Structure and Characterization of the Human Tissue Inhibitor of Metalloproteinases-2 Gene*

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We report here the characterization of the human tissue inhibitor of metalloproteinases-2 (TIMP-2) gene. The gene is 83 kilobase pairs (kb) long with exon-intron splicing sites located in preserved positions among the three members of the TIMP family. A 3.8-kb genomic DNA fragment flanking the 5'-end of the gene contains several regulatory elements including five Sp1, two AP-2, one AP-1, and three PEA-3 binding sites. Despite the presence of a complete AP-1 consensus at position −281, the promoter did not respond to 12-O-tetradecanoylphorbol-13-acetate treatment. However, 12-O-tetradecanoylphorbol-13-acetate response was generated by insertion of a similar AP-1 consensus at position −71, indicating the importance of the positioning of this motif. The promoter contains a typical CpG island; however, methylation of this island did not seem to influence gene expression. Analysis of the 3'-end of the gene revealed that the two mRNAs for TIMP-2 (1.2 and 3.8 kb) differ by the selection of their polyadenylation signal sites, but selection of these sites does not affect RNA stability. In summary, the TIMP-2 gene has several features observed in housekeeping genes, and differs significantly from TIMP-1 and TIMP-3 genes. These differences are likely to explain the specific roles that these inhibitors play in the regulation of matrix metalloproteinases.

Matrix metalloproteinases (MMP)1 are a large family of secreted neutral endopeptidases with a broad spectrum of proteolytic activity for several components of the extracellular matrix (ECM). Among this family three groups have been well characterized based on their substrate specificity and include collagenases, gelatinases, and stromelysins (1). These enzymes have been implicated in physiological and pathological conditions associated with breakdown of the ECM such as trophoblastic implantation, embryonic development, angiogenesis, osteoarthritis and tumor invasion (2, 3). The activity of these proteases in the ECM is regulated by specific inhibitors known as tissue inhibitors of metalloproteinases (TIMP). So far, the TIMP family consists of three members TIMP-1, −2, and −3, characterized in different species. The human TIMP-1 gene has been localized on the chromosome X (4), whereas the TIMP-2 and TIMP-3 genes have been assigned to chromosome 17 (5) and 22 (6, 7), respectively. These inhibitors inhibit the proteolytic activity of activated MMP by forming tight ($K_i \leq 10^{-9} \text{M}$) 1:1 stoichiometric inhibitory complexes with the enzyme (8). Despite the fact that the three TIMP genes share a common inhibitory activity for all members of the MMP family, there is experimental evidence indicating that they have specific functions. For example, TIMP-1 and TIMP-2 form preferential complexes with pro-MMP-9 and pro-MMP-2, respectively (9, 10), whereas TIMP-1 and TIMP-2 are present in a soluble form, TIMP-3 is associated with the ECM (11). TIMP-1 and TIMP-2 have been shown to promote the growth of erythroid precursor cells as well as a variety of normal and malignant cells, suggesting a bifunctional role for these inhibitors (12–14).

Additional evidence supporting specific roles for these three inhibitors in vivo has been provided by experiments showing their differential expression in cells and in tissues and during development. For example, in adult mice, TIMP-1 is preferentially expressed in epithelial tissues, in cartilage, and in muscles (15); and during murine embryonic development, TIMP-1 is specifically expressed in developing bone, whereas TIMP-3 is preferentially found in developing epithelia, cartilage, and muscles (16). TIMP-1 and TIMP-3 are up-regulated by 12-O-tetradecanoylphorbol-13-acetate (TPA) and TGF-β, whereas TIMP-2 is down-regulated by both agents (10, 11, 17). In macrophages, lipopolysaccharides have been reported to down-regulate TIMP-1 and up-regulate TIMP-2 (18). The promoters of the murine (19, 20) and the human (21) TIMP-1 and the murine TIMP-3 (22) genes have been fully characterized and shown to contain a TPA-responsive element (TRE), consistent with their response to TPA, whereas the human TIMP-2 (23) and TIMP-3 (7) promoters have only been partially characterized. Altogether, these observations suggest that each individual member of the TIMP family has a specific physiological function.

These observations led us to examine the molecular basis for some of these differences, by characterizing the human TIMP-2 (hTIMP-2) gene. In this report, we provide evidence supporting...
a major role of this inhibitor in providing a stable basal level of inhibitory activity in tissues.

