Transcriptional activity of the human tissue inhibitor of metalloproteinases 1 (TIMP-1) gene in fibroblasts involves elements in the promoter, exon 1 and intron 1

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The active forms of all of the matrix metalloproteinases (MMPs) are inhibited by a family of specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Inhibition represents a major level of control of MMP activity. A detailed knowledge of the mechanisms controlling TIMP gene expression is therefore important. We have isolated a genomic clone of the human TIMP-1 gene. A 3 kbp *Xba*I fragment has been sequenced; this fragment contains 1718 bp 5′ flanking sequences, exon 1, a 929 bp intron 1 and part of exon 2. Computer analysis reveals 10 consensus sequences for Sp1, six for activating protein 1 (AP-1), six for polyoma enhancer A3 (PEA3), 12 for AP-2 and five CCAAT boxes. The region hybridizing with a murine TIMP-1 promoter fragment has been subcloned and analysed further. RNase protection identifies six transcription start points, making exon 1 up to 48 bp in length. Transient transfection of promoter–chloramphenicol *O*-acetyltransferase reporter constructs into primary human connective tissue fibroblasts shows that a 904 bp fragment that hybridizes to a murine TIMP-1 promoter fragment contains a functional promoter. Constructs of $-738/+95$ to $-194/+21$ are inducible with serum or phorbol ester to a similar extent to the endogenous TIMP-1 gene. These results and further mapping with 5′ deletion mutants from the $-738/+95$ region have demonstrated that an AP-1 site at $-92/-86$ is essential for basal expression of the gene. Point mutations within this region have further confirmed the role of this site, along with a more minor role for a neighbouring PEA3 site, in basal expression. Deletions from the 3′ end also implicate a region across the exon 1/intron 1 boundary and especially $+21$ to $+58$ in basal expression. The $+21/+58$ region contains a putative binding site for the transcription factor leader-binding protein 1 (LBP-1). Gel-shift analysis shows that protein binds specifically to this region, but competition studies suggest that it is unlikely to be LBP-1.

INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of enzymes involved in the turnover and degradation of extracellular matrix [1]. Controlled matrix turnover is essential for a number of physiological processes including uterine involution, embryogenesis, angiogenesis and wound healing. Furthermore, aberrant matrix turnover is involved in a number of pathologies including rheumatoid arthritis and osteoarthritis, tumour invasion and metastasis, corneal ulceration and liver fibrosis [2].

The active forms of all of the MMPs are inhibited by a family of specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [3]. Inhibition represents a major level of control of MMP activity and is thus a therapeutic target [4]. A detailed knowledge of the mechanisms controlling TIMP gene expression is therefore important.

The expression of TIMP-1 in connective tissue cells is regulated by cytokines and growth factors. A number of agents induce TIMP-1 expression including all-*trans*-retinoic acid, transforming growth factor β, interleukin 6, interleukin 11, leukaemia inhibitory factor and oncostatin M [5–8]. Where investigated, the control of TIMP-1 gene expression in connective tissue cells is at the level of transcription [9].

Previous work with the murine TIMP-1 gene promoter has identified serum- and phorbol ester-responsive elements including an activating protein 1 (AP-1) and polyoma enhancer A3 (PEA3) element proximal to the transcription start points. However, sequences more distal in the 5′ flanking region, as well as those within intron 1, are likely to have a role ([10,11], and references therein). Furthermore a 2 kbp fragment of the murine TIMP-1 gene (containing 5′ flanking DNA, exon 1 and intron 1 sequences) linked to β-galactosidase was required for normal developmental expression in the mouse [12]. More recently a viral transactivator of HTLV-1, Tax 1, was shown to induce TIMP-1 expression in human T-cell lines. Again, the proximal AP-1/PEA3 elements were involved in this induction [13].

In this study we have isolated a genomic clone of the human TIMP-1 gene, and a 3 kbp *Xba*I fragment has been sequenced. A

Abbreviations used: AP-1, activating protein 1; CAT, chloramphenicol *O*-acetyltransferase; FCS, fetal calf serum; LBP-1, leader-binding protein 1; MEM, minimal essential medium; MMP, matrix metalloproteinase; PEA3, polyoma enhancer A3; TIMP, tissue inhibitor of metalloproteinases; tsp, transcription start point.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Y09720.

904 bp *Kpn*I fragment hybridizing with a murine TIMP-1 promoter fragment has been subcloned and its activity has been examined in human connective tissue fibroblasts, where the gene is normally expressed. The transcription start points have been identified and transient transfection studies in such cells have been used to map the basal promoter.

EXPERIMENTAL

Cloning of the human TIMP-1 gene promoter

A human TIMP-1 genomic clone was isolated from a human X-chromosome library LA0XNL01 in the vector Charon 35 (ATCC). Screening was performed with the human cDNA and a clone of the murine TIMP-1 promoter region (−223/+47; [11]). A clone of 14.5 kbp (TIMP A1-1) was isolated and further restriction-mapped (see Figure 1). A 3 kbp *Xba*I fragment containing the 5′ flanking region was subcloned into pBluescript KS$^-$; this was further subdivided with *Kpn*I digestion to yield a 0.9 kbp fragment that hybridized with −223/+47 of the murine gene. The nucleotide sequence of the 3 kbp *Xba*I fragment was determined for both strands by the chain-termination method [14].

