The Human Tissue Inhibitor of Metalloproteinases (TIMP)-1 Gene Contains Repressive Elements within the Promoter and Intron 1*

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Expression of the TIMP-1 (tissue inhibitor of metalloproteinases-1) gene is tightly controlled during embryonic development and in the adult animal. Previous studies have focused on elements within the gene promoter which activate transcription of the gene. Here, we identify two regions of the gene which repress transcription: An element upstream of the basal gene promoter at −1718/−1458, represses expression of a reporter gene by approximately 50%; addition of the first intron to any promoter-reporter construct also strongly represses gene expression. The TIMP-1 gene has a short first exon which is transcribed but not translated, with the translation start site located in exon 2. Deletion analysis through intron 1 reveals a number of potential regions which might mediate its effect. Protein binding studies and mutational analyses reveal that a repressive element at +684/+748 binds Sp1, Sp3, and an unidentified Ets-related factor to suppress transcription.

The matrix metalloproteinases 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, tumor invasion and metastasis, corneal ulceration, and liver fibrosis (2).

The active forms of all of the matrix metalloproteinases are inhibited by a family of four specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).1 Inhibition represents a major level of control of matrix metalloproteinase activity and as such, is a therapeutic target (3). 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, leukemia inhibitory factor, and oncostatin M (4–8). Where represses gene expression. The intron to any promoter-reporter construct also strongly represses expression of the TIMP-1 gene. Flenniken and Williams (9) found that a construct containing around 1.3 kb of murine Timp-1 5′-flanking sequence, exon 1, and most of intron 1 linked to a lacZ reporter in transgenic mice was sufficient to reproduce the spatial and temporal expression of the TIMP-1 gene in developing mouse embryos. In contrast to this, transgenic mice carrying lacZ linked to 2.7 kb of Timp-1 5′-flanking sequence, but lacking intron 1, display a subset of the correct pattern of expression (e.g., appropriate expression in the developing vertebral column, and absence in the liver), but also inappropriate expression of the lacZ reporter in sites such as the spinal cord.2 Thus sequences within intron 1 are likely to repress Timp-1 gene expression.

This study investigates in more detail the properties of the first intron in the human TIMP-1 gene, and identifies a further repressive element upstream within the gene promoter. We report that the whole of the first intron strongly represses expression of a reporter gene, while deletion analysis reveals a number of potential regions which might mediate its effect. Protein binding studies and mutational analyses reveal that a repressive element at +684/+748 binds Sp1, Sp3, and an unidentified Ets-related factor to suppress transcription. Furthermore, a region upstream of the promoter between −1718 and −1458 also represses expression of a reporter gene.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Cloning and sequencing of a fragment −1718/1188 of the human TIMP-1 gene has been previously described (10). Constructs were made within pBLCAT3 of −1718/+988 (containing both splice acceptor site and translation start site ATG); −1718/+978 (containing the splice acceptor site, but no translation start site); −1718/+965 (containing neither splice acceptor nor translation start site), as outlined in Fig. 1. Deletion sets were generated from the −1718/+988 using Bal-31 exonuclease to digest from the 3′ end (after BamHI digestion), followed by blunting and then removal of the digested insert using XhoI. The digested insert was then subcloned into pBLCAT3 digested with BglII, blunted, and re-cut with XhoI. Deletions from the 5′ end were also made using Bal-31 exonuclease after XhoI digestion of −1718/+988, followed by blunting and removal of the digested insert with BamHI; inserts were then subcloned into pBLCAT3 digested with SalI, blunted, and re-cut with BamHI. Creation of a 3′ end at +95 for the 5′ deletion set was achieved by PstI digestion, purification of the digested insert DNA, and subcloning back into pBLCAT3 digested with PstI. Point mutations were introduced using oligonucleotide-based polymerase chain reaction methodology (QuikChange, Stratagene) and identical oligonucleotides to those used in electrophoretic mobility shift assays (Table I). All mutations were confirmed by sequencing.