**EXPERIMENTAL PROCEDURES**

**Isolation of TIMP-2 Genomic Clones**—A human TIMP-2 cDNA probe containing the full (1,055-nucleotide) sequence (24) was used to screen a human placenta library in the cosmid vector pLCM15 (Stratagene, La Jolla, CA) and a human chromosome 17 library in the cosmid SuperCos I vector (originally provided by Dr. Larry Deaven at Los Alamos National Laboratory). The human chromosome 17 library was screened by hybridization to duplicate high density clone arrays on nylon filters as described elsewhere (25). For the human placenta library, a total of 3.6 × 105 plaque forming units were plated on a nylon membrane (Bond-N, Amersham Corp.) and 20 plates in the presence of EDTA (final concentration 50 mM), and the samples containing digested DNA were ethanol precipitated and redissolved in 10 mM Tris pH 8.0 and 1 mM EDTA containing the last exon) derived from a EMBL 3 library (23) and oligoprobes corresponding to TIMP-2 cDNA sequences extending from amino acid 21 to 27 (oligo YDC1: 5'-GATTTCTTCTAAGGTGAGTCA-3'). These oligonucleotides correspond to TIMP-2 cDNA sequences located in corresponding exons 2 and 3 for TIMP-1 (26). Seven among these eight positive clones from the human placenta library and 12 positive clones from the human chromosome 17 library. The positive clones from the tertiary screening were then examined by Southern blot analysis, after digestion with EcoRI, using a 5'-end probe (a 2.6 kb PsI1 fragment containing exon 1), a 3' end probe (a 1.4 kb EcoRI fragment containing the last exon) derived from a EMBL 3 library (23) and oligoprobes corresponding to TIMP-2 cDNA sequences extending from amino acid 21 to 27 (oligo YDC1: 5'-GATTTCTTCTAAGGTGAGTCA-3') and amino acid 76 to 81 (oligo 1332: 5'-CTTTCCTCACAACGCTCAG-3'). These oligonucleotides correspond to TIMP-2 cDNA sequences located in corresponding exons 2 and 3 for TIMP-1 (26). Seven among these eight positive clones from the human placenta library were found identical and hybridized with the 5'-end TIMP-2 probe. One was positive with the 3'-end probe. None of these eight clones hybridized with oligoprobes corresponding to putative exons 2 and 3. Among the 12 clones isolated from the chromosome 17 library, none hybridized with the 5'-end probe, but eight (including clone 27F6) hybridized with both oligoprobes YDC1 (putative exon 2) and 1332 (putative exon 3) and with the 3'-end probe. One clone (clone 67H4) hybridized with oligoprobe YDC1 only. One clone derived from the human placenta library and containing exon 1 (clone 1.3.3) and two clones derived from the chromosome 17 library (clones 67H4 and 27F6) were then selected to obtain the entire map of the human TIMP-2 gene.

**Restriction Mapping of Genomic Clones**—We used a modified method of Wahl et al. (27) to map the positions of the EcoRI sites in the TIMP-2 genomic clones. Genomic sequences from these positive clones including the flanking T3 and T7 promoter sequences were excised from either the pWE 15 vector or the SuperCos I vector by digestion with NotI. These genomic fragments were then subjected to partial digestion with EcoRI (1 μg of DNA digested in the presence of one unit of enzyme) for 5, 10, 20, and 30 min at 37 °C. The reaction was blocked by the addition of EDTA (final concentration 50 mM), and the samples containing digested DNA were electrophoresed in a 1% agarose gel prior to transfer onto a nylon membrane and to hybridization in the presence of [γ-32P]ATP-labeled T3 and T7 oligonucleotides. Southern analysis and polymerase chain reaction using primers derived from different exon sequences of the hTIMP-2 cDNA were also performed to determine the size of the partial EcoRI fragments confirmed by gel analysis of genomic DNA.

**Cell Culture**—Human fibrosarcoma HT1080 cell line and mouse NIH3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in 60-mm tissue culture plates in the presence of minimum essential medium containing 10% (v/v) fetal bovine serum, 2 mm l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C under a 5% CO2 atmosphere. Two human bladder carcinoma cell lines (EJ and RT4; Ref. 28) were cultured as described previously.