RNase protection assay

RNase protection was performed by hybridizing 1 μg of poly(A)$^+$ RNA from MRC-5 human fetal lung fibroblasts (or a tRNA control) with a [α-^{32}P]UTP-labelled anti-sense riboprobe of 817 nt from within the 0.9 kbp *Kpn*I fragment above. Hybridization was performed overnight at 42 °C in 80% formamide/40 mM Pipes (pH 6.4)/0.4 M NaCl/1 mM EDTA. This was followed by digestion with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) in 10 mM Tris/HCl (pH 7.5)/0.3 M NaCl/5 mM EDTA for 60 min at 30 °C. Protected fragments were then analysed on a sequencing gel next to a known DNA-sequencing reaction ([15], pp. 7.71–7.78).

Plasmid construction

Fragments of the human TIMP-1 gene promoter were subcloned into pBLCAT3 [16]. Initially, four constructs were made with *Pst*I (−738/+95), *Sau*3A (−564/+21), *Sma*I (−194/+58) and *Sma*I/*Sau*3A (−194/+21) sites. Further to this, deletion sets were generated from the −738/+95 fragment with exonuclease III digestion from the 5′ end (Erase a Base; Promega), or *Bal*31 exonuclease from the 3′ end ([17], vol. 1, p. 7.2.8). Point mutations were introduced by using oligonucleotide-based PCR methodology. All mutations were confirmed by sequencing.

Cell culture

MRC-5 cells were obtained from the European Cell Culture Collection; human foreskin fibroblasts were isolated by enzymic digestion as previously described [18]. Cells were maintained in minimum essential medium (MEM) supplemented with 1% non-essential amino acids, 10% (v/v) fetal calf serum (FCS), 100 i.u./ml penicillin, 100 μg/ml streptomycin and 20 units/ml nystatin (MEM/10% FCS). For basal conditions, 10% FCS was replaced with 0.1% BSA (MEM/0.1% BSA).

Transient transfection and reporter gene assay

Transient transfection with constructs in pBLCAT3 was performed into primary human skin fibroblasts by a modification of the method of Chen and Okayama [19]. Briefly, cells were plated at approx. 5000 cells/cm^2 into 60 mm dishes and allowed to adhere for 24 h in MEM/10% FCS. DNA precipitates containing 20 μg of plasmid DNA per plate were allowed to form in the culture medium during a 16 h incubation in an air/CO_2 (97:3) atmosphere at 37 °C. Cells were then washed three times with HBSS and medium was replaced with MEM/0.1% BSA (for stimulation experiments, MEM/10% FCS or MEM/0.1% BSA/10 nM PMA). After a 48 h incubation at 37 °C in air/CO_2 (19:1), cells were harvested by scraping. Extracts for chloramphenicol *O*-acetyltransferase (CAT) assay with an ELISA method (Boehringer-Mannheim) were prepared by freeze–thawing, and protein concentration was determined by the Bradford assay (Bio-Rad). Three plates of cells were used for each plasmid tested in each experiment. Experiments were performed at least three times with at least two different preparations of each plasmid.

Transfection efficiency was monitored with the Hirt assay [20]. Here, a nuclear extract from the transfected cells was probed with a CAT-specific cDNA on a slot blot. This result was used to normalize CAT levels where differences in transfection efficiency were observed.

Electrophoretic mobility shifts assay

Nuclear extracts were prepared from 5×10^7 human skin fibroblasts cultured in MEM/0.1% BSA as above for 48 h by a modification of the method of Dignam et al. [21]. Extracts typically contained 5–10 μg/μl protein.

Oligonucleotides were annealed in equimolar amounts and end-labelled with [γ-^{32}P]ATP followed by desalting through Sephadex G-25 spin columns. Binding reactions (10 μl) contained 4 mM Hepes, pH 7.9, 5 mM glycerol, 184 mM NaCl, 0.3 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.04 mM EDTA, 0.1 mM PMSF and 1 μg of poly(dI-dC). Probes (10 fmol) were added to the binding reaction (1 μl of nuclear extract) and incubated at room temperature for 15 min before separation on non-denaturing 7% (w/v) polyacrylamide gels. Gels were dried and autoradiographed. The oligonucleotides used were as follows: 37mer, 5′-GATCCAGCGCCCAGAGAGACACCAGAGGTAAGCAGGG-3′; 37m1, 5′-GATCCAGCGCCACTAGCGCCACCAGAGGTAAGCAGGG-3′; 37m2, 5′-GATCCAGCGCCACTAGCGCCACACTAGGTAAGCAGGG-3′; HIV, 5′-CTAACCAGAGAGACCCAGTAC-3′; random, 5′-CAAAGGGAATAAGTACTGGGCTGTTC-3′.

