Identification of an initiator-like element essential for the expression of the tissue inhibitor of metalloproteinases-4 (*Timp-4*) gene

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We have used real-time quantitative reverse transcriptase PCR (TaqMan[®]) to quantify the expression of the four tissue inhibitor of metalloproteinases (*Timp*) genes in mouse tissues during development and in the adult. Among the four *Timp* genes, *Timp-4* shows the most restricted pattern of expression, with highest RNA levels in brain, heart and testes. These data indicate that in the brain, *Timp-4* transcripts are temporally regulated during development, becoming more abundant than those of the other *Timps* after birth. Cloning of the *Timp-4* gene confirmed a five-exon organization resembling that of *Timp-2* and *Timp-3*, and like all *Timps*, *Timp-4* is located within an intron of a synapsin gene. Ribonuclease protection analysis and 5'-rapid amplification of cDNA ends PCR identified multiple transcription starts for *Timp-4* from brain and heart mRNA. The promoter region of *Timp-4* was functional in transient transfection analysis

in mouse C3H10T1/2 fibroblasts, where it directed basal expression that was non-inducible by serum. The TATA-less promoter contains consensus motifs for Sp1 and an inverted CCAAT box upstream of an initiator-like element that is in close proximity to a transcription start site. Mutation of the CCAAT box caused a 2-fold increase in reporter expression. More significantly, mutation of the Sp1 motif or initiator-like element almost completely abolished reporter expression. This first functional characterization of the *Timp-4* promoter shows it to be distinct from other members of the *Timp* family and provides insights into potential mechanisms controlling the tight spatio-temporal expression pattern of the gene.

Key words: promoter, quantitative RT-PCR, tissue inhibitor of metalloproteinase (TIMP), transcription.

INTRODUCTION

Timely breakdown and remodelling of the extracellular matrix (ECM) is an essential process in development, morphogenesis and reproduction [1]. ECM degradation is also associated with a variety of physiological and pathological processes such as angiogenesis, wound healing, tumour metastasis and joint injury and repair [2]. The tissue inhibitors of metalloproteinases (TIMPs) regulate ECM integrity by interacting with and inhibiting the matrix metalloproteinase (MMP) family of proteases [3]. The TIMP family consists of four proteins that interact via their N-terminal domains with the MMP active site in a 1:1 molar stoichiometry and block proteolytic activity. Although the four TIMPs have many shared activities, they also have unique characteristics, including distinctive spatio-temporal expression patterns and biochemical properties, suggesting that they may have specialized physiological roles [4,5].

The most recently identified member of the TIMP family, TIMP-4 [6,7], shares with other TIMPs the ability to block tumour-cell invasion *in vitro* and, at least in some instances, it also blocks tumour growth and metastasis *in vivo* [8,9]. However, TIMPs are multifunctional proteins with effects on cell growth and apoptosis as well as the control of ECM metabolism [4,10]. TIMP-4 also exerts diverse effects depending on the cell or tissue

involved. Thus TIMP-4 induces apoptosis in cardiac fibroblast cells *in vitro*, as well as inhibiting invasion and migration [11]. Recombinant TIMP-4 inhibited the growth of G401 Wilms' tumour cells *in vitro* [8], but stimulated the growth of MDA-231 breast carcinoma cells, potentially via up-regulation of Bcl-2 and Bcl-X_L [12]. Moreover, intramuscular delivery of a TIMP-4 expression construct in nude mice prevented subsequent growth of subcutaneous tumours arising from inoculated G401 cells, but enhanced mammary tumour growth [12].

One important area in which individual TIMPs display significantly different behaviour is their effects on the activation of pro-gelatinase-A (MMP-2 [13]). With some exceptions TIMPs are broadly interchangeable in their abilities to inhibit the active forms of MMPs [5], but they also enter into specific complexes with the pro-enzyme forms of the gelatinases (TIMP-1 with pro-gelatinase-B/MMP-9; TIMP-2, -3 and -4 with pro-gelatinase-A/MMP-2). These pro-gelatinase-TIMP complexes involve interactions between the C-terminal domain of the TIMPs and the C-terminal haemopexin-like domains of the gelatinases, and they are important in controlling activation of the latent enzyme via proteolysis. TIMP-2 can act as an adaptor protein that docks pro-MMP-2 to the cell surface by formation of a trimolecular complex with membrane type-1-MMP (MT1-MMP, MMP-14); an adjacent free MT1-MMP can then activate pro-MMP-2

Abbreviations used: ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1-MMP; RT-PCR, reverse transcriptase PCR; p.c., post-coitum; RPA, ribonuclease protection assay; MEM, minimal essential medium; FCS, fetal calf serum; EMSA, electrophoretic mobility-shift assay; RACE, rapid amplification of cDNA ends; AP-1, activator protein 1; Inr, initiator-like.

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[13–16]. TIMP-4, like TIMP-2, is able to interact with pro-MMP-2 via its C-terminal domain [17,18], and it is also a potent inhibitor of MT1-MMP [19,20], but unlike TIMP-2, it does not permit formation of a trimolecular complex, and thus does not facilitate MT1-MMP-mediated pro-MMP-2 activation [18,19]. Thus the TIMP-4/TIMP-2 balance may be a significant factor in controlling the activation of MMP-2 [20].

The expression pattern of *Timp-4* is distinct from that of the other TIMPs and shows very restricted localization to neural, cardiac, breast and muscle tissues [7,21,22]. Cardiovascular expression of *Timp-4* may be significantly increased in restenosis in response to vascular balloon injury [21]. Recent work has shown that *TIMP-4* expression in human malignant gliomas is negatively correlated with the degree of malignancy, with the higher-grade tumours exhibiting reduced *Timp-4* expression [9].