**Analysis of the TIMP-2 Promoter Activity**—All the plasmids used to carry out the expression of the hTIMP-2 promoter were constructed using standard recombinant DNA technology (29). A 2.6-kb PsI1 genomic fragment flanking the 5'-region of the hTIMP-2 gene and extending into the first exon was isolated, cloned into pBluescript (Stratagene, La Jolla, CA), and sequenced in both strands by the dideoxynucleotide method of Sanger et al. (30). Fragments from a 2.3-kb PsI1-NotI fragment extending from nt −2243 to nt +34 (from the transcription initiation site, TIS) was subcloned in orientation upstream of a promoterless luciferase reporter gene in pGL2-basic vector (Promega, Madison, WI) to make plasmid pTIMP2-2243. This vector was then used to generate by digestion with exonuclease III (Erase-A-Base system) a series of plasmids containing progressive upstream deletions from the 5'-end of the inserted TIMP-2 promoter. Seven deletion mutant clones were selected and sequenced on their 5'-end to identify the extent of the deletion (pTIMP2-2088, −1936, −1827, −1501, −1135, −585, and −276). These deletion constructs were then used in transient transfection assays in mouse NIH3T3 and human HT1080 cells. The reporter gene constructs were transfected using a standard calcium phosphate precipitation method (31). As control for transfection efficiency, the plasmid pSVβ-galactosidase (Promega) was co-transfected with each construct. After 48 h, cells were harvested and lysed. Luciferase activity was measured as described by de Wet et al. (32), and β-galactosidase activity was carried out using the Galacto-light kit according to the instructions of the manufacturer (Tropix, Bedford, MA). Transfections were performed in duplicate and at least three separate experiments using 10 μg of test plasmid and 1.5 μg of control plasmid for each 60-mm plate. Data were expressed as percentage of the relative activity (ratio of luciferase over β-galactosidase activities) obtained with the full-length promoter construct (pTIMP2-2243). When indicated, cells were treated with TPA using the following procedure. Twenty-four h after transfection, the culture medium was changed for medium supplemented with 0.5% fetal bovine serum (rather than 10%), and the cells were cultured for another 32 h. TPA (dissolved in ethanol) was then added to the culture medium at a final concentration of 100 ng/ml. After 16 h, cells were harvested and analyzed for luciferase and β-galactosidase expression as described above.

**Mutations of the AP-1 Element in the hTIMP-2 Promoter**—Mutations in the hTIMP-2 promoter were achieved using the overlap extension method of Horton and Pease (33). We used an oligonucleotide corresponding to a sequence located between positions −702 and −678 as a sense oligonucleotide (5'-CAATGCCTCTGTCGATCATTGCTG-3') and an oligonucleotide corresponding to a sequence located in the pGL2-basic vector as an antisense oligonucleotide (5'-CTATCTCTTGAAG-AGCAGTGC-3'). Mutations were constructed in the TIS in nt −281 with two overlapping sense and antisense oligonucleotides corresponding to a sequence (AGACGCCAG) that substituted two thymidines in the TRE consensus (TGACTGAG). This plasmid was designated pTIMP2-2243/281. Mutation that introduced a TRE between two thymidines in positions −71 and −72 was accomplished using overlapping oligonucleotides corresponding to the sequence (AGACTGAC). This plasmid was designated pTIMP2-2243-281.

**DNase Footprinting**—DNase footprinting assays were carried out using a BamHI-NarI fragment (nt positions −519 to −188) of the hTIMP-2 promoter encompassing the AP-1 binding consensus sequence (positions −288 to −281). This fragment was [γ-32P]ATP-end-labeled at the NarI site using Klenow enzyme and gel-isolated. This labeled fragment was then incubated with DNase I (1 unit/μl, diluted 1/3000 to 1/5000) at 22 °C in the presence or absence of purified c-Jun homodimer. DNase I digestion was visualized by autoradiography at −80 °C.

**Analysis of Methylation**—The level of methylation of the cytosines within the human TIMP-2 promoter was determined using restriction endonucleases HpaII and MspI as described by Chandler et al. (34) in addition to other restriction endonucleases whose activity is affected by methylation of the cytosines in their restriction sites (HpaII, NarI, and NotI). A BamHI-NarI fragment (positions −520 to +169) obtained by restriction digestion of the 2.6-kb PsI1 genomic fragment was used as probe for blot analysis of genomic DNA.

**Actionmycin D Treatment**—Confluent cultures of HT1080 cells were treated with actionmycin D (final concentration 16 μM) dissolved in dimethyl sulfoxide (Sigma). At indicated times RNA was extracted and processed for Northern blot analysis.

**RNA Extraction and Northern Blot Analysis**—Cytoplasmic RNA was isolated using the method of Chirgwin et al. (35). Poly (A) RNA was
obtained using the Poly(A)tract® mRNA isolation system (Promega). Cytoplasmic RNA samples (20 μg) were electrophoresed on a formaldehyde-containing agarose (1%) gel and blotted onto a nylon membrane. Quantitative analysis of the mRNA was performed by measuring the intensity of the radioactive signals on a GS-250 Molecular Imager (Bio-Rad).