RESULTS

Cloning of the 5′ region of the human TIMP-1 gene

A 3 kbp *Xba*I fragment of the human TIMP-1 genomic clone (Figure 1) was subcloned into pBluescript KS$^-$. The nucleotide sequence of this fragment was determined by the dideoxy method and confirmed by sequencing both strands. This sequence was subjected to computer analysis with the GCG package. The fragment contained a putative exon 1 (sequences present at the 5′ end of the cDNA before the translation start site, followed by a splice junction donor site), as well as 1718 bp of 5′ flanking sequence, a 929 bp intron 1 sequence and the 5′ end of exon 2. Murine TIMP-1 has previously been reported to have an untranslated first exon [22], and this seems to be true of the human gene. The 3 kbp fragment contained 10 consensus sequences for Sp1, six for AP-1, six for PEA3 and 12 for AP-2, and five CCAAT boxes (Figure 2). There is no strong consensus TATA box, but a sequence (ATTTAT) about 20 bases upstream of the transcription start point might be a TATA box. Alignment of this sequence with published sequences for the 5′ ends of either

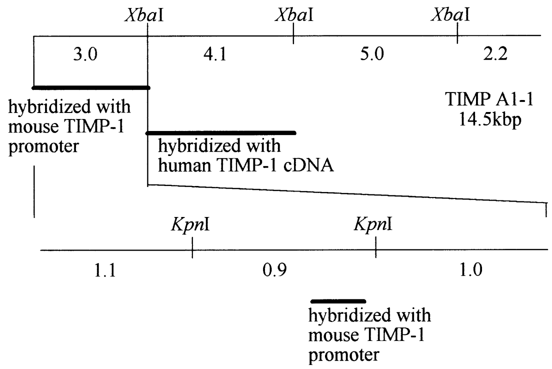

Figure 1 Genomic clone of human TIMP-1 gene

A clone of 14.5 kbp was isolated from a human X chromosome library. A 3 kbp *Xba*I fragment was subcloned and sequenced. Hybridization with the human TIMP-1 cDNA or the murine −223/+47 promoter fragment identified a 0.9 kbp *Kpn*I fragment, which was subcloned for further analysis.

mouse or rat TIMP-1 gives approx. 70 % identity. There are a number of insertions and deletions; intron 1 is 704 bp in the mouse gene, but 929 bp in the human.

Identification of transcription start points

The transcription start points (tsps) were identified by RNase protection with a riboprobe spanning the putative exon 1 between the 3′ *Kpn*I site and an upstream *Nae*I site. By using poly(A)$^{+}$ RNA from MRC-5 fibroblasts, six protected fragments were detected, ranging from 37 to 48 nucleotides in length (Figure 3). This indicates multiple trancription start points in the human TIMP-1 gene. The most 5′ start point is designated +1 in this study.

Transient transfections

The 3 kbp *Xba*I fragment was further cleaved with *Kpn*I to yield three fragments of approx. 1 kbp each. One of these fragments hybridized with −223/+47 of the murine TIMP-1 gene, and this fragment was analysed further.

Initially four fragments of the human TIMP-1 promoter were subcloned into pBLCAT3 and transiently transfected into MRC-

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TCTAGAGTCG ACCTGCAGCC CAAGCTTGGA TCTGACCAAT ATGGTGAAAC CCCCCTCTCT ACTAAAACTA  -1649

CAAAAATTAG CTGGGGGTGG TGGTGGTGTG GCTGTAATCC CAGCTACTCA GGAGGCTGAG ACAGGAGAAT  -1579
                                              AP-1       Sp1
CTCTTGAACC CCGGAGGCGG AGGTTGCAGT GAGCTGAGAT CCGCCACTGC ACTCCAGCCT AGGTGACAGA  -1509
              Sp1
GCAAGACTCT TGACAAAAAA AAAAAATAGA TTTAGAGGGT ACAGCTGCAG TTTTGTGACA TGGATATATT  -1439

ACATAGTGGT GAAGTCTGGG CTTTTAGTGT CCCCATCAAC CAAATAGTGT ACATTGTACC CATTAGGCAA  -1369

TTTCTCATCC CTCACATTCC TCCCACCCAG AATAGTGCTG GATACGCAGT AAGCACTCCA TAAGGGCTGC  -1299

TGGTAGTTTT TGTTGTGTTC TTGTTATTGG GCTATCATCC CTGAGTTAAT CAAACTTAAT TAGCAAGGGA  -1229
                                                AP-1
GTATGGTGAG TGAATGAGAT AATTTATACA AACACTTTAG CACATAGTAC ACAGAAAGTG CTCAATAAGT  -1159

GGTAGCCATC ATCACTACAA TTATTATTAT TGATCTATCC TCCTCGAATT AAGGATGTTA ATCAGAACCC  -1089
                                                            AP-1
CAGGGAAGGT TCCTGCATTG TCAGAAAGCT GGTGGGCAAG GATTGCCCCA CCCCACCCCC CACCAGGGTA  -1019

CAGGTCCATG GGGAGGGGGC AGGGCAGGAT GGGGTGGGGA TTAGTTTTCT ACTGACCCAC TCACTTGCTT  -949

CTTTCACCAT CATTCATGGC CACCCCCTTC CCAATGCTCA TTCATTCACC CGCACCATTC CCTGCTTGCT  -879

AGGACGGTGC CACTCCTGAG TTACAACCCT GGTGCCAGGT AGCCTCTGTT TGAATCCCGG CCATGCCTGT  -809

GATCTTAGGA AAATTCTACA GTTCCTCTAC GTACCCCATA TTTCCTCATC GTAAAACGGG AATAAGAACC  -739
    PEA3                                    PEA3
GGTACCCATC TCAGAGATTT GTTGTGAGCT TTGAGTGAGA TAAACTATGC TGAGTGCCTG GTATACAGTA  -669