Due to the importance of the *TIMP* gene family in the regulation of ECM remodelling and cell growth, a detailed analysis of the mechanisms of regulation of murine *Timp-4* is warranted. In this study, we have performed the first detailed murine developmental-stage and tissue-distribution analysis of all four *Timp* genes using real-time quantitative reverse transcriptase PCR (RT-PCR; TaqMan®). Also, we have carried out an initial identification and characterization of the *Timp-4* promoter. These data identify several features of the architecture of the *Timp-4* promoter that are unique in the *Timp* family, which may help us to understand the distinctive expression pattern of this gene. In particular, the TATA-less promoter contains an Sp1-binding motif upstream of an initiator-like element, both of which are required for promoter function.

EXPERIMENTAL

Animals and tissue collection, and RNA isolation

Female CD-1 mice (Charles River, Lachine, Quebec, Canada), 7–8 weeks of age, were paired with CD-1 males to generate pregnant animals for collection of staged embryos, newborn pups and postnatal stages of mouse development. Noon of the day of the vaginal plug was designated as day 0.5 post-coitum (p.c.). Animals were killed on days 9.5, 11.5 and 13.5 p.c. to recover whole embryos and brains were dissected from the embryos on days 15.5 and 17.5 p.c., and from newborn mice. Brains were also dissected from postnatal mice aged 3, 7, 14, 21 and 28 days. Other tissues were collected along with brains at postnatal stages, and in some cases from 8 week-old (56 day) mice that were reproductively mature. All tissues were frozen on dry ice and stored at -70°C . Total RNA was extracted using the modified guanidinium isothiocyanate method of Chomczynski and Sacchi [23].

Quantitative real-time RT-PCR

Quantification of *Timp* expression was performed using the Applied Biosystems ABI prism 7700 sequence-detection system (TaqMan®). Initially, 1 μg of total RNA was reverse transcribed in a 20 μl reaction using 2 μg of random hexamers and Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Oligonucleotide primers and fluorescently labelled TaqMan® probes (Table 1) were designed using Primer Express 1.0 software (Applied Biosystems). In order to control against amplification of genomic DNA and to ensure that the PCR signal was generated from cDNA, primers were placed within different exons, close to intron/exon boundaries (Table 1). BLASTN searches were conducted on all primer/probe nucleotide sequences to ensure gene specificity. The identity of PCR products was confirmed by direct sequencing of amplicons.

Table 1 Sequences of PCR primers and sequence-specific probes for each *Timp* target

FAMTM and TAMRA are the fluorescent label and quencher for quantitative RT-PCR; FAM is 5'-located, TAMRA is 3'-located.

TaqMan® primers and probes	Sequence
<i>Timp-1</i>	
Forward primer	5'-CATGGAAGCCTCTGTGGATATG-3'
Reverse primer	5'-AAGCTGCAGGCACTGATGTG-3'
Probe	5'-FAM TM -CTCATCACGGCCGCTAAGGAAC-TAMRA-3'
<i>Timp-2</i>	
Forward primer	5'-CCAGAAGAAGAGCCTGAACCA-3'
Reverse primer	5'-GTCCATCCAGAGGCACTCATC-3'
Probe	5'-FAM TM -ACTCGCTGCCCTGCTCCCTTGC-TAMRA-3'
<i>Timp-3</i>	
Forward primer	5'-GGCCTCAATTACCGCTACCA-3'
Reverse primer	5'-CTGATAGCCAGGATACCAAAA-3'
Probe	5'-FAM TM -TGCTACTGCTGCTGCTTTTGTGACCTCCA-TAMRA-3'
<i>Timp-4</i>	
Forward primer	5'-TGCAGAGGGAGAGCCTGAA-3'
Reverse primer	5'-GGTACATGGCACTGCATAGCA-3'
Probe	5'-FAM TM -CCACCAGAACTGTGGCTGCCAAATC-TAMRA-3'

The 18 S ribosomal RNA gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. TaqMan® 18 S ribosomal RNA primers and a 5'-VICTM-labelled probe were used according to the manufacturer's instructions (PE Applied Biosystems). PCR reactions for all samples were performed in duplicate in 96-well optical plates using 0.5% cDNA, 12.5 μl of 2 $\times$ TaqMan® Universal PCR mastermix (PE Applied Biosystems), 100 nM probe, 200 nM of each primer and water to a final volume of 25 μl . Thermocycler conditions comprised an initial holding stage at 50°C for 120 s followed by 95°C for 10 min. This was followed by a two-step TaqMan® PCR programme consisting of 95°C for 15 s and 60°C for 60 s for 40 cycles.

To determine the number of RNA copies in the samples, standard curves were prepared as follows [24]. The full-length cDNA of each *TIMP* gene was cloned into the pBluescript KS(–) vector (Stratagene, Amsterdam, The Netherlands), plasmids were linearized with an appropriate endonuclease, and sense RNA for each gene was transcribed *in vitro* using either a T7 or T3 polymerase (Roche, Lewes, East Sussex, U.K.). Plasmid DNA was then digested with RNase-free DNase I (Roche), the RNA precipitated and resuspended in RNase-free water, and the amount synthesized was determined by spectrophotometry using a GeneQuant *pro* (Amersham Bioscience, Little Chalfont, Bucks, U.K.). Knowing the sequence and length of the synthesized RNA, the molecular mass of each RNA was calculated, and the number of molecules synthesized was determined. We then reversed transcribed 1 μg of *in vitro*-transcribed RNA, and 10-fold serial dilutions of cDNA were prepared covering concentrations ranging from the equivalent of 10^{10} to 10^1 copies of RNA. These dilutions were subject to real-time PCR as described above. During PCR the TaqMan® probe emits a fluorescence signal, which increases in intensity in direct proportion with the amount of specific amplified product. The ABI Prism 7700 instrument measures the cycle-to-cycle changes in fluorescence in each sample. Data are initially expressed as a threshold cycle (C_T) at which an increase in fluorescence above a baseline signal can first be detected. The fewer cycles it takes to reach the C_T , the greater the initial template copy number. A standard curve for

C_T versus input RNA was prepared, and using this curve the amount of starting RNA in each sample was determined.

