Structure of the human *TIMP-3* gene and its cell cycle-regulated promoter

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The gene encoding tissue inhibitor of metalloproteinases-3 (*TIMP-3*) is regulated during development, mitogenic stimulation and normal cell cycle progression. The *TIMP-3* gene is structurally altered or deregulated in certain diseases of the eye and in tumour cells. A detailed knowledge of the *TIMP-3* gene and its regulatory elements is therefore of paramount importance to understand its role in development, cell cycle progression and disease. In this study, we present the complete structure of the human *TIMP-3* gene. We show that *TIMP-3* is a TATA-less gene, which initiates transcription at one major site, is composed of five exons and four introns spanning a region of ~ 30 kb, and gives rise to three distinct mRNAs, presumably due to the usage

of alternative polyadenylation signals. Using somatic cell hybrids the *TIMP-3* locus was mapped to chromosomal location 22q13.1. We also show that the *TIMP-3* 5' flanking region is sufficient to confer both high basal level expression in growing cells and cell cycle regulation in serum-stimulated cells. While the first 112 bases of the promoter, which harbour multiple Sp1 sites, were found to suffice for high basal level activity, the adjacent region spanning positions –463 and –112 was found to be a major determinant of serum inducibility. These results provide an important basis for further investigations addressing the role of *TIMP-3* in physiological processes and pathological conditions.

INTRODUCTION

Extracellular matrix (ECM) degradation is a complex process that is indispensable for normal development and tissue remodelling during repair and disease [1]. The matrix metalloproteinases (MMPs) are major ECM-degrading enzymes [2]. The active enzymes are specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs, reviewed in [3]) which constitute a family of three members (*TIMP-1*, *TIMP-2*, *TIMP-3*). Since the first identification of distinct ECM-binding chicken [4,5] and human *TIMP-3* [6], a number of groups have cloned human *TIMP-3* [7–10] and mouse *Timp-3* cDNAs [11–13] using different approaches. The necessity of deriving a detailed understanding of the structure and function of the *TIMP-3* gene is dictated by its potential role in diverse physiological and pathological processes, such as cancer, arthritis and ocular disease.

The *Timp-3* gene is highly expressed during mouse embryo implantation [14], and also shows a highly regulated expression in many developing systems (such as cartilage, epithelia, placenta and muscle) at later stages of development [11]. In addition, the *TIMP-3* gene is also under the stringent control of signal transduction and cell cycle-regulated mechanisms. Thus, *TIMP-3* is strongly induced during the recruitment of quiescent cells into the cell cycle, i.e. during the serum-induced $G_0 \rightarrow S$ transition, showing induction kinetics and other properties that are characteristic of a secondary, delayed early gene. In contrast to the vast majority of other mitogen-inducible genes, which are constitutively expressed during the normal cell cycle, *TIMP-3* expression is subject to cell cycle regulation showing a clear peak of expression in G_1 [9].

Although a relative excess of MMPs over TIMPs has been implicated in diseases such as arthritis and cancer [15,16], *TIMP-3* has recently been directly implicated in human disease. Point mutations in the *TIMP-3* gene have been identified in patients

from two unrelated families with Sorsby's fundus dystrophy (SFD), a dominantly inherited cause of adult-onset blindness [17]. Retinal *TIMP-3* mRNA levels are dramatically increased in simplex retinitis pigmentosa, another cause of blindness [18]. Furthermore, in mouse melanoma cell lines, *Timp-3* mRNA levels are higher in non-metastatic variants than in metastatic ones [13]. These examples suggest that a deregulation of *TIMP-3* expression may play a role in the onset or progression of certain diseases.

Determining the complete structure of the *TIMP-3* gene and its location within a high-resolution physical map of chromosome 22 is critical for identifying disease-related mutations of the *TIMP-3* gene. Of similar importance are studies pertaining to the structure and function of its promoter, especially in view of its unique cell cycle regulation and its potential involvement in development and disease. In this paper, we present the structure of the *TIMP-3* gene and its promoter, a high-resolution mapping of the *TIMP-3* (and thus the SFD) locus on chromosome 22, and functional studies of the *TIMP-3* promoter aiming at a delineation of regions that are involved in basal level expression and the induction of transcription during the $G_0 \rightarrow S$ transition.