RESULTS
Molecular Cloning and Mapping of the hTIMP-2 Gene—By screening two genomic libraries, we isolated three overlapping genomic clones, which contained the entire human TIMP-2 gene, spanned in approximately 83 kb (Fig. 1). The gene is composed of five exons separated by four introns of 54.8, 2.7, 9.1, and 1.7 kb. The exon-intron boundaries for these five exons have splicing sites located at positions corresponding to amino acids 17, 51, 87, and 129 (Table I). Sequence analysis of these exon-intron boundaries was consistent with conserved sequences found in splicing sites with a GT motif on the donor site and an AG motif on the acceptor site.

Characterization of the 5’-End of the hTIMP-2 Gene—The sequence of the 2.6-kb PstI genomic fragment containing the 5’-end of the hTIMP-2 gene and including part of the first exon is presented in Fig. 2. This region contains 2243 bp upstream of the major transcription initiation site (23) that includes a TATA-like motif (AATAAAA, Ref. 36) at positions 1-226 to 1-220, five consensus sequences for Sp1 (located at positions 1-2125 to 1-2121, 1-2089 to 1-2085, 1-2072 to 1-2068, 1-2056 to 1-2052, and 1-2040 to 1-2036), a complete consensus sequence for AP-1 (at positions 1-2088 to 1-2081), two binding motifs for AP-2 (at positions 1-2077 to 1-2070 and 1-2020 to 1-2013), and three PEA-3 binding sites (at positions 1-1997 to 1-1992, 1-1946 to 1-1941, and 1-1894 to 1-1889). In addition, some other consensus elements such as nuclear factor-1, NF-IL6, and myocyte-specific enhancer-binding factor-2 binding motifs were identified (37). Further analysis of the promoter region revealed the presence of two IklpS sites (1-1987 and 1-1977), a GATA site (1-1992), and three CCAAT motifs (1-1948, 1-1939, and 1-1895). The sequence also contains two putative T-rich regions (1-1983 and 1-1959), which may be involved in transcriptional regulation. The promoter also contains a number of putative transcription factor binding sites, including Oct-1 (1-2089), Oct-2 (1-2056), and Ets-1 (1-2013).

FIG. 1. Organization of the human TIMP-2 gene and the corresponding protein. The gene (derived from the three overlapping cosmid clones 1.3.3, 67H4, and 27F6 mapped with EcoRI), black boxes represent the translated parts of exons 1–5 and lines represent introns. The 5’-UTR and 3’-UTR regions are represented by white boxes. The initiator ATG codon and the TAA stop codon are shown in exons 1 and 5, respectively. In the protein, the signal peptide is indicated by a black box, and the 12 conserved Cys residues are shown by solid circles.

FIG. 2. Nucleotide sequence of the 2.6-kb PstI genomic fragment containing the hTIMP-2 promoter. The sequence includes part of the first exon. Positions of nt indicated on the left are numbered from the major transcription initiation site (nt +1, shown by an arrow). Consensus sequences including a TATA-like motif, five Sp1, two AP-2, one AP-1, and three PEA-3 binding sites in addition to other binding sites are underlined. The initiator ATG is shown at positions +303 to +305. Sequences extending from the 3’-end PstI site to position −519 (BanHI site) were previously published (23).

TABLE I
The exon-intron junctions of the human TIMP-2 gene

| Exon | Intron-exon junction | Exon size | Intron size |
|------|----------------------|-----------|-------------|
| 1    | GTG TGA G            | nt 54.8   | kb (15.6°17.5°) |
| 2    | GTAC GGG TGG TGG     | 101       | 2.7 (0.16°7.0°) |
| 3    | ATT AAT AGG CCC      | 109       | 9.1 (0.16°7.0°) |
| 4    | ATG GCG TGC         | 125       | 1.7 (0.95°0.8°) |
| 5    | AGC GCT TCA         |           |             |

a Size of the introns of the mouse TIMP-1 gene as described by Coulombe et al. (26).
b Size of the introns of the human TIMP-3 gene as described by Hammani et al. (41) and Wick et al. (7).
thermore, the most proximal region of the hTIMP-2 promoter extending from nt +1 to nt −300 has a G/C content of 76% and contains a typical CpG island (23). The promoter activity of 5′-end flanking region of the hTIMP-2 gene was examined by transient transfection assay in mouse NIH3T3 cells (Fig. 3). Deletion of the region extending from nucleotide −2243 to −276, which includes most of the binding consensus, did not significantly affect the promoter activity in NIH3T3 cells. Furthermore, the activity of the largest promoter construct (pTIMP2-2243) was in the same range as the activity of the smallest promoter construct (pTIMP2-276), suggesting an absence of involvement of the various binding consensus elements in the basal expression of the gene and confirming, as previously reported (23), that the short −276 bp region encompassing a single Sp1 binding site and an AP-2 binding site contained all the elements required for basal expression.