                 ↓NaeI
GGTGCTGTAT AAATGCCGGC TATTTGCCTG TGTTATTTGA GACCCTGGCT TTGGCTCCTG GCCACCTGAG  -599

                                ↓Sau3A
TTCCAGTCTC AGTTCTGCCA TGTATTGACT CTGTGATCCT GGGTAAGTCA CTTAACCACT CCGTGCCTCA  -529

GTTTTCCCCA TTTTGTATTC CTCCCCTTTC ACCTGCCTTA TCTCCCTCCA CTGCTGCTAC TTAATTTGTT  -459

TCCTCTCTGC CACCCCTCAC CAGCATGTCA GACATACAAA ACAAGGGATT TTTGTGTGCT TGGCACACAG  -389
PEA3
TAGATGCACA ATAAATGTTG AAGGGCTGAA CTAATTTGGG TTTGAGTCAT AGGGAGCTTG GGGGATGTGG  -319
                                              AP-1
GTGATTGGAT AGATTCTGGA GACTTTAGGG GACTGGGCCG GGGGAAATGC GGCCTCTAAG CTCTCCGCTG  -249
 CCAAT-box
                                                         ↓SmaI
AGGCGGCTTG GAAGGAATAG TGACTGACGT GGAGGTGGGG GAGGTGGCTG GCCCGGGCGA GGCCCAGGGA  -179

GAGGGAGAGG AGGCGGGTGG GAGAGGAGGA GGGTGTATCT CCTTTCGTCG GCCCGCCCCT TGGCTTCTGC  -109
             Sp1                                         Sp1
ACTGATGGTG GGTGGATGAG TAATGCATCC AGGAAGCCTG GAGGCCTGTG GTTTCCGCAC CCGCTGCCAC  -39
                  AP-1           PEA3
                                     +1↓  ↓ ↓ ↓ ↓   ↓          ↓Sau3A
CCCCGCCCCT AGCGTGGACA TTTATCCTCT AGCGCTCAGG CCCTGCCGCC ATCGCCGCAG ATCCAGCGCC  +32
  Sp1                                     --------------------------------
                           ↓SmaI                                    ↓PstI
CAGAGAGACA CCAGAGGTAA GCAGGGCCCG GGGTGGCCCA GCAGGGACCG GAGCTGGGCT GCAGCTTGGG  +102
LBP1-------LBP1--
TGCTGGCCAC CAGGCCCGTG ACTCCCGTGC CAGATGCCTG TCTACTCAGC TTGGCTGCGG TACCAGGACC  +172
                                              AP-1
CTGGGCTAGT CTAGGGGGAA GAGGGTCGGA GGCTGGAACT GCTTTCCCAA CCCCGAGGCT CCAAACTCCC  +242
  Sp1
TAGAACCCCT GACATCCGCC CCCAATTCCC CCAAACCCAT GACCCCCTAA TATCACAAAT GCTTTCCAAA  +312
           PEA3 and Spl
TCCCCCCGCA AATTCCTTCA TCCCGAAATT TCCCTCATCG CCCCCTAAAT ACCCACCGCT AACCCCTGCA  +382

GCTCCCTAAA CTCCCCCAGC TCCCCAATCC CCAGTTCCCC AGCTCCCAAA CTTCTTTTGC CCTCAAATTC  +452
                        CCAAT-box
CTCAAGTATC CCCATTGCCT TAAGCCCCCA AATTCCCCCA ACCCCTTCAA CCTCCAAACT CCCCCAGCTC  +522

CCTAAACCCC CAACCTCCTC CAAATTCCTC AACCTCCCTA AATTCCCCCA GCCTCTTATA TCTCTTAATA  +592
                                                 Sp1
TACACAACCC CTAAACTCTG CCGTCTCCAA ACTCCTCCAG CCCCCAATCC CAAATTCCTC ACCCCCAAAC  +662
                                             CCAAT-box
TCCTCAACCT CCAGAATTCC CCAATCCCCT CATTCCTCAA ACTTCCTCAG CCCCCTTCTC CCCCAAGTGC  +732
                     CCAAT-box             PEA3 and Spl
CACAACCCCA TAACCCCGCA ACTCACTCAG TCCCTCAATA ATTTAAACTC TCCCAACTCT CTAAACCCTA  +802

AATTCCTCTG CGCTCCCCAA TCCCCTTAAA ATCCCTAAGC TTACCAACCC CTCAAGCCCA AATCCAGCCC  +872
              CCAAT-box
CCTAATCCCC CCCATAGGCT GCCCCGGGGC AAGATGGGGT GGATGACCTG CCCAGGTCAG CAGATCAGCT  +942