MATERIALS AND METHODS

Cloning of the human *TIMP-3* gene and its 5' flanking region

The insert of the *TIMP-3* cDNA clone pSAhT3 [6] was random-primer labelled with [³²P]dCTP and used as a probe to screen the chromosome 22 cosmid library LL22NCO3 'N' in the Lawrist 16 vector [19,20]. Eight positive clones were identified and one clone, N130D3, was selected for further analysis and a physical map of the clone was obtained by restriction mapping and Southern blot analysis; appropriate fragments were subcloned into pBluescript SK⁺ for further restriction mapping and se-

Abbreviations used: DTT, dithiothreitol; ECM, extracellular matrix; FCS, fetal calf serum; MMP, matrix metalloproteinase; SFD, Sorsby's fundus dystrophy; *TIMP-3*, tissue inhibitor of metalloproteinase-3 gene; UT, untranslated; YAC, yeast artificial chromosome.

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Figure 1 Complete structure of the *TIMP-3* gene

(a) The genomic clones used to derive the gene structure. Restriction sites are identified for *EcoRI* (E), *EcoRV* (ERV), *HindIII* (H), *SalI* (S) and *XhoI* (X). The genomic probe used for Northern blot analysis is indicated. (b) The exon-intron structure of the *TIMP-3* gene. Exons are shown by the heavy line (with numbers) and introns by the thin line. The locations of the translation initiation codon (ATG) and termination codon (TGA) are also shown. The 3'-end of exon 5 varies according to polyadenylation signal usage and is shown by the hatched box beyond the first identified and utilized poly(A) signal. (c) The derivation of the *TIMP-3* protein from the *TIMP-3* exons. The solid box represents the signal peptide; the open box represents the mature protein. Conserved cysteine residues are indicated by the solid circles. Scale bars denote 4 kb for (a) and (b) and 22 amino acid residues (aa) for (c).

quence analysis. Dideoxynucleotide sequencing [21] with internal primers based on the *TIMP-3* cDNA sequence or flanking primers was carried out and sequences were analysed with the DNASTAR software programs. Intron sizes were estimated by PCR with intron-spanning primers or by restriction mapping and Southern blot analysis with exon-specific probes.

For cloning of the 5' flanking region, approximately 10⁶ plaques of a genomic library from human WI-38 lung fibroblasts in λ -Fix (Stratagene) were screened using the 5' 295 bp of *TIMP-3* coding sequence as a probe [9]. A recombinant phage containing a 13 kb insert of the *TIMP-3* gene was identified and used for further analysis. The phage DNA was digested by *SacI* and a 4.7 kb fragment containing the 5' flanking region (pm5pro-bs4.7, Figure 1a) was cloned into pBluescript SK⁻ (Stratagene) and the promoterless luciferase vector pXP2 (kindly provided by S. K. Nordeen, University of Colorado, Denver, CO, U.S.A.) [22]. The nucleotide sequence for the 5' flanking 1500 bp was determined for both strands by the chain-termination method.

Northern blotting

A Northern blot with Poly(A⁺) RNA from eight adult human tissues (Clontech, U.S.A.) was hybridized to [³²P]dCTP-labelled *TIMP-3* probes as recommended by the manufacturer. One probe used was a 500 bp cDNA probe (a 500 bp fragment from the 5'-end of pSAhT3; [6]) corresponding to the coding region of the mRNA. The second probe was a genomic fragment from cosmid N130D3 (see Figure 1).

High-resolution chromosomal localization

Sublocalization of the *TIMP-3* gene on chromosome 22 was performed by Southern blot hybridization using the cDNA clone pSAhT3 [6] as a probe against a panel of somatic cell hybrid DNA containing defined regions of this chromosome as well as a chromosome 22 YAC library, to further refine the previously reported chromosomal mapping of *TIMP-3*.

Cell culture

WI-38 cells were obtained from the American Type Culture Collection (A.T.C.C.), and were cultured in Dulbecco-Vogt modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. NIH 3T3 cells were kindly provided by R. Treisman (ICRF, London, U.K.) and cultured as described for WI-38 cells.