Transient Transfection and Reporter Gene Assay—Transient transfection using constructs in pBLCAT3 was performed into primary hu-
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Fig. 1. Construction of plasmids containing intron 1. -1718/+1188 of the human TIMP-1 gene contains upstream sequences, exon 1 (transcribed but not translated), intron 1, exon 2 (containing the translation start site), and some intron 2 sequence. Three constructs containing intron 1 were made: -1718/+988 containing both splice junction acceptor site and translation start site ATG; -1718/+978 containing the splice junction acceptor site, but no translation start site; -1718/+965 containing neither splice junction acceptor nor translation start site. Deletion sets were then constructed using Bal-31 exonuclease digestion of -1718/+988.

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human skin fibroblasts using the FuGene 6 transfection reagent (Roche Molecular Biochemicals). Briefly, cells were plated at approximately 5000 cells/cm² into 60-mm dishes or 6-well plates and allowed to adhere for 24 h in MEM, 10% fetal calf serum. FuGene 6 (3 μl/dish or well) was diluted into 100 μl of MEM (serum free) and incubated at room temperature for 5 min. DNA (1 μg/dish or well) was added, mixed, and incubated at room temperature for 10 min. 100 μl of the FuGene 6/DNA/MEM mixture was then added dropwise to each dish or well and incubated overnight at 37 °C in 5% CO₂ atmosphere. Cells were then washed two times with Hanks’ balanced salt solution and medium was replaced with MEM, 0.1% BSA. After a 48 h incubation at 37 °C in 5% CO₂ cells were harvested by scraping. Extracts for CAT assay using an enzyme-linked immunosorbent assay method (Roche Molecular Biochemicals) were prepared by freeze-thawing and protein concentration was determined using the Bradford assay (Bio-Rad). Each construct was tested in triplicate within a single experiment and experiments were performed at least three times with at least two different preparations of each plasmid.

Transfection efficiency was monitored using the Hirt’s assay (11). Here, a nuclear extract from the transfected cells was probed with a CAT-specific cDNA on a slot blot.

Cell Culture—Human foreskin fibroblasts were isolated by enzymatic digestion as described previously (10). Cells were maintained in MEM supplemented with 1% non-essential amino acids, 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 units/ml nystatin.

Nuclear Extracts—Nuclear extracts were prepared from 5 × 10⁶ human skin fibroblasts cultured in 0.1% BSA as above for 48 h using a modification of the Dignam method (12). Cells were scraped into ice-cold phosphate-buffered saline, pelleted at 500 × g, and resuspended in 1 ml of phosphate-buffered saline containing 0.1% Nonidet P-40 for approximately 30 s. After centrifugation at 12,000 × g for 10 s, supernatant was removed and the nuclear pellet was resuspended in 3 volumes of high salt buffer (25 mM HEPES, pH 7.8, 500 mM KCl, 0.5 mM MgSO₄, 1 mM dithiothreitol) containing EDTA-free Complete protease inhibitors (Roche Molecular Biochemicals). The suspension was incubated on ice for 20 min with occasional vortex 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 5–10 μg of protein/μl.

Electrophoretic Mobility Shift Assay (EMSAs)—Oligonucleotides were annealed in equimolar amounts and end-labeled with [γ-³²P]ATP followed by desalting through Sephadex G-25 spin columns. Binding reactions (10 μl total volume, incubated for 1 h at 4 °C) contained radiolabeled probe (approximately 40,000 cpm), 4 μl of nuclear extract and 1 μg of poly(dI-dC) in 1 × binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 5 mM MgCl₂, and 5% glycerol). Antibody supershift/inhibition analyses, a further incubation with 1 μl of antibody was performed for 1 h at 4 °C or 25 °C. Antibodies were from Santa Cruz Biotechnology (anti-Sp1, sc-59x; anti-Sp3, sc-644x; anti-Ets, sc-112x; anti-c-Myc, sc-517x). It should be noted that in EMSAs, antibodies may either further retard the migration of a protein-DNA complex (supershift) or block binding of the protein to the DNA (inhibition) depending on epitope recognized and binding affinity (13). Recombinant Sp1 was from Promega, 1 μl (which equals 1 footprinting unit of the topographically expressed Sp1 solution) was added to each binding reaction. Binding reactions were electrophoresed on a 7% polyacrylamide non-denaturing gel in 1 × TBE (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.