GGGCCGGGGC TGCCTGACAT CCCCCTTCCC CACAGAACCC ACCATGGCCC CCTTTGAGCC CCTGGCTTCT  +1012
                                    ----------------------------------
GGCATCCTGT TGTTGCTGTG GCTGATAGCC CCCAGCAGGC CTGCACCTGT GTCCCACCCC ACCCACAGAC  +1082
----------------------------------------------------------------------
GGCCTTCTGC AATTCCGACC TCGGTGAGTC CTCACCCCAC TCAGCCCACA CACTCTGCCT TGGTTTCCCT  +1152
------------------------------------------Sp1---------------------------
TTCAGTCCAG TGGGGCTCAC CATGGCACTT ACCTCT                                   +1188
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Figure 2 Nucleotide sequence of the 3 kbp genomic clone of human TIMP-1

The clone contains exon 1 with six tsps identified by RNase protection (bold arrows); 1718 nucleotides of 5′ flanking sequence; a 929 nucleotide intron 1, and the 5′ end of exon 2. Numbering is from the most 5′ tsp as +1. Consensus sequences for transcription factor binding are underlined (solid) and labelled. Exons are underlined with a broken line. Restriction sites used for subcloning are marked.

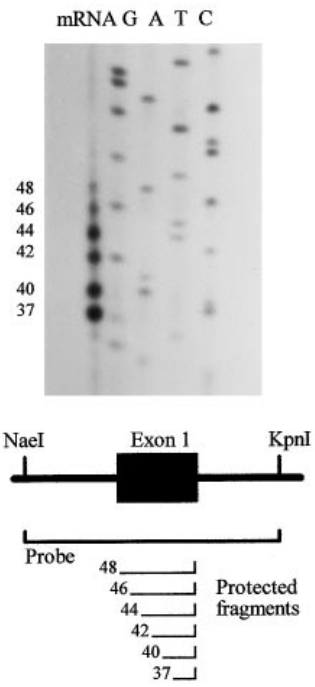

Figure 3 Localization of transcription start points in the human TIMP-1 gene

An RNase protection assay was performed by hybridizing 1 μg of poly(A)$^+$ RNA from MRC-5 cells with a riboprobe corresponding to the sequence from the *Kpn*I to *Nae*I sites in the 0.9 kbp clone (see the Experimental section). Six protected fragments were identified and sizes were assigned from a standard sequencing ladder. A tRNA control yielded no protected fragments (results not shown).

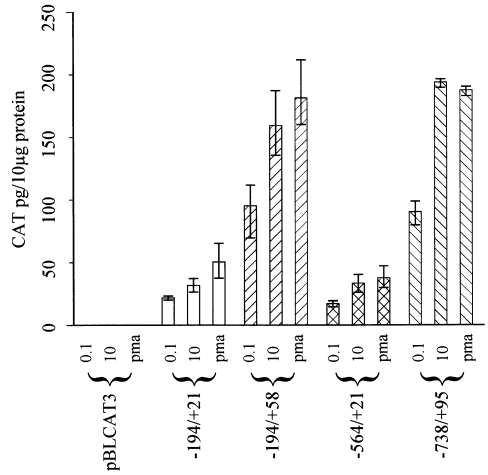

Figure 4 Transient transfection in MRC-5 cells

Plasmids as shown were transiently transfected into MRC-5 cells at passage 22 as described in the Experimental section. Cells were maintained in medium supplemented with 0.1% BSA (0.1), 10% (v/v) FCS (10) or 10 nM PMA in 0.1% BSA (pma) before harvest and CAT assay. Hirt's assay was used to monitor transfection efficiency. Results are plotted as means ± S.E.M.

5 cells or primary human skin fibroblasts; similar results were obtained in both cell types, and all subsequent experiments were performed in the skin fibroblasts. Figure 4 shows representative results from three experiments in MRC-5 cells, demonstrating that all constructs supported transcription under basal conditions. The $-738/+95$ and $-194/+58$ constructs gave approximately equal levels of CAT protein, which was 3–5-fold higher than the $-564/+21$ and the $-194/+21$ constructs. This suggests that the 37 bp region between $+21$ and $+58$ is important in basal transcription. However, all four constructs could be induced with either 10% FCS or 10 nM PMA by a similar amount, 1.5–3.3-fold and 1.9–3.2-fold respectively. This level of induction is similar to that of the endogenous TIMP-1 protein in this system as measured by specific ELISA (results not shown).

To map the regions in the promoter responsible for basal transcription, we made deletion sets from each end of the $-738/+95$ insert. Figure 5 shows a typical experiment with the 5′ deletion set. Progressive deletion of -738 to -102 gave a general increase in CAT protein, suggesting that this area might negatively regulate human TIMP-1 gene transcription. However, levels of CAT from constructs with end points in this region fluctuated appreciably, making it impossible to locate a specific negative element. Deletion from -102 to -80 led to a sharp decrease in CAT protein of 16–27-fold, whereas further deletion to -26 had no further effect and deletion through the transcription start sites to $+15$ brought CAT protein down to below detectable levels. Hence the sequence between -102 and -80 is important in basal transcription.