Primer extension analysis

³²P-labelled primer (0.3 pmol; 5'-CCCGGCGAAGGAGCAG-GAGC-3') and total cellular WI-38 RNA (20 μ g) or Poly(A⁺) RNA from WI-38 cells (2 μ g), serum stimulated for 6 h with 10% FCS, were denatured for 10 min at 65 °C and then incubated at 37 °C for 30 min. Primer extension was carried out in a total volume of 50 μ l containing 50 mM Tris, pH 8.3, 75 mM KCl, 10 mM dithiothreitol (DTT), 3 mM MgCl₂, 400 μ M dNTPs, 2 units of RNasin and 400 units of Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (Gibco-BRL). After incubation for 1 h at 37 °C, the reaction was stopped with EDTA followed by RNase treatment. The DNA was precipitated, redissolved and separated by electrophoresis on a 6% polyacrylamide/7 M urea gel.

Construction of reporter gene constructs and luciferase assays

Deletion constructs were generated either by using restriction sites in the *TIMP-3* sequence to subclone fragments (Δ -1300, Δ -1010) or by exonuclease III digestion using a nested deletion kit (Pharmacia-LKB) (Δ -925, Δ -660, Δ -463, Δ -112). Deletions are named after the 5'-most nucleotide of upstream sequence present in the constructs relative to the transcription start site. All *TIMP-3* fragments were cloned into the pXP2 vector [22]. The plasmids used in Table 2 have been described previously (pT81 [23]; p5xTRE [24]; pRSV-LTR [25]; cyclin D1 Δ -973 [26]). NIH 3T3 cells were transfected by the DEAE-dextran technique [27,28] using relatively dense cultures. This proved to be advantageous, because the cells became quiescent more rapidly and efficiently compared with sparser cultures and the protein content in quiescent, cycling and stimulated cells varied by a factor of <2. Per dish (5 cm diameter) 7.5 $\times$ 10⁵ cells were plated 24 h prior to transfection of 7 μ g of DNA. To synchronize cells in G₀, cells were maintained in serum-free medium for 2 days. Stimulation was carried out for the indicated times with 20% FCS. PBS washed cells were lysed in 250 μ l of lysis buffer [1% (v/v) Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT] and 100 μ l of protein extracts were assayed in 360 μ l of luciferase assay buffer [25 mM glycylglycine (pH 7.8), 15 mM potassium phosphate (pH 7.8), 15 mM MgSO₄, 4 mM EGTA, 2 mM ATP, 1 mM DTT] in the presence of 165 μ M luciferin. Assays were carried out in an AutoLumat LB953 (Berthold) luminometer and evaluated as described [26]. For each DNA, experiments were repeated three times with two independent preparations of plasmids to account for experimental variations. All constructs gave similar transfection efficiencies, as shown by the co-transfection of reporter plasmids carrying the cytomegalovirus (CMV) or the simian virus 40 (SV-40) early enhancer/promoter regions (results not shown).

RESULTS AND DISCUSSION

Structure of the human *TIMP-3* gene

A cosmid clone (N130D3) was found to contain the entire *TIMP-3* gene and 5' flanking region (Figure 1). The *TIMP-3*

Table 1 The exon–intron boundaries of the *TIMP-3* gene

Intron sequences are shown in lowercase type; exon sequences are in uppercase type, and are arranged as codons in the appropriate reading frame. Only the partial sequence of each exon adjacent to the splice site is shown. In the column for exon and intron sizes, the sizes of the corresponding exons and introns of the murine *Timp-3* gene [39] are shown in parentheses.

Exon	Intron–exon–intron junctions	Exon size	Intron size*
1	agcggcaATG ACC CCT TGG AAC TCC GAC ATC Gnn†	118 bp	17–18 kb (> 15 kb‡)
2	ctctgcagTG ATC CGG GCC CAG ATG AAGtaggtaat	83bp (83bp)	7.5 kb (6.0 kb)
3	ccacacagATG TAC CGA GG CTG CTG ACA Ggtaatgg	112bp (112bp)	700 bp (650 bp)
4	tcctgtagGT CGC GTC TAT TGT AAC TGC AAGgtaag	122bp (122bp)	1.0 kb (1.0 kb)
5	aattgcagATC AAG TCC TG polyadenylation signal	Variable§	

* This column defines the size of the intron immediately following the exon in the relevant row. Introns of the *TIMP-3* gene were not sequenced in their entirety. The sizes of the *TIMP-3* introns were estimated by a combination of restriction mapping, Southern analysis and PCR.