Isolation of *Timp-4* genomic clones

Approx. 7×10^5 pfu of a lambda phage mouse genomic DNA library (mouse strain 129) were screened using a random-prime ^{32}P -labelled *Timp-4* cDNA as a probe [7] according to standard procedures [25]. Seven hybridizing plaques were identified and subsequently plaque-purified. Lambda DNA was prepared from the purified clones and subjected to restriction-digestion analysis using ^{32}P -labelled oligonucleotide primers corresponding to the 5' and 3' sequences of the mouse *Timp-4* cDNA. Two clones were identified as spanning the entire coding sequence, one of which was used for restriction digestion with *EcoRI* and *BamHI*, followed by gel purification of *Timp-4* restriction fragments and ligation into pBlueScript KS(–), all according to standard procedures [25]. Two subclones, pBSEE5.0 and pBSHH4.3, were found to contain the mouse *Timp-4* promoter sequences based on restriction mapping and sequence analysis. Together with a further subclone, pBSEE8.0, these constructs were shown to contain the full *Timp-4* gene.

Plasmid construction

Timp-4 promoter fragments were cloned into the reporter vector pGL3-Basic (Promega, Southampton, U.K.). A *Timp-4* fragment, –1026/–21, was isolated by *BamHI*/*SacI* digestion of the *Timp-4* genomic plasmid pBSEE5.0 and initially cloned into a similarly digested pBlueScript II SK(–) vector (Stratagene) to generate the plasmid pBS-T4 –1026/–21. The same *Timp-4* 1.0 kb fragment was subcloned into a *BglII*/*SacI*-digested pGL3-Basic vector to generate the antisense *Timp-4* promoter construct pGL3-T4 –21/–1026. To create the construct pGL3-T4 –1026/–21, pBS-T4 –1026/–21 was restriction-digested with *KpnI* and *SacI* and the resultant 1.0 kb *Timp-4* promoter fragment subcloned into a similarly digested pGL3-Basic vector. The 250 bp (–21/+239) *SacI* fragment of pBSEE5.0 was ligated into the *SacI*-linearized plasmid pGL3-T4 –1026/–21 to make the construct pGL3-T4 –1026/+239. Similarly, the same fragment was subcloned into *SacI*-linearized pBS-T4 –1026/–21 to form pBS-T4 –1026/+239 for ribonuclease protection assay (RPA) analysis. The orientation of the *SacI* fragment was determined for both constructs by restriction-digest analysis and DNA sequencing. The *Timp-4* ATG present within the construct pGL3-T4 –1026/+239 was mutated to TAG using the QuikChange methodology (Stratagene) to produce the new reporter pGL3-T4 –1026/+239mATG. This was to prevent aberrant translation beginning with the *Timp-4* ATG. The construct pGL3-T4 –281/–21 was generated by PCR amplification, using *Pfu* DNA polymerase (Stratagene), of pGL3-T4 –1026/–21 with 5' and 3' primers containing *KpnI* and *SacI* restriction sites, respectively. Mutant constructs mSp1, mCCAAT and mInr were generated again using QuikChange methodology.

RPA

A [^{32}P]UTP-labelled antisense riboprobe of 1265 nt (–1027/+238) was synthesized using T3 RNA polymerase and *KpnI*-digested *Timp-4* plasmid, pBS-T4 –1026/+239. This sequence spanned the junction of exon 1 with intron 1 (positions +139 and +140). RNase protection was performed by hybridizing 1 μg of poly(A)⁺ RNA from mouse brain or heart (Clontech), or a tRNA control, with the riboprobe. Hybridization was performed overnight at 55 °C in 80 % formamide, 40 mM Pipes, pH

6.4, 0.4 M NaCl and 1 mM EDTA. This was followed by digestion with RNase A (40 $\mu\text{g}/\text{ml}$) and RNase T1 (2 $\mu\text{g}/\text{ml}$) in 10 mM Tris/HCl, pH 7.5, 0.3 M NaCl and 5 mM EDTA for 60 min at 30 °C. Protected fragments were then analysed on a sequencing gel next to a ^{32}P -end-labelled DNA ladder.