Computer analysis revealed an AP-1 site at $-92/-86$ and a PEA3 site at $-78/-73$; point mutations were made in either or both of these sites in the context of the $-102/+95$ construct, which gives high basal expression. Figure 6 shows that either deletion of the AP-1 site (as above) or point mutation (to render the site functionally inactive) greatly decreased basal expression; point mutation in the PEA3 site gave approx. 60% of wild-type expression; interestingly, point mutation in both AP-1 and PEA3 sites gave higher expression than for the AP-1 mutation alone.

Figure 7 shows a typical experiment with the 3′ deletion set. Deletion of intron 1 sequences between $+95$ and $+60$ led to a decrease in CAT protein, deletion to $+26$ resulted in a further decrease in CAT protein, and deletion through to -34 had no further effect. This reinforces the results from the initial restriction site-generated constructs (above) that sequences between $+26$ and $+60$ are important in basal transcription although the deletion set results suggest that sequences to $+95$ are also involved.

Electrophoretic mobility-shift assays

Computer analysis of the region between $+21$ and $+58$ revealed a putative leader-binding protein 1 (LBP-1)-binding site (two consensus half-sites). LBP-1 is a transcription factor that binds to the initiation site of HIV-1; it has been shown to either up-regulate or repress transcription, depending on site and context bound; it binds as a multimer across two or more half-sites [23]. By using an oligonucleotide probe corresponding to this sequence ($+21/+58$) and nuclear extracts from human skin fibroblasts in a mobility-shift assay (Figure 8, upper panel), four protein–DNA complexes were identified. Competition with unlabelled oligonucleotides demonstrated that bands 1, 2 and 3 are not specific because they were competed for equally by relevant and irrelevant oligonucleotides. Band 4 was competed for by 37 ($+21/58$), but less well by 37m1 ($+21/+58$ with a point mutation in the 5′ LBP-1 consensus half-site) and less well still by 37m2 ($+21/+58$ with a point mutation in both LBP-1 half-sites). An LBP-1-binding oligonucleotide from HIV-1 did not compete well for this band. HeLa cell nuclear extracts, known to contain LBP-1,

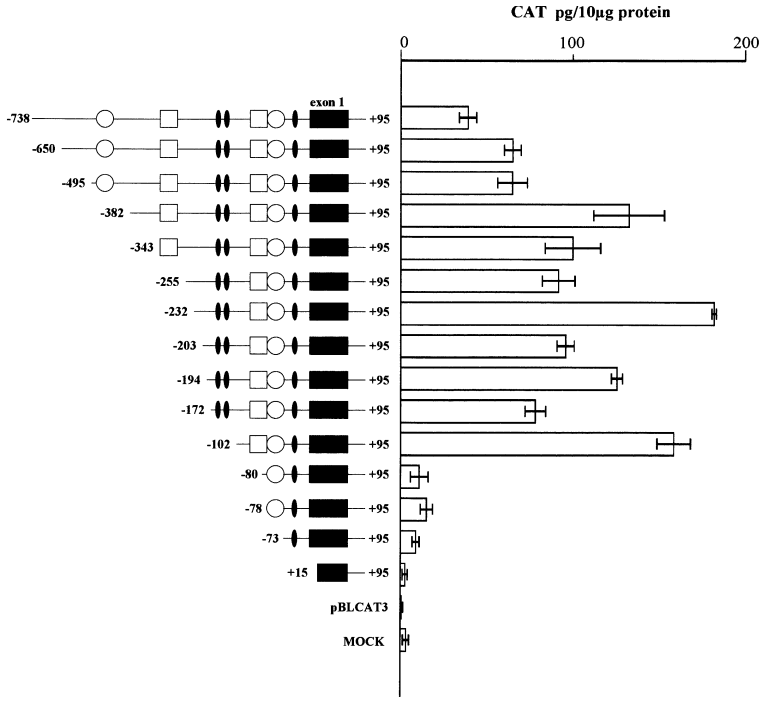

Figure 5 Transient transfection of a 5′ deletion set in human skin fibroblasts

The 5′ deletion mutants shown were transiently transfected into hsf10 human skin fibroblasts at passage 10. Cells were maintained in medium supplemented with 0.1% BSA before harvest and CAT assay. Hirt's assay was used to monitor transfection efficiency. Results are plotted as means ± S.E.M. Consensus sequences for transcription factor binding are indicated as follows: ○, PEA3; □, AP-1; solid oval, Sp1.

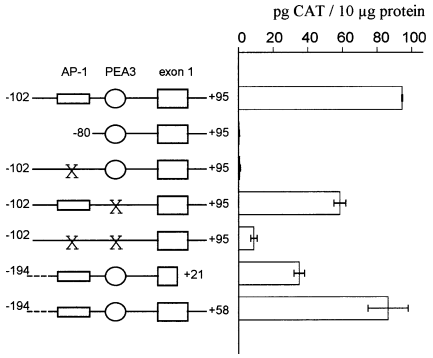

Figure 6 Transient transfection of point mutants in human skin fibroblasts

Point mutations were made in the context of the −102/+95 construct; AP-1 mutation alters TGAGTAA to GGACTAA and PEA3 mutation alters AGGAAG to AAAAAG. Constructs were transfected in hsf15 human skin fibroblasts at passage 8. Constructs −194/+58 and −194/+21 are included for comparison. Cells were maintained in medium supplemented with 0.1% BSA before harvest and CAT assay. Hirt's assay was used to monitor transfection efficiency. Results are plotted as means ± S.E.M.