DNase I Footprinting—A +470/+970 fragment was generated by restriction digestion of −1718/+988 with EcoRI and Sp1 and gel purified. The DNA fragment was end-labeled with polynucleotide kinase, followed by digestion with BgII to yield a +470/+970 fragment. Binding reactions were in a 50-μl volume containing approximately 40,000 cpm of labeled DNA, 25 μg of nuclear extract, 2 μg of poly(dI-dC), and final concentrations of 4 μg HEPES, pH 7.9, 84 mM NaCl, 0.3 mM MgCl₂, 0.1 mM dithiothreitol, 0.04 mM EDTA, and 5% glycerol. The binding reaction was allowed to proceed for 20 min at 4 °C prior to the addition of 5 μl of a 5 mM CaCl₂, 10 mM MgCl₂ mixture. After incubation for 1 min, 6 units of DNase 1 was added and the reaction allowed to proceed for a further minute prior to the addition of 140 μl of a DNase stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% (w/v) SDS, 64 μg/ml yeast RNA). The reaction was then extracted with phenol/chloroform (1:1), precipitated with ethanol, and resuspended in 3 μl of loading dye (deionized formamide containing 10 mM EDTA, 0.8% (w/v) bromphenol blue, and 0.3% (w/v) xylene cyanol). Samples were heated to 75 °C for 2 min and separated on an 8% polyacrylamide, 8 M urea sequencing gel. Control reactions substituted bovine serum albumin for nuclear extract. G/A ladder was prepared by boiling the labeled DNA fragment in loading dye for 1 h. After electrophoresis, the gel was dried and subjected to autoradiography.

RESULTS

Repressive Elements in the Human TIMP-1 Gene—Our previously published data described transcription from a −738/+95 construct of the human TIMP-1 gene containing exon 1 and the first 47 base pairs of intron 1 in pBLCAT3 (10). Fig. 2 shows that addition of further 5' sequence up to −1458, has no effect on the levels of CAT reporter under basal culture conditions when transiently transfected into primary human skin fibroblasts. Addition of a further 5' sequence up to −1718 gives approximately a 50% reduction in gene expression.

Three constructs containing intron 1 were made: −1718/+988 containing both the 3' splice junction acceptor site and the translation start ATG of exon 2, in-frame with the coding region of the CAT gene itself; −1718/+978 to contain the 3' splice site, but no ATG from the TIMP-1 gene; −1718/+965 to contain neither 3' splice site, nor TIMP-1 ATG. In transient transfection experiments, these three constructs all repressed expression of the CAT reporter down to the level of the empty pBLCAT3 vector as shown for the −1718/+988 construct in Fig. 2.

Intron 1 Contains a Number of Potential Regulatory Ele-
mats—Starting with the −1718/+988 construct, a series of 3′ deletion mutants were created using Bal-31 exonuclease digestion to progressively remove intron 1 sequences in the context of the −1718 5′ end. These were transiently transfected into primary human skin fibroblasts and expression of CAT measured under basal culture conditions. Fig. 3 shows that deletion to −1718/+748 has no effect on CAT expression, but further deletion to −1718/+684 reproducibly increases CAT levels by approximately 4–5-fold. Expression remains high in −1718/+654, −1718/+649 (data not shown), and −1718/+637. Deletion to −1718/+561 reduces CAT expression to background levels again. This pattern is repeated with another region of increased expression across −1718/+413 and −1718/+397, and reduced expression in −1718/+348. Expression then rises again from −1718/+317 through until the shortest construct of −1718/+95. In constructs containing intron 1 (with a 3′ end at +988), deletion from the 5′ end from −1718 to −320 does not increase expression of the reporter gene (data not shown).