Cell culture, transfections and reporter assays

Mouse fibroblast cell line C3H10T1/2 was maintained in an air/CO₂ (95:5) atmosphere in minimal essential medium (MEM) containing 10 % (v/v) fetal calf serum (FCS) and supplemented with 1 % non-essential amino acids, 20 units/ml nystatin, 100 i.u./ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Promoter/reporter constructs were transiently transfected into C3H10T1/2 cells using FuGENE6 transfection reagent (Roche) as per the manufacturer's instructions. Briefly, cells were plated on 6-well plates at a density of 8.75×10^3 cells/cm² in MEM supplemented as described above plus 10 % FCS. The following day the cells were transfected with 1 μg of reporter DNA previously mixed with 3 μl of FuGENE6 and 0.1 ml of MEM. After 24 h the cells were washed with Hanks balanced salt solution and the media replaced. After 24 h the cells were harvested into a lysis buffer and the supernatant assayed with a luciferase reporter assay kit (Roche). All data were normalized for protein concentration as determined by a BCA assay (Pierce). A Hirt assay [26] and/or co-transfection of a constitutive chloramphenicol acetyltransferase (CAT) reporter (roux sarcoma virus–CAT), followed by a CAT ELISA (Roche), were used to monitor transfection efficiencies. For the serum-stimulation experiment, 24 h after transfection cells were washed in Hanks balanced salt solution and serum-starved (MEM supplemented as above but with no FCS) for a further 24 h. After starvation the cells were washed twice with Hanks balanced salt solution and either returned to starvation conditions or to MEM supplemented as above and containing 10 % FCS for 6 or 24 h periods, prior to harvest and analysis as described above.

Electrophoretic mobility-shift assay (EMSA)

Timp-4 initiator-like (Inr) and mutant Inr oligonucleotides were annealed in equimolar amounts. The *Timp-4* Inr probe was end-labelled with [γ - ^{32}P]ATP followed by desalting through Sephadex G-25 spin columns. Binding reactions (10 μl total, incubated for 20 min at 4 °C) contained radiolabelled probe (approx. 20000 c.p.m.), 2 μl of nuclear extract and 1 μg of poly(dI-dC) · poly(dI-dC) in 1 × binding buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol and 5 % glycerol). Binding reactions were electrophoresed on a 6 % polyacrylamide non-denaturing gel in 1 × Tris-buffered saline (90 mM Tris/HCl/90 mM boric acid/2 mM EDTA). Gels were pre-run for at least 30 min and run at 10 V/cm at 4 °C to maximize stability of the protein–DNA complexes. Gels were dried and autoradiographed.

Nuclear extracts

Nuclear extracts were prepared from C3H10T1/2 mouse fibroblasts cultured in 10 % serum as above, using a modification of the method by Dignam and co-workers [27]. Briefly, cells from a 15 cm-diameter dish were scraped into ice-cold PBS, pelleted at 500 *g* and resuspended in 1 ml of PBS containing 0.1 % Nonidet P-40 for approx. 30 s. After centrifugation at 12000 *g* for 10 s, supernatant was removed and the nuclear pellet was resuspended in 3 vol. of high-salt buffer (25 mM Hepes, pH 7.8, 500 mM KCl, 0.5 mM MgSO₄ and 1 mM dithiothreitol) containing EDTA-free Complete proteinase inhibitors (Roche). The suspension was

incubated on ice for 20 min with occasional mixing and then centrifuged at 12 000 *g* for 2 min at 4 °C. Supernatant was divided into aliquots, frozen on dry ice and stored at -70 °C. Protein concentration in the nuclear extract was determined by Bradford assay (Bio-Rad) and was typically 2–5 mg of protein/ml.

RESULTS

Timp expression analysis

We performed a detailed tissue-distribution analysis of *Timp-1*–*Timp-4* in developing and adult mouse tissues using real-time

quantitative RT-PCR (Figure 1). These data confirm and extend previous findings of the high degree of spatio-temporal regulation of the gene family and the distinctness of each gene's pattern of expression. However, calibration of the TaqMan® quantitative RT-PCR data with synthetic *Timp* transcripts allowed, for the first time, determination of the absolute transcript abundance of the four *Timps*. In general, *Timp-1* transcript levels were lower than those of the other *Timps*, the most striking expression of *Timp-1* being seen in the uterus, decidua and ovary, where a role for the gene in reproduction has been proposed [28–32]. Unlike *Timp-2* and -4, and to a lesser extent *Timp-3*, little *Timp-1*

Figure 1 Real-time quantitative RT-PCR analysis of *Timp* gene expression in mouse tissues of different developmental stages

RNA was isolated and analysed by real-time quantitative RT-PCR and the number of RNA molecules in each sample were determined using a standard curve, as described in the Experimental section. Averaged data from two independent PCR reactions are depicted. Brain, heart, kidney, liver and muscle-tissue RNA samples were taken from embryonic, newborn or developing mice at the indicated days p.c. or postnatal. Testes, thymus and spleen RNA was isolated from the tissues of adult animals. RNA was isolated from the reproductive tissues (placenta, uterus, decidua and ovary) of pregnant mice at the indicated days p.c. The key is as follows, A, 9.5 days p.c.; B, 11.5 days p.c.; C, 13.5 days p.c.; D, 15.5 days p.c.; E, 17.5 days p.c.; F, newborn; G, postnatal day 3; H, postnatal day 7; I, postnatal day 14; J, postnatal day 28; K, postnatal; L, adult.

Figure 2 Genomic organization of *Timp-4*

Timp-4 is encoded within five exons (black rectangles) and the gene is encompassed completely by the clones pBSHH4.3, pBSEE5.0 and pBSEE8.0 spanning a total of approx. 13.5 kb of murine chromosome 6 [36]. White rectangles show the location of two consecutive exons of the murine synapsin 2 gene (*Syn2*), which is encoded in the antisense orientation in relation to the *Timp-4* gene (arrows). The locations of constructs pGL3-T4 -1026/-21 and pGL3/pBS-T4 -1026/+239 used for promoter analysis are shown below the main restriction map. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sac*I.

expression was observed in the brain at any developmental stage. *Timp-2* was widely expressed, although the liver showed the lowest expression at all developmental stages (this was the case for all four *Timp* genes). Expression of *Timp-2* in the brain was low until day 15.5 p.c., after which levels rose to a maximum on postnatal day 3, subsequently declining. *Timp-2* expression in the placenta, uterus and ovary closely mirrored that of *Timp-1*. The highest levels of *Timp-3* expression were in the kidney, heart, ovary and thymus.