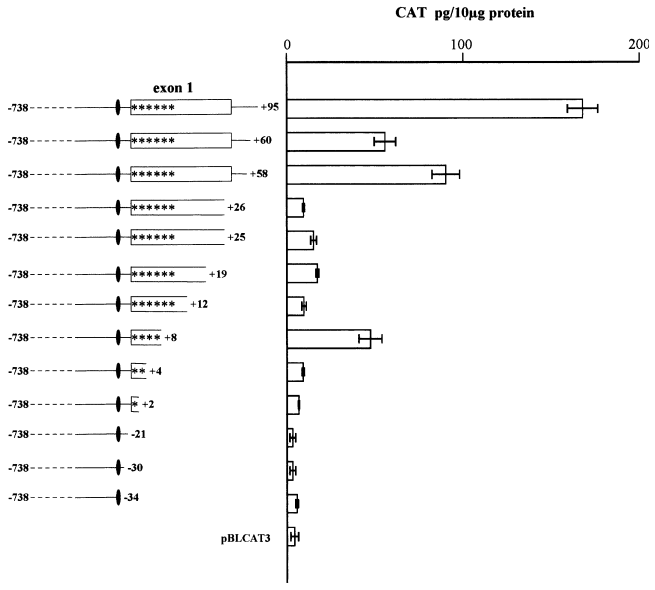

Figure 7 Transient transfection of a 3′ deletion set in human skin fibroblasts

The 3′ deletion mutants shown were transiently transfected into hsf10 human skin fibroblasts at passage 10. Cells were maintained in medium supplemented with 0.1% BSA before harvest and CAT assay. Hirt's assay was used to monitor transfection efficiency. Results are plotted as means ± S.E.M. Symbols: *, transcription start point; solid oval, Sp1-binding consensus sequence.

bound to the 37mer (Figure 8, lower panel), giving a complex of the same mobility as band 4 from the skin fibroblasts, as well as at least two other more retarded bands. Proteins from both skin fibroblasts and HeLa cells bound to the LBP-1-binding oligonucleotide from HIV-1, with one band from skin fibroblasts of corresponding mobility to that from HeLa. We conclude that one or more proteins are binding to the region across +21/+58 and that mutation within the LBP-1 consensus sequences affects this binding, but a lack of competition from the HIV-1 oligonucleotide suggests that the protein is not LBP-1.

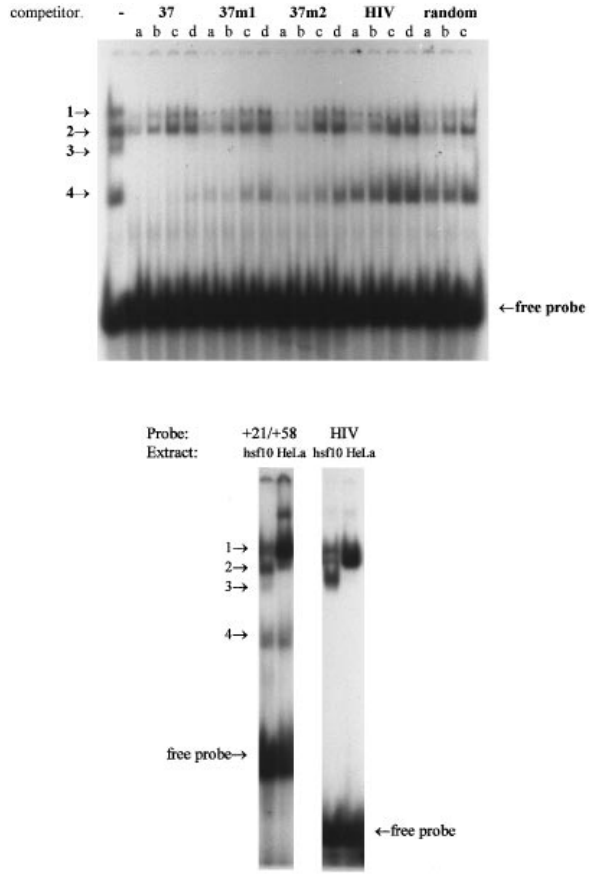

Figure 8 Electrophoretic mobility-shift assays with +21/+58 as a probe

Upper panel: an electrophoretic mobility-shift assay was performed as described in the Experimental section with human skin fibroblast (hsf10) nuclear extracts and a 37mer oligonucleotide probe (+21/+58 of the human TIMP-1 promoter). Competition was with various unlabelled oligonucleotides: 37, +21/+58; 37m1, +21/+58 with mutation in 5′ LBP-1 half-site; 37m2, +21/+58 with mutation in both LBP-1 half-sites; HIV, LBP-1-binding sequence from HIV-1; random, random sequence (a, 100-fold, b, 50-fold, c, 20-fold, d, 10-fold excess unlabelled oligonucleotide). Lower panel: hsf10 or HeLa nuclear extracts were bound to the TIMP-1 probe as above or to a 21mer oligonucleotide probe (HIV, LBP-1-binding sequence from HIV-1).