It is possible that the absence of CAT protein from transfections of reporter constructs lacking the 3′ splice junction acceptor of intron 1 of the TIMP-1 gene might result from a failure to splice intron 1 sequences from the primary transcripts. Although additional ATG sequences within intron 1 are not involved in changes in expression (see “Discussion”), it is still possible that intronic sequences in an extended 5′-untranslated region ahead of the reporter cassette might affect translational efficiency. In order to address this question, we examined splicing of transcripts derived from the intron 1 deletion reporter constructs using reverse transcriptase-polymerase chain reaction with a forward primer in exon 1 of the TIMP-1 gene and a reverse primer in the CAT gene, −1718/+988 which contains the native splice junction donor and acceptor sites of intron 1 of the TIMP-1 gene, splices correctly using these sites. All of the other constructs contain the splice junction donor site, but not the acceptor, but the two constructs we tested, −1718/+748 and −1718/+684, still splice using cryptic acceptor sites. We examined this in more detail since this was the region our study focused upon (see below), and sequenced the polymerase chain reaction products to ascertain the exact site of the splice junction acceptor site used. The construct −1718/+748 splices using the AG at +712, while the construct −1718/+684 splices using the AG at +676 (data not shown). Thus, the level of production of CAT protein from the intron 1-deleted constructs is not attributable to the absence of the 3′ splice junction acceptor.

DNase I Footprinting Identifies Protein Binding Across +712/+738—A +470/+970 fragment of the human TIMP-1 gene was subjected to DNase I footprinting in order to identify protein-binding regions which might contribute to changes in expression seen in the deletion analysis. Nuclear extract was prepared from quiescent human skin fibroblasts. Fig. 4 shows a broad footprint across +712/+738 using nuclear extract compared with the bovine serum albumin control. Computer analysis of this region identifies three consensus sequences for transcription factor binding; one at +725/+734 for c-Myb, one at +708/+716 for Sp1, and one at +703/+710 for c-Ets.

Electrophoretic Mobility Shift Assay Demonstrates Sp1 and Ets Binding—Using oligonucleotides −698/+743 across the footprinted region, and oligonucleotides +698/+726 and +719/+743 to separate out the Ets and Sp1 sites in the former from the c-Myb site in the latter, EMSA was performed. Initially, binding and competition studies with all three oligonucleotides were performed (Fig. 5), identifying three low mobility bands (bands a, b, and c) as specific to the +698/+726 region and a further band (band d) as specific to the +719/+743 region.

This initial data was pursued by supershift/inhibition analysis using antibodies against the transcription factors identified in the footprinting above. Fig. 6A shows that in the context of +698/+743 oligonucleotide, anti-Sp1 antibody blocks binding of a low mobility doublet (bands a and b), while in the context of the +698/+726 oligonucleotide, the same antibody also blocks binding of a third band of slightly faster mobility (band c). Anti-Ets and anti-c-Myb antibodies have no effect on the shift seen with these oligonucleotides. The pattern of three bands (a, b, and c) in Fig. 6A is described in the literature as...
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Fig. 4. DNase I footprinting of +470/+970 of the human TIMP-1 gene. A +470/+970 fragment of the human TIMP-1 gene was subjected to DNase I footprinting. Nuclear extract was prepared from quiescent primary human skin fibroblasts, and compared with BSA. A GA ladder is shown to position the protected areas, and the full sequence, along with consensus sequences for known transcription factors is also shown.

deriving from the binding of both Sp1 and Sp3 transcription factors (e.g. Refs. 14–17). Fig. 6B shows that in the context of +698/+726, at 25 °C, anti-Sp1 antibody blocks the formation of band a, and partially blocks band b; anti-Sp3 antibody blocks formation of bands b and c. This suggests that band a contains Sp1, band b contains both Sp1 and Sp3, and band c contains Sp3. Some cross-reactivity of the anti-Sp1 antibody for Sp3 may explain its ability to block formation of band c at 4 °C as seen in Fig. 6A.

