...CCGCCCTTTTGTGA -413

GGCCGATGAGGTAATGCGGCTCTGCCATTTGGTCTGAGGGGCGGCCCCAACAGCCGA...GGCGGGTCCCGGGGGCCAGCGCTATATCTACTCGGC -312
GGCCGATGAGGTAATGCGGCTCTGCCATTTGGTCTGAGGGGCGGCCCCAGCTCCCGAGGGGCTGGTCCCTCGCGCC...GATATCTACTCTGC -317

CGCCAGGCAGACGGCGCAGAGCGGCAGGCAGGCGGCGGCGCTCAGACGGTTCTCTCTCTCTTTGCTCTCCAGCTCTGCTCCTTGCCTGCGCGG -212
AGCCCTGGACTCTCGCTCACAGTGGGCAGCGGGCA.GCCGCCAGCGCAAGA.....ATCTTCTTCTCCCGCTTCTCCGCTTCCCGATCCCTTCCCGG -225

GAGGCCGCCCCGCGAGTCTTGGCCAGCGCCAGGCAGCTGCTGCGCCCC...ATCCCGTCCCGCGGGC.ACTCGGAGGGCAGCGCGCCGGAGGCCAA -116
GAGGCCACTCGTTGGTCCGCGGACTCG.TGTTCCAGCGACCCCTTGCCACTTAGTCCTGTCGCGGGCTACTTGAAGGCATTCGCCCGGAGCTCAT -126

GGTTTGCC.....CGCAGGCCCGG.....CGGGGAGCGAGCTCGGGTGCAGCAGCCCCCGCGCGCACGGCAACTTTGAGAGGGGAGCAGCA -28
CGTTGCCACCGTGCACAGTGCCTGTTAAACCAGCGAGTGAGCTCGGACTGTAGCATCAGCGCTAGC...CTCGCAACTTTGAAGAAAAGCGGCA -29

+1
GCCCCGCGAGCGG.CCGCAGCAGCGCAATGACCCCTTGGCTCGGGCTCATCTGTCTCTGGCCAGCTGGAGCCTGGGGGACTGGGGCGCGAGGCGTGC +72
GTCCCCGACGGACCACAACAGCTACCATGACTCCCTGGCTTGGCTTGTCTGTCTCTGAGCTGTGGAGCCTTGGGCACTGGGGCGCGGAAGCGTGC +72

Figure 2 (See facing page for legend.)

STÖHR ET AL.

human TIMP3 with published mouse *Timp3* sequences (Sun et al. 1995).

G + C content and observed versus expected frequency of CpG dinucleotides were determined along the entire 1184 bp of the 5'-flanking region of mouse and human TIMP3 (Fig. 2A). The segments around nucleotides + 1 to -580, especially, show high averages of > 50% G + C and values of > 0.6 for observed versus expected frequency of CpG dinucleotides, thus, fulfilling the criteria for the presence of true CpG islands in both species (Larsen et al. 1992) (Fig. 2A). Outside of this region, around nucleotides -1184 to -580, is a sharp decline in both the G + C content as well as in the observed versus expected CpG frequency similar to the observed suppression of CpGs in bulk genomic DNA (Fig. 2A).

Alignment of the putative human and mouse TIMP3 promoter regions revealed an overall sequence similarity of 75% (Fig. 2B). Significant sequence similarities were found in the area around nucleotides -522 to -307. In this stretch of DNA, we identified also three conserved putative Sp1 consensus binding sites at positions -452 to -447, -425 to -420, and -370 to -365 and a conserved TATA box at position -324 to -320 (numbers are relative to the human TIMP3 start codon). The longest human TIMP3 cDNA clone isolated starts at nucleotide -283 (Byrne et al. 1995), whereas the longest mouse cDNA begins at nucleotide -309 (Leco et al. 1994). A major transcription initiation site at position -316 has been defined for the mouse *Timp3* gene (Sun et al. 1995) (Fig. 2B). Other putative transcription factor binding sites (additional SP1 and AP1, PEA3, CTF/NF-1) were identified but did not appear to be highly conserved between human and mouse populations.