Of all the *Timp* genes, *Timp-4* showed the most restricted expression, being confined largely to the brain, heart (most strikingly on postnatal day 7) and testes. These data are in accord with other recent analyses of *Timp-4* expression by Northern blotting and conventional RT-PCR [7,33,34]. *Timp-4* expression in the brain was first detected at day 15.5 p.c. with levels then rising through to postnatal day 21, significantly later than the maximum *Timp-2* expression. The levels of TIMP-4 protein during brain development determined by Western-blot analysis followed a pattern very similar to that of the mRNA (results not shown). Thus in the brain, expression of *Timp-2* and *Timp-4* rises in parallel in late embryonic and early postnatal stages, with *Timp-4* becoming the dominantly expressed gene at later times.

***Timp-4* genomic organization**

A *Timp-4* genomic clone was isolated from a mouse 129 embryonic stem cell genomic DNA library. The gene was further subcloned, restriction-mapped (Figure 2) and partially sequenced. The exon structure of the mouse gene was determined by sequence analysis using primers designed for all the expected murine exons based on the known human gene organization (Table 2). As expected, the *Timp-4* genomic organization was identical to that of the human homologue, with all available cDNA sequence being accommodated in five exons, all with consensus boundary sequences (Table 2). All *Timp* genes identified to date reside in an antisense orientation within an intron of a synapsin gene, including the recently identified *Drosophila Timp* [35]. Further sequence analysis of the *Timp-4* genomic clones identified two exons of mouse *Syn2* between -2900 and -2300 (all numbering is relative to the translation start site). Therefore, we inferred that the *Timp-4* promoter must lie downstream of the -2300 *Syn2* exon.

***Timp-4* promoter is TATA-less and lacks many identifiable promoter landmarks**

Since *Timp-4* shows a very restricted pattern of expression and inducibility ([9], and K. J. Leco, unpublished work), we wanted to define the *Timp-4* promoter and ascertain the major control elements. Initially we mapped the transcription start points of *Timp-4* by 5'-rapid amplification of cDNA ends (RACE) PCR. The positions of the endpoints determined from a series of 5'-RACE PCR clones are identified in Figure 3, which shows the sequence of approx. 1 kb lying upstream from the *Timp-4* translation start site. An RPA indicated two major transcription start points located approx. 155 and 225 bp from the 3' splice junction of exon 1, using mRNA from mouse heart and brain tissues (Figure 4). The positions of these sites were consistent with the termini identified from RACE PCR.

The putative promoter region surrounding the transcription start sites possesses few identifiable transcription-factor-binding motifs, although single motifs for GATA-, myogenin-, Ets-, Sp1- and CCAAT-binding proteins were found by screening the sequence through the TRANSFAC databases [37]. The sequence also lacks a classical TATA box, but does contain a sequence ATAAA at -100/-96 that may act as such. However, this sequence can be encompassed within an Inr motif that is close to one of the transcription-initiation sites. This initiator-like sequence (TCATAAA; -102/-96) diverges somewhat from the consensus Inr motif (YYANWYY) [38].

***Timp-4* promoter shows expression in C3H10T1/2 cells**

To determine whether the sequence identified was the *Timp-4* promoter, several promoter luciferase reporter constructs were generated. Constructs containing the sequences -1026 to -21 or +239 (Figure 5A) were initially transfected into mouse C3H10T1/2 fibroblast cells. All constructs showed a strong level of reporter expression over the empty vector (Figure 5B). The addition of exon 1 and part of intron 1 (-20 to +239) to the promoter construct -1026/-21 reduced reporter levels by approximately a third. This reduction may be due to aberrant splicing of the constructs (which contain a 5' but no 3' splice junction) or the presence of a repressor element within this additional sequence. Interestingly, no significant differences could

Table 2 Intron/exon structure alignment of human and murine *Timp-4*

Intron sequences are shown in lower-case letters and exon sequences are shown in upper-case letters. Bold letters indicate splice-site sequences.

Exon	Size (nt)	Human versus mouse intron/exon/intron sequence	Intron size
Human 1	—	TCG GCA CTT G gtg agtccga	1.2 kb
Mouse 1	—	TCG GCT CTA G gtg agtcctat	1.0 kb
Human 2	98	ttcctatgc ag TG ATT CGG ...	0.45 kb
Mouse 2	98	gttactttt ag TG ATA CGG ...	0.42 kb
Human 3	115	ttgcctcga ag ATG TTC AAA ...	2.3 kb
Mouse 3	115	ctgcctttt ag ATG TTT AAA ...	2.7 kb
Human 4	125	ctcccttga ag GT CAG GTC ...	0.7 kb
Mouse 4	125	ctctccaca ag GC CAG ATT ...	0.74 kb
Human 5	634	tgactct ag ATC ACC ACC ...	
Mouse 5	459	cacgaact ag ATC ACC ACT ...	