Fig. 7 demonstrates that recombinant Sp1 does not bind to the +698/+743 or +698/+726 oligonucleotides when added to the binding reaction in isolation. However, strong binding is observed to both of these oligonucleotides when recombinant Sp1 is added in the additional presence of nuclear extract. Furthermore, the binding of both Sp1 present in the nuclear extract and the recombinant protein is only partially abrogated by a mutation in the Sp1 site in the context of the +698/+743 oligonucleotide, but completely abrogated by the same mutation in the context of the +698/+726 oligonucleotide. This suggests that other nuclear factors are responsible for tethering the Sp1 to the DNA via binding to the +726/+743 region of the longer oligonucleotide. Furthermore, Fig. 7 shows that Sp1 and Sp3 bind to the same site, since addition of recombinant Sp1 competes away bands b and c, with concomitant increase in band a, the major Sp1-containing complex found in Fig. 6B.

Fig. 8 shows that binding of nuclear extract to a +719/+743 oligonucleotide in the additional presence of an anti-Ets anti-body, leads to a strong supershifted band; anti-Sp1 or anti-c-Myb antibodies have no effect (data not shown). Since this region has no canonical sites for the binding of Ets factors, we undertook a linker scan method to identify the region responsible for the supershift. Strings of five residues were replaced with adenosines across the +719/+743 sequence, as shown in Table 1. These were then used in EMSA with nuclear extract alone, or in the additional presence of anti-Ets antibody. Although some of these mutants show increased binding of proteins from nuclear extracts, it is clear from Fig. 8 that the supershift is lost in mutants 1, 2, and 5. This localizes binding to the +719/+726 region (5′-TCTCCC-C3′), with additional contributions from the +731/+735 region (5′-GCCAC3′).

Functional Analysis of the +698/+743 Sequence—In order to establish whether transcription factor binding identified in the footprinting and EMSA studies was responsible for the increase in gene expression observed upon deletion of the +748/+684 sequence, we undertook a point mutational analysis of this region in the CAT constructs. In the context of −1718/+988, the mutation which abrogates Sp1 binding in the gel shift analyses increases expression of the reporter gene approximately 3-fold (Fig. 9). Interestingly, a mutation in this region which destroys the Ets site at +703/+710 also increases expression of the reporter gene by a similar amount, as does a mutation which destroys the Ets site at +703/+710, but increases binding of Sp1 and Sp3 in EMSA experiments. Since the splice junction acceptor site used by the −1718/+748 construct is at +712, and this is destroyed by the mutation which abrogates Sp1 binding, we ascertained that the mutations in the context of −1718/+988 had no effect on splicing. Reverse transcriptase-polymerase chain reaction shows that transcripts from all of the mutant constructs splice in an identical manner to the wild-type −1718/+988 construct (data not shown). Hence, the increase in expression of CAT from these mutants is not due to alterations in splicing.

DISCUSSION

Our previous data (10) addressed the identity of the minimal promoter for the human TIMP-1 gene, utilizing a −738/+95 fragment as our starting point. However, our genomic clone contained upstream sequences to −1718 and downstream sequences through the first intron and into exon 2 (+1188). In order to study the function of these sequences, more distal to the transcription start point, we performed deletional analyses from both 5′ and 3′ ends of a −1718/+988 construct (containing the translation start point ATG within exon 2 at the 3′ end). In order to increase expression from a CAT reporter, thereby enhancing the sensitivity of the experiment, the 5′ deletion set was assessed in a +95 context (downstream sequences being highly repressive, see below). Of the 5′ sequence, the removal of the −1718/−1458 region, increased the basal expression of a CAT reporter gene by approximately 2-fold; no further differences were observed with deletions to −738. Interestingly, this region contains an Alu repeat at −1687/−1487. Alu repeats are sequences interspersed throughout the human genome with an average spacing of 5 kb, with the total number of Alu repeats estimated at more than 500,000 in the haploid human genome (18). Their function is unclear, but they often delineate genes, and hence, the −1687/−1487 Alu repeat may represent the 5′ boundary of the human TIMP-1 gene. Alternatively, they have been frequently identified in the regulatory regions of genes, both associated with transcriptional enhancers and silencers (19), suggesting a possible role in modulating gene expression. However, until further deletional and mutational analyses of the −1718/−1458 region are performed, the possible role of Alu in expression of the human TIMP-1 gene remains purely speculative.
The addition of intron 1 to the -1718/+95 construct repressed basal expression of the CAT reporter to background levels. Since exon 1 of the TIMP-1 gene is transcribed, but not...
translated, with the translation start site ATG at the beginning of exon 2, we made constructs both with and without the intron 1/exon 2 junction, and recombinant Sp1 (Promega). Oligonucleotides with mutation in the Sp1-binding site (ΔSp1) are listed in Table I. Bands a—c contain Sp1 and/or Sp3 as described in the text.