Role of the AP-1 Element in Basal Expression and Response to TPA—The presence of a complete AP-1 binding consensus (TGAGTCAG) at position −288 to −281 in the hTIMP-2 gene suggested that this element could play a regulating role in the transcription of the gene. We first examined by DNaseI footprint analysis whether this consensus could bind the c-Jun homodimer protein (Fig. 4). The data showed the presence of a clear zone of protection, which appeared in a dose-dependent manner with the addition of c-Jun homodimer (Fig. 4, lanes 2–5). Thus, the AP-1 consensus was found to bind AP-1 in vitro. To determine whether the AP-1 consensus was involved in basal expression of the hTIMP-2 gene, a mutation that replaced the TGAGTCAG consensus by a nonfunctional AGAGGCAG consensus (38) was generated (Fig. 5A). This mutation resulted in a 22 and 38% decrease in the basal expression of the reporter gene in HT1080 and NIH3T3 cells, respectively (Fig. 5B), suggesting some involvement of the AP-1 consensus in basal gene expression.

We then examined whether the presence of this consensus could affect the promoter activity after treatment with TPA in transient transfection assays. The data (Fig. 5C) indicated no significant changes in reporter gene expression after TPA treatment. Since in many TPA-responsive genes the TRE is found in close proximity to the TATA box (39), we postulated that the presence of the TRE at the −276 position of the TATA box in the hTIMP-2 promoter may be responsible for the lack of response to TPA. To test this hypothesis, we inserted by mutagenesis, an AP-1 binding consensus TGAGTCAT between positions −72 and −71, in closer proximity of the TATA box. This mutation not only resulted in a 2–3-fold increase in basal expression of the hTIMP-2 promoter but was also associated with an additional 2-fold increase in activity after TPA treatment (Fig. 5C). Thus, the position of the AP-1 consensus in close proximity to the TIS seems to be an important factor influencing its activity, and the particular position of this consensus in the hTIMP-2 promoter may be responsible for a lack of up-regulatory function.

Characterization of the 3′-end of the hTIMP-2 Gene and RNA Stability—The characterization of the 3′-end of the hTIMP-2 gene brought novel information on the molecular basis for the presence of two TIMP-2 mRNAs of 1.2 and 3.8 kb as previously shown by us (23) and others (10). The size of the 1.2-kb mRNA

**Fig. 3.** Promoter activity of the 2.3-kb 5′-flanking region of the hTIMP-2 gene. Left side represents a map of the deletion constructs obtained from the full-length hTIMP-2 promoter region (pTIMP2-2243) by exonuclease III digestion as described under “Experimental Procedures.” The arrow indicates the position of the TIS. Also represented are some consensus sequences in the hTIMP-2 promoter including the TATA box and Sp1, AP-1, and PEA-3 binding sites. These constructs were inserted upstream of the promoterless luciferase reporter gene in the pGL2-basic vector. The right side represents the activity of the constructs shown on the left using transient transfection assays in NIH3T3 cells as described under “Experimental Procedures.” Data were calculated as a relative luciferase (Luc) over β-galactosidase (Gal) activity and were expressed as a percentage ± S.D. of the activity of the pTIMP2-2243 construct. Data represent the mean of four separate experiments done in duplicate.

**Fig. 4.** DNase protection assay showing the binding of c-Jun homodimer to a region of the hTIMP-2 gene encompassing the AP-1 binding sequence. The DNase footprinting analysis was done as described under “Experimental Procedures,” and the fragments generated were analyzed in a 7 M urea, 5% acrylamide gel. Lane 1, no protein; lane 2, with 50 ng of c-Jun; lane 3, with 100 ng of c-Jun; lane 4, with 200 ng of c-Jun; lane 5, with 500 ng of c-Jun. The position of the footprint was determined by comparison with a known DNA sequence (shown on the left) in the hTIMP-2 promoter upstream from the AP-1 consensus sequence (from nt position −519). This sequence located in a region less G/C-rich and easier to sequence was used to position the footprint based on the known sequence of the promoter. Nucleotide sequences of the hTIMP-2 promoter corresponding to the footprint region are indicated on the right, with the AP-1 binding consensus shown in a box.
Furthermore, the 0.3-kb with the 1.2-kb mRNA but hybridized with the 3.8-kb mRNA (Fig. 6). The data show that both fragments failed to hybridize downstream, were used as probes in Northern blot analysis one derived from a 2.8-kb EcoRI fragment containing exon 5 (0.3-kb this site, one derived from the distal end of a 1.4-kb (1,069nt). Twogenomic DNA fragments located downstream of gene and the polyadenylation signal in the 3′ UTR of exon 5 (top panel). Polyadenylation signal consensus sequences (AATAAAA) are indicated by asterisks. X, XhoI; E, EcoRI.