† The complete sequence of intron 1 has not been determined.

‡ The exact size of intron 1 of mouse *Timp-3* has not been defined.

§ The size of exon 5 depends on the polyadenylation signal used.

gene consists of five exons spanning a distance of approximately 30 kb (Figure 1) and bounded by consensus splice donor and acceptor sequences (Table 1). Exon 1 contains both the transcription and translation start site, whereas the translation termination codon and 3'-untranslated (UT) region are located in exon 5. A comparison of the human and mouse genes shows that they are very similar, both in the exon structures as well as in the sizes of their introns (Table 1). Intron 1, in particular, is very large, being 17–18 kb in size (the exact size of the mouse intron 1 is unknown).

We have sequenced exon 5 upto the polyadenylation signal found in three of the five published cDNA sequences for *TIMP-3* [7,8,10]. The determination of a single transcription start site in this paper suggests that usage of this polyadenylation signal defines the smallest *TIMP-3* transcripts to be 1.2 kb. On the other hand, Northern blot analysis has shown that the most prominent transcripts of *TIMP-3* are 2.4, 2.8 and 4.5–5.0 kb in size [6–8]. Furthermore, Apte et al. [6] have isolated a cDNA clone with a 1.0 kb 3'-UT region that does not have a poly(A) tail at the 3'-end, and Wick et al. [9] have reported a 2.2 kb *TIMP-3* cDNA. In the absence of alternate splicing, this would be evidence of alternative polyadenylation signal usage. This is in agreement with the presence of multiple poly(A) signals in the mouse *Timp-3* cDNA [13], which have all been localized within exon 5 of the *Timp-3* gene (S. Apte, B. R. Olsen and G. Murphy, unpublished work). Northern blot analysis of human RNA shows that a cDNA probe from the protein coding region of the mRNA identifies the three major *TIMP-3* transcripts (Figure 2a; the 1.2 kb transcript is not seen, presumably due to its low prevalence), yet the genomic probe corresponding to the putative 3'-most region of exon 5 identifies only the longest transcript of 4.5–5.0 kb (Figure 2b). Both the size and the relative hybridization intensities are similar with both probes, indicating that they are detecting identical 4.5–5.0 kb transcripts (Figure 2). We therefore believe that, as in the mouse *Timp-3* gene, exon 5 encodes the entire 3'-UT region, which is a maximum of 3 kb in length and contains multiple polyadenylation signals.

High-resolution chromosomal mapping of the *TIMP-3* gene

Since it was known that *TIMP-3* mapped to 22q12.1-13.2 from previous work [6], only a subset of chromosome 22 somatic cell hybrids was screened (Figure 3). These were from a panel of hybrids that divides the long arm of chromosome 22 into 25 regions and has been used to assign over 300 markers on

Figure 2 Northern blot analysis of *TIMP-3* expression in various adult human tissues

Northern blot analysis was performed using either (a) a *TIMP-3* cDNA probe (a 500 bp *Pst*I + *Eco*RI fragment from pSAhT3) or (b) the genomic probe shown in Figure 1. Lanes 1 to 8 contain 2 µg each of poly(A⁺) RNA from the heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). RNA size markers in kb are shown between the two blots. The 2.4 and 2.2 kb RNA species seen in (a) are absent in (b), demonstrating that the longest 5.0 kb RNA species results from the use of a distal polyadenylation signal.

chromosome 22 (M. L. Budarf, unpublished work). A positive signal on GM10888, which is a chromosome 22-only hybrid demonstrates the specificity of the cDNA probe. D6S5 and C1-3/5878, whose breakpoints define the proximal and distal boundaries of region 15 respectively, are both negative for *TIMP-3*, localizing the gene to bin 15. All tested hybrids that contain bin 15 gave a positive signal (Figure 3). These results confirm and further refine the previous chromosomal localization to 22q13.1. In addition, a yeast artificial chromosome (YAC), identified using a *TIMP-3* probe, is positive for another marker D22S44, which has also been mapped to bin 15. This YAC is part of a large YAC contig which extends from bin 12 to bin 15 [29] (a contig is a continuous sequence of DNA that has been assembled from overlapping cloned DNA fragments).