Table I

| Primer name          | Sequence (5’ → 3’)                     |
|----------------------|----------------------------------------|
| +698/+743            | CTCAAACTTCCTCAAAGATGGCCACACCCCAT      |
| +698/+726            | CTCAAACTTCCTCAAAGATGGCCACACCCCAT      |
| +719/+743            | TCTCCCAAGTGCCACACCCCAT                |
| +698/+743 ΔSp1       | TCTCCCAAGTGCCACACCCCAT                |
| +698/+726 ΔSp1       | TCTCCCAAGTGCCACACCCCAT                |
| +698/+743 ΔEts/Sp1(up)| TCTCCCAAGTGCCACACCCCAT                |
| +698/+743 ΔEts       | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(1)      | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(2)      | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(3)      | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(4)      | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(5)      | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(6)      | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(7)      | TCTCCCAAGTGCCACACCCCAT                |
| +719/+743 5A(8)      | TCTCCCAAGTGCCACACCCCAT                |

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translated, with the translation start site ATG at the beginning of exon 2, we made constructs both with and without the intron 1/exon 2 junction, and with and without the exon 2 ATG; the level of expression from all of these constructs was low. We had predicted this pattern from previous studies with transgenic mice: a lacZ transgene driven by 1.3 kb of the murine Timp-1 5’-flanking sequence, exon 1 and most of intron 1 (~1350/+775 of the murine Timp-1 gene) was reported to be sufficient to reproduce the spatial and temporal expression of the Timp-1 gene during murine embryonic development (9); in contrast, our own studies showed that a transgene driven by 2.7 kb of murine Timp-1 5’-flanking DNA, part of exon 1, but lacking the intron 1 (~2700/+47), display only a subset of correct developmental expression, with inappropriate expression in sites where the Timp-1 gene itself is silent. This is despite the fact that these same sequences (~2700/+47), driving a CAT re-
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Fig. 9. Transient transfection of point mutants in human skin fibroblasts. Plasmids as shown were transiently transfected into primary human skin fibroblasts as described under “Experimental Procedures”; mutations are listed in Table I. 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 mean ± S.E.

porter in stably transfected cells, recapitulate the responses of the native gene to phorbol ester, serum, transforming growth factor-β, epidermal growth factor, and dexamethasone in vitro. The intrinsic sequences are therefore implicated in repression of the Timp-1 gene in the mouse, and this is supported by the fact that constructs of −2700/+775 and −223/+775 of the murine Timp-1 gene in a CAT reporter vector show greatly reduced expression compared with constructs with end points at +190.3 It should be noted here that the murine intron 1 is 704 base pairs, compared with the human intron 1 of 929 base pairs. There are several examples of regulatory elements, both positive and negative, within introns, most commonly the first intron; this has perhaps been best described in the collagen gene family (Ref. 20, and references therein). These may be important in physiology and pathology, e.g., a G/T polymorphism in an Sp1 site within the first intron of the Col1A1 gene, has been associated with reduced bone mineral density and a tendency for osteoporotic fracture (21).