is consistent with the positions of the TIS in the 5′-end of the gene and the polyadenylation signal in the 3′-UTR of exon 5 (1,069 nt). Two genomic DNA fragments located downstream of this site, one derived from the distal end of a 1.4-kb EcoRI fragment containing exon 5 (0.3-kb XhoI-EcoRI fragment) and one derived from a 2.8-kb EcoRI fragment located further downstream, were used as probes in Northern blot analysis (Fig. 6). The data show that both fragments failed to hybridize with the 1.2-kb mRNA but hybridized with the 3.8-kb mRNA. Furthermore, the 0.3-kb XhoI-EcoRI fragment weakly hybridized with an additional 1.7-kb mRNA. The data suggest that the hTIMP-2 mRNAs differ by the selection of their polyadenylation signal sites and indicate the presence of an additional mRNA of 1.7 kb in a small amount. Consistently, sequencing of the 2.8-kb EcoRI fragment revealed the presence of five polyadenylation signal consensus (AATAAAA) located between 3.25 and 4.75 kb downstream of the TIS. To determine whether the length of the hTIMP-2 mRNA could influence RNA stability, we performed RNA analysis after treatment with actinomycin D in HT1080 cells (Fig. 7). These experiments indicated no significant difference in the half-life of the two hTIMP-2 mRNAs (32 and 26 h for the 1.2- and 3.8-kb TIMP-2 mRNAs, respectively), and showed that both mRNAs had a half-life longer than the human β-actin mRNA (20 h).

Regulation of the hTIMP-2 Gene by Methylation—The presence of a CpG island in the most proximal region of the hTIMP-2 promoter (Fig. 8A) led us to examine whether methylation of cytosines in the promoter could affect gene expression. For these analyses, we selected two human bladder carcinoma cell lines (EJ and RT4) because of the presence (EJ) or the absence (RT4) of TIMP-2 expression (Fig. 8B). The methylation level of the promoter region in these cells was first examined by Southern blot analysis of genomic DNA digested with PstI and HpaII or MspI and probed with a 0.7-kb BamHI-HpaI promoter fragment (Fig. 8, A and C). Whereas both HpaII and MspI digest 5′-CCGG-3′ sequences, only MspI can cleave these restriction sites when the internal cytosine is methylated. The data revealed a higher degree of DNA cleavage with MspI than with HpaII, suggesting the presence of multiple

**Fig. 5. Mutation of the AP-1 binding site and its effect on basal expression and response to TPA.** Panel A shows the mutation performed on the AP-1 binding consensus (TGAGTCAG) present in the hTIMP-2 promoter, as described under “Experimental Procedures.” The positions of the various consensus elements are represented by symbols as shown in Fig. 3. Panel B represents the basal activity of the pTIMP2-2243 and pTIMP2-2243/355kbp-1 constructs in transient transfection assays with HT1080 and NIH3T3 cells as described under “Experimental Procedures.” Panel C represents the activity of the pTIMP2-2243 and pTIMP2-2243/355kbp-1 constructs after treatment with TPA (100 ng/ml) as described under “Experimental Procedures.” Data in panels B and C were calculated as luciferase (Luc) over β-galactosidase (Gal) activity and were expressed as a percentage of the activity of the pTIMP2-2243 wild type construct. Data represent the mean ± S.E. of three separate experiments done in duplicate. p values were calculated using Student’s t test; *, p < 0.02; **, p < 0.002; NS, not significant.
the hTIMP-2 promoter varies, it is unlikely that it has any
although themethylation state of the various G/C sequences in
ing and nonexpressing cells. The data suggest, therefore, that
difference in the pattern of digestion between TIMP-2-express-
lation status of each of them. All of these analysis revealed no
region, this analysis did not allow us to determine the methy-
ment. Analysis of the
methylated cytosines within the CpG island of the promoter.
the one in position
and human TIMP-3 genes revealed both similarities and differences. As is the case in many genes that belong to a same family, we found
methylated cytosines within the CpG island of the promoter.
The methylation status at some specific G/C sequences was
also determined using restriction enzymes NoI, NarI, and
FnuDII, which all have a C/G motif in their restriction se-
quence (Fig. 8A). These data showed that the unique NoI site in
the promoter is unmethylated, as indicated by the presence of
a 2.3-kb band on the Southern blot (Fig. 8C). The presence of
a 2.0-kb band after digestion with NarI also indicated that the
two more distal NarI sites are methylated (unmethylated), whereas
the one in position 191 is unmethylated, as shown by the
presence of a 0.5-kb band on the Southern blot. The methyla-
status at the most proximal site (position +303) could not be
determined because of the small size of the cleaved frag-
ment. Analysis of the FnuDII-generated fragments revealed the
presence of a large 1.9-kb fragment, indicating that the
most distal site (position 865) is methylated, whereas sites in
most proximal positions are unmethylated, although, because
of the high number of FnuDII sites in close proximity in that
region, this analysis did not allow us to determine the methy-
lization status of each of them. All of these analysis revealed no
difference in the pattern of digestion between TIMP-2-express-
ing and nonexpressing cells. The data suggest, therefore, that
although the methylation state of the various G/C sequences in
the hTIMP-2 promoter varies, it is unlikely that it has any
effect on gene expression.