↓BamHI

-1026 GGATCCAGCC TCCTAACCCCT CCCCGGGCAG CCATTCCAGC AGCTTCTTTT CATTCAAATT CTGGAGCAG TTGCTGACAA

-946 ACAGTGGACA AGGCTTGGGT CACAATGAGT GCCTGGCTTG TTTGCTAAGC GGCTCCACCT TCTGCAACAT GGCAGTCTCC

Myogenin

-866 ACTGGGCAAT GATGTGCAGT AGGCGCTCTG AGGTCCAAAG TTTGCCCTGA GCTACAGGAC ACCCCTGTTT TAAGCTCCAC

-786 AGAAGAAGCT GGGCCCCGCT CCCCCCCCCC CCCCCCCCCC CGTAGCGGTT AACTAAGGCA CTGGCTGTAA GAAAGGGTTT

-706 GAAAGCACTG GCTACTTTCT CTGACATCCT TCCCTACCAC CCCCCACAGC CCCCACCCCC ACCCTTGGCT GACAGCGCCA

-626 AGACACACAC ACCCCAACCT TCTCTGTCTT GTTCCCTGAC TGTTTGCTGT TGCTTTGGAG CCTCGTAAAT AGGGCATTGA

-546 AAACACTTCC TGCAGCTGAA AGAAACCCCTG AGTAGCTTGT CTCACTCCCA GTATGCAGCT CAGCAAGGGG TCTGTCCTCC

-466 TCTGTCACTG TCTCTTTTGC CTGTTGTAAT TCTGTCTGCC TCTGACTTGG CCTGTGTCTC TCTGTGTCTA TGCTTCCCTT

-386 CACTAGCTCT TGCTCTTAGT GCTCTCCCTC ATCTCTCTGC CTTTCTTAAG TTCTCTTCAC TGGTCTTTGC TTTTTTTTTT

-306 TTTTTTTTTT TTTATCTCTC TGGCCCCTAA CTGCAGAATC TTCCTCTACT TCCAGGCTCT TTTCGGAATC TGCAGGTTTC

GATA Ets

-226 CCTCTGCAAC TTCCCAACTG GGTTTGTGTT TCTGGTCAGG AGGGCCTTGG GTCTGTGGC TGTGGGTGGC CTGGTGCCAA

* ↓HindIII

-146 AGGAGGGAGG AGCCCATGGG GCGGTGATTG GCTCTAAAGC CACCTCATAA AGACTGGGGC TGCACGAAGC TTTCTGGAGG

Sp1 CCAAT/NF1-box Inr

* SacI↓

-66 AGTCTCGGAG GGGCTGTTTG TTACGACAGG CAGACCCAC GAGCTCAGCT GGGGCTCTGC AGTGTCTATC CTGGAGTCC

AP-4 M P W S P

+15 CTGGCTGCG CTGAGCTGGG CGTGTGTGCT GAGGCTGCTG GCTTTGCTGT GGCCCCCTGG ACGGGGTGAG GCGTGCAGCT

L A A L S W A L V L R L L A L L W P P G R G E A C S C

+95 GTGCGCCTGC GCACCCCCAG CAGCACTTCT GCCACTCGGC TCTAGgtgag tccatggtcc tgaggaccac agcaggggtt

A P A H P Q Q H F C H S A L G

SacI↓

+175 qccagtaqqa aacqtqqtqt tqqqcagaqc tttaqaccgc qagctctgaqg qaaqqqqqtqg aqctc +239

Figure 3 Nucleotide sequence of the promoter of *Timp-4* through into intron 1

Timp-4 DNA sequence of the promoter construct pGL3 — 1026/ + 239 (GenBank accession no. AY072631). Useful restriction-enzyme sites used in the cloning of the promoter are shown above the sequence and marked with arrows. Single-letter amino acid code is included from the *Timp-4* ATG (+1) present within exon 1. Intron 1 is indicated in lower case. Transcription start points, as identified by 5'-RACE PCR (see main text), are indicated by a * symbol. Potential transcription-factor-binding sites, as identified by TRANSFAC analysis [37], are shown below their underlined binding motifs.

Figure 4 RPA shows multiple *Timp-4* transcription start sites

RNase protection was performed by hybridizing poly(A)⁺ RNA from mouse brain or heart (Clontech), or a tRNA control, with a [³²P]UTP-labelled *Timp-4* antisense riboprobe. The control lane contained no RNA. The final lane (Probe) contained the *Timp-4* antisense riboprobe of 1264 nt (−1026/+239) prior to RNase digestion. Hybridization was performed overnight and followed by digestion with RNase A and RNase T1. Protected fragments were then analysed on a sequencing gel next to a labelled DNA ladder.

be seen when the *Timp-4* ATG was intact or mutated to avoid the possibility of the expression of an aberrant *Timp-4*-luciferase chimera (Figure 5B). The same constructs were also active in a variety of human primary cell types, although reporter levels were greatly reduced, possibly due to human/mouse promoter incompatibility, or the fact that all cell types examined do not express *Timp-4* endogenously (results not shown). Reversal of the *Timp-4* promoter sequence −1026/−21 into the antisense orientation ablated all reporter expression (Figure 5C). Examination of the activity of the *Timp-4* promoter under basal (unstimulated) and stimulatory (10% serum) conditions for 6 or 24 h revealed no significant differences in reporter expression (Figure 5D). This reiterates previously reported data that *Timp-4* expression is unresponsive to the presence of serum [9].

Initiator and Sp1 mutations reduce *Timp-4* expression whereas CCAAT mutation increases it

To examine their contributions to overall *Timp-4* expression we mutated either the Sp1, CCAAT or Inr motifs in the context of a short (−281/−21) luciferase reporter construct and transfected these into C3H10T1/2 cells (Figure 6A). Luciferase expression from the mCCAAT construct was reproducibly 2-fold higher than that of the 'wild-type' expression vector. The same mutation in the larger −1026/−21 construct showed the same increase (results not shown). However, more striking was the significant reduction in expression upon Sp1 or Inr mutation. Either mutation reduced luciferase levels to approx. 20% of 'wild-type' expression and only negligibly above that of the basal level produced by the promoterless reporter vector control (pGL3-Basic; Figure 6A).