DISCUSSION

We have cloned the promoter region of the human TIMP-1 gene and performed an initial characterization of the features that are important for basal and inducible transcription. A 3 kbp fragment was sequenced and a 904 bp subclone that hybridized with a murine promoter fragment was chosen for initial characterization. A comparison of our sequence with previously published sequences of parts of this region reveals a number of differences (results not shown) [10,13]. Whether these represent true polymorphisms or just sequencing errors is unknown; in this study both strands were sequenced and were rechecked where differences were found. Alignment with the mouse or rat TIMP-1 promoter sequence shows areas of identity (70 % overall), but also a number of differences, including large insertions and deletions.

Multiple transcription start points were identified by RNase protection. This is in agreement with earlier work with the murine TIMP-1 gene, and also with the human TIMP-2 gene, although only one tsp was identified in the human TIMP-3 gene [22,24,25]. Our human TIMP-1 sequence does not contain a canonical TATA-box, but a related sequence is found approx. 20 bases upstream of the tsp, as in TIMP-2; likewise, the TIMP-3 gene does not contain a canonical TATA-box. The most 5′ tsp identified in the human TIMP-1 gene gives exon 1 a size of 48 bp; in the murine TIMP-1 gene, multiple tsps give a range of 37–102 bp for exon 1.

Initial transient transfections demonstrate that regions controlling basal expression are distinct from those controlling induction of the gene. For example, the −194/+58 construct gives 3–5-fold higher basal activity than the −194/+21 construct, but both are inducible 2–3-fold by serum or phorbol ester. This implicates the region +21 to +58 in basal expression, but not induction of the TIMP-1 gene in human skin fibroblasts.

Deletion from the 5′ end suggests that sequences between −102 and −738 have some negative effects on expression, but it is impossible to define specific elements. Deletion from −102 to −80 gives a large decrease in expression; this deletion results in the removal of an AP-1 element at −92/−86 while leaving a PEA3 element at −78/−73 intact. Thus this AP-1 site is strongly implicated in basal expression. A point mutation that renders these sites functionally inactive confirms that the AP-1 site is essential for basal expression. Mutation of the PEA3 site also decreases basal expression, although to a smaller extent. Logan et al. [26] have reported that the equivalent AP-1 and PEA3 sites and the proteins binding to them in the murine TIMP-1 promoter actually interact to enhance transcription driven from the whole element.

Deletion from the 3′ end demonstrates that any deletion of intron 1 sequences leads to a decrease in expression and again particularly implicates sequences between +26 and +60 in basal transcription. We have examined protein binding to the 37 bp region between +21 and +58 with mobility-shift assays. Our results indicate that there is a putative LBP-1-binding site in this region. Using electrophoretic mobility-shift assay we found evidence that protein does bind to an oligonucleotide corresponding to the +21/+58 sequence. Competition with an oligonucleotide from HIV-1, known to bind LBP-1, along with mutant oligonucleotides of the +21/+58 region, suggests that this protein binds across the LBP-1 site, but is distinct from LBP-1. Confirmation of this requires antibody to LBP-1 or recombinant protein.

Interestingly, transient transfection of −194/+21 and −194/+58 into activated hepatic stellate cells yielded opposite results to human fibroblasts with the −194/+21 construct, giving higher levels of expression than the −194/+58 region (D. A. Mann and M. J. Bahr, unpublished work). Hence it is possible that the +21/+58 region is also involved in cell-specific expression of the human TIMP-1 gene.

Edwards et al. [11] described the minimum construct for serum inducibility in the murine TIMP-1 gene as −120/+22 (numbered from our +1), whereas the addition of further 5′ sequence to −248 enhanced basal expression. This −248/−120 region does not act as an enhancer in our system. Sequences between +22 and +165 also increased basal expression in this murine system. A further study, with a system *in vitro*, demonstrated the importance of AP-1 binding 5′ to the tsp as well as CCAAT binding at the 3′ end of intron 1 in the basal expression of murine TIMP-1 [27]. In human T-cell lines the AP-1 site at −92/−86 is essential for the induction of human TIMP-1 gene expression by the viral transactivator, Tax1; however, the PEA3 site at −78/−73 is important in basal expression. Logan et al. [26] demonstrated that the proteins binding to the AP-1 site at −92/−86 might interact with those at the PEA3 site at −79/−74 to enhance transcription driven from the whole

element. In the rat TIMP-1 promoter, a $-120/+29$ fragment was defined as the basal promoter in HepG2 cells; deletion of a CCAAT/enhancer-binding protein (C/EBP) site at $-108/-100$ resulted in a marked decrease in basal expression, although comparison with our human TIMP-1 sequence shows only poor sequence similarity across this region [28].

In summary, we have isolated a genomic clone of the human TIMP-1 gene. A 3 kbp fragment at the 5′ end of this clone has been sequenced. Sequencing shows that this region contains a number of consensus sites for transcription factor binding. We have identified multiple transcription start points. Transient transfections of human TIMP-1 promoter–CAT reporter constructs into human connective tissue fibroblasts demonstrated the importance of an AP-1 site at $-92/-86$ in basal expression. Sequences between $+21$ and $+58$ are also involved in basal expression and this is separate from those regions involved in induction of the gene by serum or phorbol ester.

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