DISCUSSION

We have isolated the entire hTIMP-2 gene and described its
structural organization. Comparison of the structure of the
hTIMP-2 gene with the published structures of the murine
TIMP-1 gene (26) and the murine (40) and human (7, 41)
TIMP-3 genes revealed both similarities and differences. As is the case in many genes that belong to a same family, we found
that the exon-intron boundaries were preserved in the three
members of TIMP family. In contrast to the TIMP-1 gene, which is contained within a 4.5-kb HindIII genomic fragment, the
hTIMP-2 gene is much larger (spanning approximately 83
kb) and contains a first intron of 57 kb. The significance of the
presence of such large intron, also observed in the mouse and
the human TIMP-3 genes (7, 40, 41), is unclear, and whether
this intron contains (as is the case in the TIMP-1 gene (26))
elements capable of enhancing gene expression is unknown.

Another difference between the TIMP-1 and the TIMP-2 genes
resides in the 5′-UTR, which contains in the case of TIMP-1 a
short noncoding first intron (Fig. 9). Sequencing of the 2.6-kb
PstI genomic fragment of the hTIMP-2 gene indicated no dif-
fferences between the cDNA and the genomic sequences, con-
firming that the ATG codon was located within the first exon.
Further comparison of the structure of the TIMP genes from
different species should lead to a better understanding of their
evolutionary relationship.

In this manuscript we have extended our first study of the
hTIMP-2 promoter (23) to include a total of 2.3 kb of 5′-flanking
sequences upstream from the major transcription initiation
site. A comparison between this promoter region and similar
regions of the murine TIMP-1 and TIMP-3 genes is shown in
Fig. 9. The promoter region of the hTIMP-2 gene, like the
murine TIMP-3 gene, has a higher G/C content (76 and 70% of
G/C in the region extending from nt −300 to nt +1 for hTIMP-2
and murine TIMP-3, respectively) than the corresponding
region of the TIMP-1 gene (58% G/C content). Furthermore,
the positions of some key transcription elements are different
between the promoter region of the three members of the TIMP
family. Whereas in the TIMP-1 promoter, the AP-1 binding
consensus is proximal to the TIS (positions −59 to −53) and
closely associated with a PEA-3 element, the AP-1 binding
element in the hTIMP-2 gene is more distantly positioned from
the TIS (positions −288 to −281), and three PEA-3 elements
are located further upstream (position −721 and further up-
stream). In contrast, in the promoter region of the murine
TIMP-3 gene there are six AP-1 binding consensus elements
(located between positions −1950 and −611) with many PEA-3
elements variably dispersed in the promoter region.

The presence of an AP-1 binding site is a common feature of
many genes up-regulated by TPA. Often this element is located
in close proximity (−50 to −70 nt) of the TIS and is closely
associated with one or several PEA-3 elements to form a TPA-
and oncogene-responsive unit (43–45) as seen in the promoters
of many TPA-responsive genes including several MMP and
TIMP-1 (19–21, 38, 39, 42). We had previously demonstrated

FIG. 8. Methylation status of the hTIMP-2 promoter. A comparison between cell-expressing and nonexpressing TIMP-2 mRNAs is shown. Genomic DNA (15 μg) was isolated from cultures of human bladder carcinoma cell lines (EJ and RT4) and digested with PstI alone (−) or with PstI and HpaII, MspI, FnuDII, NarI, or NotI. Samples of digested DNA were electrophoresed, blotted to a nylon membrane, and
hybridized to a BamHI-ApoI fragment extending from nt −520 to nt +169 in the promoter region. A, map of the restriction sites for the
enzymes used. The positions of the Cpg and GpC sequences as well as
the localization of the probe are also shown. B, cytoplasmic RNA was obtained from EJ and RT4 cells and analyzed by Northern blot using
obtained from EJ and RT4 cells and analyzed by Northern blot using