Structure and cell cycle regulation of the human *TIMP-3* gene 5' flanking sequence

A genomic library of WI-38 cells was screened with a cDNA clone representing the 5' 295 bp of the *TIMP-3* coding region [9]. A recombinant phage containing a 13 kb insert was identified and used for further analysis. The nucleotide sequence of approximately 1500 bp of the 5' flanking region was determined for both strands (EMBL/GenBank data library accession number pending). The most relevant part of the sequence, as

Figure 3 Assignment of the *TIMP-3* gene to 22q13.1

The region of chromosome 22 retained by each somatic cell hybrid is indicated to the right of the chromosome 22 ideogram by the vertical bar below the cell line name. The regions defined by the complete panel are designated as bins 1–22, immediately to the right of the ideogram. The presence (+) or absence (–) of hybridization signal to the *TIMP-3* gene is indicated at the bottom of the Figure underneath each cell line. GM10888 is a human × hamster hybrid containing a normal chromosome 22 as its only intact human chromosome [32]. Rad-110a is a radiation-reduced human × hamster hybrid which contains a large portion of chromosome 22 [33]. C1-1/TW is a human × hamster hybrid made from a human cell line with an Ewing sarcoma-associated t(11;22)(q24;q12) containing a der(11) as the only relevant human chromosome [34]. D6S5 is a cell line derived from a normal human × mouse fusion which spontaneously lost the distal long arm of chromosome 22. It retains C lambda and all the BCR-related genes, but is negative for PDGFB [35]. C1-3/5878, C1-1/5878, C1-2/5878 and C1-8/5878 are human × hamster hybrids which each contain a deleted chromosome 22 (M. L. Budarf, unpublished work) due to the selection for adenylylsuccinate lyase subsequent to fusion with Ade-1 [36]. The human × mouse hybrid 1/22AM-6 (GM11224C) contains the der(1) of a constitutional 1(1;22)(q42;q13) and 1/22AM-27(GM11223C) contains the der(22) of this translocation [37]. C102/DIBA is a human × hamster hybrid which contains the der(12) of a constitutional t(12;22)(q13.31;q13.31) (M. L. Budarf, unpublished work). APR 8.5 is a human × hamster hybrid which contains a der(22) of a constitutional t(11;22)(p11;q13.2) translocation [38].

determined below, is shown in Figure 4. To identify the point(s) of transcription initiation, the 5'-end of *TIMP-3* mRNA was determined by primer extension analysis (Figure 5). This experiment led to the identification of one major start point located 364 bp 5' to the ATG start codon. Inspection of the nucleotide sequence 5' to the start of transcription showed no canonical TATA box or TATA-like sequence. We therefore conclude that *TIMP-3* is a TATA-less gene, but nevertheless initiates transcription from one major site. The previously analysed mouse

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-500 ATGGCTTCCC ATATCCCGAGA GAGTAAGAAC CAGAGAGAGA GAGAGAAAGA GAGAGAGTTT
-440 GGGTCTTTCT CCTCTGTGCC TGCTCTCTCC AGAGAAACTG GAGGGGTAGC AGTTAGCATT
-380 CCCCCGCTGG TTCCACCAAG CACAGTCAAG GTCTCTAGGA CATGGCCACC CCTCACCTGT
-320 GGAAGCGGTC CTGCTGGGGT GGGTGGGTGT TAGTTGGTTC TGGTTTGGGT CAGAGACACC
-260 CAGTGGCCCA GGTGGGCGTG GGGCCAGGCG GCAGACGAGA AGGGGCACGA GGGCTCCGCT
-200 CCGAGGACCC AGCGGCAAGC ACCGGTCCCG GCGCGGCCCC AGCCACCCCA CTCGCGTGCC
-140 CACGGCGGCA TTATTCCCTA TAAGGATCTG AACGATCCGG GGGCGGCCCC GGGCCGTTAC
-80 CCCTTGCCCC CGGCCCCGCC CCCTTTTGTG AGGCGGATG AGGTAAATGCG GCTCTGCCAT
-20 TGGTCTGAGG GGGCGGCCCC CAACAGCCCG AGGCGGGGTC CCCGGGGGCC CAGCGCTATA
+42 TCATCTGGCC GCCCAGGCAG CGGGCAGAG CGGGCAGAG GCAGGCGGCG GCGCTCAGA