Further 3′ deletional analysis through intron 1 revealed a complex pattern of increasing and decreasing reporter gene expression. We considered the possibility that an mRNA containing intrinsic sequences might contain many ATG translation start sites followed by in-frame stop codons; however, only three ATG sequences exist within intron 1 at +136/+138, +906/+908, and +915/+917, and their removal does not correlate with changes in reporter gene expression. Furthermore, we discovered experimentally that constructs −1718/+748 and −1718/+684 both undergo splicing of at least part of the intron using cryptic splice junction acceptor sites, removing the ATG at +136/+138. It is possible that intron 1 contains a number of regulatory elements, both positive and negative, and that on deletion of one of these elements, another becomes dominant, explaining the pattern of increased and decreased expression in the 3′ deletion set. This pattern is unlikely to be an artifact since more than one construct exhibits each of the levels of expression observed.

Since the first increase in expression of the reporter was observed when the +748/+684 region was deleted, protein binding to this region was assessed using DNase I footprinting. This revealed protection across a sequence containing consen-
sus binding sites for the transcription factors c-Myb, Sp1, and c-Ets. EMSA demonstrated that both Sp1 and Sp3 could bind to the Sp1 consensus at +708/+716; the ability of an anti-Sp1 antibody to block this binding at 4 °C was only partial in the context of a +698/+743 oligonucleotide, whereas, complete abrogation of binding was seen in the context of a shorter, +698/+726 oligonucleotide. This suggests that additional factors binding within +726/+743 are tethering the Sp1 factor to its cognate sequence. This is reinforced by the fact that recombinant Sp1 alone does not bind to either the +698/+743 or +698/+726 oligonucleotides; however, in the additional presence of a nuclear extract, the recombinant Sp1 binds strongly to both oligonucleotides, also displacing the Sp3 which would otherwise bind to this Sp1 consensus. It is also apparent that mutation within the Sp consensus only partially blocks binding of the Sp1-Sp3 complex or the recombinant Sp1 in the context of +698/+743, but completely blocks binding in the context of +698/+726. GC boxes (and the related GT boxes) are one of the most widely distributed motifs in promoter elements, and were originally thought to be bound only by the transcription factor Sp1 (22). More recently, further related proteins have been identified (Sp2, Sp3, and Sp4) which have similar structural features and transcriptional properties (23). While Sp1 activates transcription, the function of Sp3 is less clear, acting as an activator or repressor depending on the context and number of binding sites (17). In respect of the human TIMP-1 gene, there are at least nine Sp1 family consensus sequences within the −1718/+988 region (10). Forced overexpression of Sp1 in primary human skin fibroblasts has little effect on basal expression of CAT reporter from constructs −1718/+988, −1718/+748, −1718/+684; overexpression of Sp3 appears suppressive to these constructs (data not shown). Since this is true for the −1718/+684 construct, suppression is mediated through more than just the +708/+716 site described above.

Three sets of point mutations were introduced into the −1718/+988 construct to ascertain the role of the transcription factor binding seen in EMSA: A mutation which abrogated Sp1/3 binding to the +708/+716 site, a mutation which increased Sp1/3 binding to this site (data not shown) but destroys the neighboring c-Ets consensus at +703/+710, and a mutation which destroys the c-Ets consensus without altering Sp1/3 binding. All of these mutations increased expression in the context of −1718/+988 (and in a shorter −1718/+843 construct, data not shown). The cryptic splice junction acceptor sites at −712 and −676 are not used by these constructs, which splice correctly. Hence, it appears that any alteration in this region leads to an increase in expression, perhaps because a higher order protein complex is disrupted, e.g. with proteins binding around the transcription start point.

In summary, intron 1 of the human TIMP-1 gene strongly suppresses gene expression. However, within the intron are a number of elements which have the capacity to induce expression, and these may be involved in relieving repression at sites where the gene is expressed in vivo, and/or in response to cytokines and growth factors. Our future work aims to investigate this gene further using a transgenic mouse system.

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