The putative *Timp-4* initiator exhibits specific protein binding

Initiator elements are thought to function in part by binding a transcription factor and nucleating the assembly of a basal transcription complex. EMSA showed that a double-stranded oligonucleotide probe encompassing the putative *Timp-4* Inr was bound by at least three factors, resulting in a predominant complex (Figure 6B, lane 2, B) and two minor complexes (Figure 6B, lane 2, A and C). Excess of the unlabelled 'self' (Inr) competitor resulted in the loss of all three complexes, indicating that all are specific for the *Timp-4* Inr sequence (Figure 6B, lane 3). An oligonucleotide containing a 3 bp mutation in the Inr sequence (mInr), identical to the functional mutation in the *Timp-4* −281/−21 construct (Figure 6A), did not compete with complex A when added to the binding reaction in excess (Figure 6B, lane 4). Therefore, all complexes are specific for the *Timp-4* sequence and complex A binds explicitly at the *Timp-4* Inr element.

DISCUSSION

The four *Timp* genes show dramatic differences in their spatio-temporal patterns of expression *in vivo*, and in their responses to stimuli *in vitro*. At least *Timp-1* and *Timp-3* are regulated principally at the transcriptional level [39,40]. We have performed the first detailed tissue-distribution analysis of *Timp-1–4* in developing and adult mouse using real-time quantitative RT-PCR (Figure 1). There was strong agreement with previously reported Northern-blot, *in situ* and reverse zymography data [33,40–43]. This analysis thus provided a potent assessment of the value of the quantitative RT-PCR technique, a key aspect of

Figure 5 *Timp-4* promoter-luciferase constructs are active in transient transfections of C3H10T1/2 cells

Timp-4 promoter-luciferase constructs were transiently transfected into C3H10T1/2 cells. All luciferase activity results were normalized for protein concentration and transfection efficiencies were monitored by means of a Hirt assay [26]. (A) Schematic of *Timp-4*-luciferase constructs used in transient transfections. (B) Initial analysis of the pGL3-Basic luciferase expression vector and *Timp-4* promoter-luciferase constructs (pGL3-T4 -1026/-21, -1026/+239 and -1026/+239mATG). (C) The constructs pGL3-Basic, pGL3-T4 -1026/-21 and pGL3-T4 -21/-1026 were transiently transfected into C3H10T1/2 cells to examine the orientation dependence of the *Timp-4* promoter. (D) The constructs pGL3-Basic and pGL3-T4 -1026/+239mATG were transfected into C3H10T1/2 cells and after 24 h of serum starvation were either stimulated with 10% FCS (solid bars) or serum-starved (open bars) for 6 or 24 h.

which is the ability to determine absolute transcript abundance. Our data showed *Timp-1* expression to be consistently lower than that of all other *Timps*; the highest levels were seen in adult female mouse reproductive tissues, including ovary, uterus and decidua, the peak in uterine expression occurring in mid-gestation, as reported previously by Waterhouse et al. [28]. Expression of *Timp-2* was more widespread, with the highest levels detected in the heart, brain and reproductive organs (ovary and uterus). *Timp-3* expression was high in adult kidney, heart and ovary [44] but a new finding here was that the highest *Timp-3* transcript level was found in adult thymus, which has not been examined previously.

Of all the *Timp* genes, *Timp-4* showed the most restricted expression distribution [6,7,45]. Our data show that its expression is confined largely to the brain (from the late prenatal stage to postnatal day 28), heart (newborn and postnatal day 7) and adult testes, which supplements previous localization experiments where *Timp-4* was restricted to neural, cardiac, breast and muscle tissues [7,21,22,45]. A previous study has reported *Timp-4* to be undetectable in mouse testis by Northern blotting, although expression of all four TIMPs has been seen in human fetal testis, with strong expression of *Timp-4* in Sertoli cells [34]. It will be of interest to revisit the expression of *Timp-4* during testicular

development in the mouse using the more sensitive real-time RT-PCR technique. One of the most striking findings of the present work is that in postnatal mouse brain; *Timp-4* expression overtakes that of any other *Timp*. Recent *in situ* hybridization studies have shown that the *Timps* have distinct patterns of localization, with *Timp-4* being expressed primarily by cerebellar Purkinje cells [33]. In contrast, *Timp-2*, which is the next most abundantly expressed *Timp* in mouse brain, is predominantly expressed in the meninges and choroid plexus [43]. This means that the TIMP-2/TIMP-4 ratio probably varies dramatically at different sites in the central nervous system, which will have profound effects on local MT1-MMP activities and thus pro-MMP-2 activation. In turn, this may influence processes such as neuronal extension, migration and synapse formation during central nervous system development.

We also undertook a characterization of the *Timp-4* promoter and upstream regulatory region to shed light on potential mechanisms regulating spatio-temporal expression of the gene. The putative promoter region was shown to be functional using transfection of various promoter-luciferase constructs into mouse C3H10T1/2 fibroblasts. Reversal of the *Timp-4* promoter into the antisense orientation completely abolishes this expression, and, coupled with our RACE and RPA analyses, this confirms