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Figure 4 Nucleotide sequence of 500 nucleotides of the human *TIMP-3* promoter and 101 nucleotides of the 5' transcribed region

A further 480 nucleotides have been determined and will be deposited with the GenBank/EMBL/ DDBJ Nucleotide Sequence Databases. GC-Boxes (Sp1 sites) are double-underlined; the two half sites of a potential NF1 site are underlined; an element resembling a C/EBP site is marked by a dotted line.

Figure 5 Mapping of the 5'-end of *TIMP-3* mRNA by primer extension in WI-38 cells serum stimulated for 6 h with 10% FCS

Templates were either 20 µg of total RNA or 2 µg of poly(A⁺) RNA. tRNA: negative control using yeast tRNA instead of WI-38 RNA. G: a G-sequencing reaction was run alongside to be able to accurately determine the 5'-end of the *TIMP-3* mRNA. The major transcription initiation site is indicated by an arrow.

Timp-1 gene also lacks a TATA-box, but has multiple transcription start sites, whereas the human *TIMP-2* gene is characterized by a single transcription start site [30,31]. The *TIMP-3* upstream sequence contains a number of elements resembling binding sites for known transcription factors, including four Sp1 sites and potential NF1 and C/EBP sites (see Figure 4).

A *TIMP-3* gene fragment spanning nucleotides –1010 to +281 was linked to the bacterial luciferase gene (construct Δ-1010) and transfected into NIH 3T3 cells to test whether the isolated promoter fragment was functional in a transient expression assay. As shown in Figure 6, construct Δ-1010 was cell

Figure 6 Kinetics of induction of the Δ -1010 *TIMP-3* promoter-luciferase construct after serum stimulation of quiescent NIH 3T3 cells with 20% FCS

For details see the Materials and methods section. Abbreviation: RLUs, relative light units.

Table 2 Expression of various promoter-luciferase constructs in normally growing cells (A), and inducibility by a 4 h serum stimulation (B)

(A) Expression in growing cells ($10^{-3} \times$ RLU)

<i>TIMP-3</i> Δ -1010	189.5 $\pm$ 6.4
pRSV-LTR	308.1 $\pm$ 23.4
p5 $\times$ TRE	59.1 $\pm$ 2.2
Cyclin D1 Δ -973	27.8 $\pm$ 3.5
pXP2	0.2

(B) Induction by 10% FCS (fold)

<i>TIMP-3</i> Δ -1010	8.4 $\pm$ 0.4
p5 $\times$ TRE	2.4 $\pm$ 0.2
Cyclin D1 Δ -973	3.5 $\pm$ 0.3
pT81	1.0 $\pm$ 0.2
pXP2	1.2

cycle regulated in serum-stimulated cells that had been synchronized in G_0 . Luciferase activity increased already after 1 h reaching a 7-fold higher level compared with quiescent cells 4 h after serum stimulation. In an additional experiment the expression of four other constructs, containing either the Herpes virus *tk* promoter [23], a 5 $\times$ TRE minimal promoter [24], a RSV-LTR [25] or a 973 bp fragment of the human cyclin D1 promoter [26], were assayed for comparison in normally growing and serum-stimulated NIH 3T3 cells. The results of this experiment are shown in Table 2. In normally growing NIH 3T3 cells (Table 2A) the *TIMP-3* promoter construct Δ -1010 was expressed at approximately 3-fold higher levels than a 5 $\times$ TRE minimal promoter and at about 7-fold higher levels than the analysed cyclin D1 promoter construct, while the RSV-LTR reporter plasmid showed an approximately 2-fold higher activity than the *TIMP-3* promoter construct. This indicates a relatively strong transcriptional activity of the human *TIMP-3* promoter. In addition, as shown in Figure 6 and Table 2(B), the *TIMP-3* promoter construct proved to be inducible in cells serum stimulated for 4 h to 7- to 8-fold higher levels compared with quiescent cells, which is 3.5- or 2.4-fold stronger than the induction