FIG. 9. Comparison between the 5′-flanking regions of the	hree TIMP genes. Putative transcriptional regulatory elements are shown in the promoter of the hTIMP-2 gene and compared with those
found in the murine TIMP-1 gene (mTIMP-1; Refs. 20 and 26) and the
murine TIMP-3 gene (mTIMP-3; Ref. 22). The positions of these ele-
ments are determined from the position of the major TIS in the three
genes. The first two exons in the murine TIMP-1 gene and the first exon
of the hTIMP-2 and murine TIMP-3 genes are represented by boxes in
which the coding part is shown as a black box with the position of the
initiator ATG codon indicated. A 0.8-kb sequence corresponding to the
5′-end of the human TIMP-1 gene has been recently published (21) and
contains features similar to those found in the murine TIMP-1 gene.
that a 715-bp-long promoter sequence of the hTIMP-2 gene containing the AP-1 consensus failed to respond to TPA (23). We now demonstrate that a longer construct containing three PEA-3 elements in addition to the AP-1 binding consensus also failed to respond and also provide evidence that the AP-1 consensus binds the c-Jun homodimer in vitro. We therefore postulated that the failure of this element to respond to TPA may be related to its position, far more distant (281 bp) from the transcription initiation site than in the case of most TPA-inducible genes (39, 42). By mutagenesis experiments, we demonstrated that insertion of a consensus for AP-1 at position −71 resulted in an increase in basal gene expression and also in an additional increase in expression after treatment with TPA, clearly confirming the importance of the position of the AP-1 consensus not only in basal gene expression but also in TPA inducibility. It is conceivable that such positioning prevents interaction of the Jun and Fos transactivation factors (AP-1) with the transcription initiation complex. We had previously shown that elimination of a 124-bp SmaI fragment of the hTIMP-2 promoter containing the AP-1 consensus resulted in a 2-fold increase in basal gene expression (23), suggesting the presence of inhibitory sequences in this region. Since the AP-1 consensus has been previously shown to repress gene expression in some cases (46, 47), we examined whether the AP-1 consensus in the hTIMP-2 promoter could suppress expression. Our mutagenesis data clearly show that this is not the case, since mutation of the AP-1 consensus in its original position did not result in an increase of basal expression. Rather, a small but significant decrease in expression was observed, suggesting some involvement of the AP-1 site in basal expression.

The high G/C content of the hTIMP-2 promoter suggests that its activity could be controlled by methylation of the cytosine residues as shown in several G/C-rich promoters (48) including its activity could be controlled by methylation of the cytosine since mutation of the AP-1 consensus in its original position did not result in an increase in basal gene expression and also in an additional increase in expression after treatment with TPA, clearly confirming the importance of the position of the AP-1 consensus not only in basal gene expression but also in TPA inducibility. It is conceivable that such positioning prevents interaction of the Jun and Fos transactivation factors (AP-1) with the transcription initiation complex. We had previously shown that elimination of a 124-bp SmaI fragment of the hTIMP-2 promoter containing the AP-1 consensus resulted in a 2-fold increase in basal gene expression (23), suggesting the presence of inhibitory sequences in this region. Since the AP-1 consensus has been previously shown to repress gene expression in some cases (46, 47), we examined whether the AP-1 consensus in the hTIMP-2 promoter could suppress expression. Our mutagenesis data clearly show that this is not the case, since mutation of the AP-1 consensus in its original position did not result in an increase of basal expression. Rather, a small but significant decrease in expression was observed, suggesting some involvement of the AP-1 site in basal expression.

The high G/C content of the hTIMP-2 promoter suggests that its activity could be controlled by methylation of the cytosine residues as shown in several G/C-rich promoters (48) including the murine TIMP-3 (22). Whereas Sun residues shown in several G/C-rich promoters (48) including its activity could be controlled by methylation of the cytosine since mutation of the AP-1 consensus in its original position did not result in an increase in basal gene expression and also in an additional increase in expression after treatment with TPA, clearly confirming the importance of the position of the AP-1 consensus not only in basal gene expression but also in TPA inducibility. It is conceivable that such positioning prevents interaction of the Jun and Fos transactivation factors (AP-1) with the transcription initiation complex. We had previously shown that elimination of a 124-bp SmaI fragment of the hTIMP-2 promoter containing the AP-1 consensus resulted in a 2-fold increase in basal gene expression (23), suggesting the presence of inhibitory sequences in this region. Since the AP-1 consensus has been previously shown to repress gene expression in some cases (46, 47), we examined whether the AP-1 consensus in the hTIMP-2 promoter could suppress expression. Our mutagenesis data clearly show that this is not the case, since mutation of the AP-1 consensus in its original position did not result in an increase of basal expression. Rather, a small but significant decrease in expression was observed, suggesting some involvement of the AP-1 site in basal expression.
Structure and Characterization of TIMP-2 Gene

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