Figure 6 *Timp-4* promoter Inr characterization

(A) Plasmids pGL3-Basic and *Timp-4* promoter-luciferase constructs pGL3-T4 -281/-21 (wild-type; WT), pGL3-T4 -281/-21mSp1 (mSp1), pGL3-T4 -281/-21mInr (mInr) and pGL3-T4 -281/-21mCCAAT (mCCAAT) were transiently transfected into C3H10T1/2 cells. After 48 h cell extracts were prepared and assayed for luciferase expression. All luciferase activity results were normalized for protein concentration and transfection efficiencies were monitored by means of a Hirt assay [26]. The wild-type (boxed) and mutant Sp1, Inr or CCAAT motif sequences are depicted. (B) EMSAs using oligonucleotides across *Timp-4* -110/-85. EMSA was performed as described in the Experimental section using nuclear extract from mouse fibroblastic cell line C3H10T1/2. Competition was performed with a 100-fold excess of each unlabelled oligonucleotide over the labelled probe to identify specific binding. Specific bands are labelled A-C, as described in the text.

that we have indeed identified the promoter of *Timp-4*. Sequence analysis and mapping of exon/intron boundaries show the *Timp-4* genomic organization resembles that of *Timp-2* and -3, being encoded within five exons, all of which code for protein, and is distinct from *Timp-1* which is encoded by six exons, the first being untranslated. Furthermore, as described, *Timp-4* is located within an intron of *Syn2*. However, with this initial characterization of the *Timp-4* promoter, it is clear that, despite similarities in genomic structure, the overall organization, sequences and motifs of all four *Timp* promoters differ considerably (Figure 7). The *Timp-4* promoter lacks a classical TATA-box sequence. TATA-less genes often initiate transcription from several start points, and indeed our RPA analysis does show that *Timp-4* transcription begins at at least two points. The *Timp-4* promoter sequence contains long stretches of cytosine (21) and thymine (23) residues of unknown function and relatively few identifiable transcription-factor-binding consensus motifs. However, potential *cis* elements for binding factors myogenin, GATA and Ets family members can be identified.

Ets gene expression plays a key role during mammalian development and is associated with changes of ECM protein expression. The expression of several Ets family members coincides with our observed *Timp-4* expression [46,47].

Most tissues of either mesodermal or endodermal origin express one or more GATA-4, -5 or -6 factors at some point

during development. GATA factors regulate tissue-specific expression of many genes via interactions with other transcription factors, and can also repress transcription through interaction with transcriptional repressors such as CtBP2 (C-terminal-binding protein-2), via FOG-2 ('friend of GATA-2') [48]. It is interesting to note that GATA-6 represses proliferation of vascular smooth-muscle cells, and in response to mechanical stimuli GATA-6 mRNA levels are down-regulated at a time when *Timp-4* levels have been shown to increase [21,48].

Close to the *Timp-4* transcription start points are a consensus Sp1/GC box, CCAAT motif and a putative initiator (Inr) motif. Transcription from TATA-less promoters can initiate from Inr sequences that are often associated with a nearby Sp1 motif. Only *Timp-3* and *TIMP-2* contain sequences homologous to TATA boxes. All four *Timp* gene promoters contain potential Sp1 and other CpG-rich sequences, but overall there appears little organizational conservation of these motifs. Unlike *Timp-1*, -2 and -3, the *Timp-4* promoter region described here contains no activator protein 1 (AP-1) motif. The AP-1 motif of *Timp-1* is crucial to both basal expression of the gene and inducible expression *in vitro* [49]. The *Timp-2* AP-1, and possibly all six AP-1 motifs of *Timp-3*, appear(s) to be involved in the basal expression of their respective genes.

The *Timp-4* Inr-like sequence diverges somewhat from the consensus Inr (5'-TCATAAA-3' versus 5'-YYANWYY-3'), as

Figure 7 Comparison of *Timp* promoter organization

Schematics of the four *Timp* promoters (*Timp* represents mouse; *TIMP* is human). Elongated open rectangles represent the 5' UTR of each gene, arrows show the 5' transcription start points, and solid rectangles (after the ATG) represent coding sequence. T indicates a putative TATA box. Transcription-factor motifs: solid diamond, AP-1; open square, PEA3/Ets; solid square, GC box; star, nuclear factor- κ B; parallelogram, Sp1; solid circle, initiator; solid triangle, GATA; open oval, CCAAT.

designated by the terminal deoxynucleotide transferase gene [38]. Mutation of the *Timp-4* Inr resulted in an almost complete abolition of reporter expression in transiently transfected mouse cells, thus confirming the importance of this sequence in basal *Timp-4* expression. Furthermore, the mutation did not alter the ATAAA of the Inr sequence, implying that this sequence is not a potential TATA box. The core promoter of a gene transcribed by RNA polymerase II is recognized by the basal transcription factor TFIID. This supplies the scaffolding upon which the rest of the transcriptional machinery can assemble. TFIID is a multi-subunit complex that usually contains TBP (TATA-binding protein) and several TBP-associated proteins, which may play a role, e.g. as transcriptional co-activators. Interestingly, recent data have demonstrated that transcription initiation can be stimulated by several activators in a TFIID-independent manner, implying the existence of alternative co-activators other than TBP-associated proteins. Indeed several putative co-activator complexes, which contain many of the same polypeptides, have recently been identified. Many of these proteins are also components of the Sp1 co-factor CRSP (co-factor required for Sp1 activation) [50]. Such a novel co-activator may be involved in *Timp-4* expression due the close proximity of the Sp1 motif to the transcription-initiation site.

In summary, we have identified the basic architecture of *Timp-4* and demonstrate the functional importance of Sp1 and Inr-like elements in basal gene expression. Further work will be required to examine and identify the factors that can bind specifically to the *Timp-4* Inr-like element and the functional complexes that bind the putative CCAAT and Sp1 motifs. In addition, the presence of Ets/PEA3 and GATA sites further marks the distinctive organization of the *Timp-4* promoter, identifying them as prime candidates for further studies of the tight spatio-temporal control of *Timp-4* expression.

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