The presence of a potential TATA box in TIMP3 is interesting in light of the fact that in the other two family members—murine *Timp1* (Edwards et al. 1992) and human TIMP2 (DeClerck et al. 1994)—no such canonical TATA box has been identified. It has been suggested that TIMP1 and TIMP2 belong to the TATA box-deficient, G + C-rich promoter group (Edwards et al. 1992; DeClerck et al. 1994), which previously has been found to direct transcription of housekeeping genes (Dyan 1986), although in some cases tissue-specific transcription may be possible also (Oliva et al. 1991). TIMP3 expression appears to be different from TIMP1 and TIMP2 in that it has been detected in many organs but seems to be restricted to certain tissue components, for exam-

ple, the epithelial cells (Apte et al. 1994b). Thus, TIMP3 may be considered an intermediate between constitutively expressed genes and tissue- or cell-specific expressed genes.

Taken together, we have determined the gene structure of human TIMP3 and have shown that the 5 exons of this gene span ~55 kb of genomic DNA. We have provided sequence information on all exon/intron boundaries that will greatly facilitate the mutational analysis of TIMP3 in human genetic diseases. Besides its involvement in SFD, TIMP3 might also play a critical role in other human retinopathies (Jones et al. 1994), as well as in some debilitating disorders where loss of ECM integrity is a principal feature (Docherty et al. 1992). In addition, we have sequenced 1200 bp of the 5'-flanking region of the human TIMP3 gene and, by sequence alignment to the homologous region in mouse (Sun et al. 1995), have identified several highly conserved putative regulatory elements. Although these sites will have to be confirmed by binding assays and functional studies, those predictions may greatly facilitate the further characterization of the TIMP3 promoter region.

METHODS

For library screening TIMP3 cDNA probes were obtained by RT-PCR amplification of total human retinal RNA with three primer pairs designed according to published TIMP3 cDNA sequences (Silbiger et al. 1994). Primers used were 5'TIMP (5'-TTCTCCTCCTCCTTGC-3')/1FF (5'-ACGATGTCGGAGTTGCA-3'), 1F (5'-TGGAGCCTGGGG-GACTGG-3')/1R (5'-CTGGGAGAGGGTGAGCTGG-3'), and 2F (5'-CAACTTCGTGGAGAGGTGGG-3')/2R (5'-AGGGTCTGGCGCTCAGGG-3'). High-density gridded filters of chromosome 22-specific cosmid libraries were kindly provided by the Reference Library Data Base of the Imperial Cancer Research Fund, London, UK (constructed in Lawrist 4) and by Peter Scambler and Sara C.M. Daw, Institute of Child Health, London, UK (constructed in Lawrist 16 by the Lawrence Livermore National Laboratories, Livermore, CA).

Overlaps between positive clones were detected by Southern blot hybridizations of T3-, SP6-, and T7-endclone fragments to *EcoRI*-restricted digests of the cosmid DNAs. Exon-containing cosmid fragments were subcloned into the plasmid vector pBluescript SK(-) (Stratagene). Double-stranded sequencing was carried out using the dideoxy chain termination method with vector-specific T3 and T7 primers as well as internal exon-specific primers 5'TIMP, 1FF, 1F, 1R, 2F, and 2R. Exon/intron boundaries were identified by alignment of the genomic sequence with the published TIMP3 cDNA (Silbiger et al. 1994) using MacVector sequence analysis software (release 4.0). The identified splice sequences were in excellent agreement with published consensus sequences. The distribution of G/C content and observed versus expected frequency of CpG dinucleotides along the human and mouse 5' flanking se-

quence was plotted using the Wisconsin GCG package (version 8, 1994). For a window size of 200 bp the criteria for a CpG island are C + G > 50% and observed versus expected CpG > 0.6. Optimal alignment of the putative human and mouse promoter regions were achieved by using the GCG program BESTFIT.

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Note

The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL data bank under accession numbers U33110, U33111, U33112, U33113, and U33114.

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