Figure 7 (a) Transient expression analysis of terminally truncated *TIMP-3* promoter-luciferase constructs in normally growing NIH 3T3 cells and (b) analysis of the same constructs as in (a) in quiescent (G_0) versus cells serum stimulated for 4 h with 20% FCS

(a) Plasmids were named to indicate the 5' truncation, e.g. Δ -1300 contains 1300 bp upstream of the initiation site. Experiments in (a) and (b) were performed three times with two independently prepared sets of plasmids. S.D. values are indicated. The absence of an error bar means that the S.D. was too low to be shown on the graph. For details see the Materials and methods section. Abbreviation: RLUs, relative light units.

observed with a 5 $\times$ TRE-luciferase reporter construct or the cyclin D1 promoter construct, respectively. In contrast, a Herpes simplex *tk* promoter-luciferase construct (pT81) showed no induction by serum. From these results we conclude that the 1010 bp of 5' flanking sequence analysed in this experiment contains most, if not all, of the elements required for both efficient basal level transcription and induction by serum.

Deletion analysis of the *TIMP-3* promoter: control of basal level activity and serum induction

In order to identify functionally relevant regions in the *TIMP-3* promoter we generated a series of terminal truncations and in a first experiment analysed for basal level expression in transient luciferase assays in NIH 3T3 cells. Deletions are named after the 5'-most nucleotide of upstream sequence present in the constructs relative to the transcription start site. The results of this study are shown in Figure 7(a) and led to the following three major conclusions: (i) The strongest expression was observed with construct Δ -1010. Truncation of a further 85 bp (Δ -925) led to an approximately 2-fold decrease in activity, indicating that the

region between positions -1010 and -925 contains one or more elements involved in basal level transcription. (ii) Further 5' truncations down to position -112 only slightly affected transcription, suggesting that the sequence between position -925 and -112 is of minor importance for basal level transcription in normally growing cells. The first 112 bases of the promoter are sufficient to confer high basal level expression in growing cells. This region harbours multiple Sp1 sites. Consensus Sp1 sites have also been noted in both the *Timp-1* and *TIMP-2* promoters. (iii) As indicated by the results obtained with Δ -1300, the region between -1300 and -1010 seems to have a strongly negative effect on transcription. The molecular basis underlying this effect is not clear so far.

The next experiment addressed the serum inducibility of the various 5' deletion constructs. For this purpose, cells were synchronized in G_0 by serum deprivation for 48 h and then stimulated with 20% FCS for 4 h. The results in Figure 7(b) show that the expression profile was similar to that obtained with normally cycling cells. Expression levels in quiescent cells were reduced about 2-fold compared with normally cycling cells, and were induced 2.9- to 8.5-fold 4 h post serum stimulation. Like in the experiment in Figure 7(a) the region between -1300 and -1010 had a negative effect on transcription (construct Δ -1300), but not on the factor of serum induction. In addition, the highest luciferase activity was seen with construct Δ -1010. Further truncation of 5' sequences up to position -660 led to only a 1.5-2-fold decrease in activity, but these constructs (Δ -1010, Δ -925, Δ -660) retained a clear induction by serum in the range of 6- to 8-fold. Truncation of another 200 bp down to -463 (Δ -463) led to a further approximately 2-fold decrease in overall serum-induced activity, but again this deletion had no influence on the factor of induction relative to quiescent cells. The only clear decrease in serum inducibility was observed for construct Δ -112 which showed an only 3-fold induction, corresponding to a 50-65% reduction. These results indicate that (i) the region between -463 and -112 contains element(s) that are required for efficient serum inducibility, and (ii) that at least two regions are involved in enhancing basal transcription, i.e. the region already identified in growing cells (-1010/-925; see Figure 7a) and an additional region between nucleotides -660 and -463.

We believe that these results provide an important basis for further investigations addressing the role of *TIMP-3* in development, cell cycle regulation, tumour progression and certain hereditary diseases.

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