Differential functional significance of AP-1 binding sites in the promoter of the gene encoding mouse tissue inhibitor of metalloproteinases-3

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Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular-matrix-associated protein that suppresses tumorigenicity or invasion in several model systems. We have identified, by *in vitro* footprinting, six AP-1 (activator protein-1) or AP-1-like binding sites in the mouse TIMP-3 promoter that bind purified c-Jun homodimers. Electrophoretic mobility shift assays revealed that the non-consensus fifth AP-1 binding site (AP-720; nt –720 to –714) had the strongest binding activity for recombinant c-Jun protein, and that the fourth binding site (AP-763; nt –763 to –754) and AP-720 showed strong binding activity for cellular nuclear proteins. Antibody supershift and blocking experiments suggest that AP-720, but not AP-763, binds authentic AP-1 components. Transient transfection reporter assays of deletion constructs showed that the region

spanning AP-720 has the highest transcriptional activity, and that sequences 5' to this region (nt –2846 to –747) may contain negative regulatory elements. The deletion construct containing about 500 nt 5' to the transcriptional start, but no AP-1 sites, showed lower but significant activity, suggesting both AP-1-dependent and -independent regulation of the mouse TIMP-3 promoter. Mutational inactivation of AP-720 abolished the activity increment that distinguished the reporter construct containing both AP-720 and sixth AP-1 binding site (AP-617; nt –617 to –611) from that containing only AP-617. In summary, we report here that both AP-1 and non-AP-1 elements contribute to activity, with the non-consensus AP-1 site at –720 showing the greatest functional significance among the AP-1 sites.

INTRODUCTION

Mutational activation of oncogenes and mutational inactivation of tumour suppressor genes have been shown to be key elements in multistage carcinogenesis. We have used variants of the mouse JB6 epidermal cell to investigate the complicated molecular mechanisms of tumorigenesis. The JB6 cell lines were originally established from untreated Balb/c primary epidermal cell cultures and consist of three phenotypically distinct clonal cell variants: (i) promotion-resistant (P[–]) cells that are resistant to tumour-promoter-induced transformation (CI30.7b), (ii) promotion-sensitive (P⁺) cells that are sensitive to tumour-promoter-induced transformation (CI22 and CI41), and (iii) P⁺-derived neoplastically transformed (Tx) cells (RT101) [1]. This system, therefore, has permitted the study of preneoplastic progression (P[–] to P⁺) and preneoplastic-to-neoplastic progression (P⁺ to Tx) in tumour-promoter-induced transformation [1–3]. The molecular mechanism for tumorigenesis in the JB6 model is partially understood. P⁺ cells display activator protein-1 (AP-1)-dependent tumour-promoter-inducible transactivation of gene expression, whereas P[–] cells have a defect in AP-1 transactivation [4]. Elevated AP-1 activation appears to be required for both the induction and maintenance of tumour phenotype, as blocking of AP-1 produces reversion to earlier stages [5]. Neither point mutation of the *H-ras* or *p53* genes nor structural alterations in *p53* are involved in tumorigenesis in JB6 cells [3].

By using the recently developed technique of differential mRNA display [6], five differentially expressed clones were isolated from JB6 cell variants [7]. Interestingly, one of these mRNAs was specifically expressed in preneoplastic JB6 (P[–] and

P⁺) cells, but was not expressed in multiple neoplastic (Tx) cells. This isolated clone showed 96% amino acid sequence identity to the recorded sequence of human tissue inhibitor of metalloproteinases-3 (hTIMP-3) and 100% identity to mouse TIMP-3 (mTIMP-3).

Matrix metalloproteinases are zinc-containing enzymes that are involved in the degradation of the extracellular matrix, and the activities of these proteinases are controlled by specific inhibitors such as the TIMPs [8]. Three members of the TIMP family have been reported and have been shown to regulate matrix degradation as well as the suppression of tumorigenicity or invasion in several model systems [9–12]. TIMP-1 and TIMP-2 are relatively well characterized, while hTIMP-3 [13–18] and mTIMP-3 [7,19–22] have been more recently cloned and identified. Unlike TIMP-1 and TIMP-2, TIMP-3 is an extracellular-matrix-associated protein with a distinct pattern of expression [16,20–24].

The fact that mTIMP-3 transcripts are expressed in pre-neoplastic but not neoplastic mouse JB6 cells suggests a role for mTIMP-3 in neoplastic progression in this system. The lack of expression of mTIMP-3 in neoplastic JB6 cells is due neither to mutation of the gene nor to lack of transcription factors [22]. Altered methylation of the mTIMP-3 gene may contribute to the complete down-regulation of gene expression seen in neoplastic cells [22]. However, the mechanism by which this gene is regulated in cells that express mTIMP-3 is currently unknown. Recently we have cloned the full-length mTIMP-3 cDNA and the promoter of the gene [22]. Sequencing of 2.9 kb upstream of the mTIMP-3 transcriptional start site revealed six AP-1 or AP-1-like binding sites (–1958 to –1950, –1348 to –1342, –1117 to –1111,

Abbreviation used: TIMP-3, tissue inhibitor of metalloproteinases-3; hTIMP-3, human TIMP-3; mTIMP-3, mouse TIMP-3; P[–] and P⁺ cells, JB6 cells that are resistant and sensitive respectively to tumour-promoter-induced transformation; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; TRE, PMA ('TPA')-responsive element; SV40, simian virus 40; NF- κ B, nuclear factor- κ B.

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–763 to –754, –720 to –714 and –617 to –611) [22]. In the present paper, we have addressed questions concerning the functional activities of these AP-1 binding sites in the regulation of the mTIMP-3 promoter. This is, to our knowledge, the first reported attempt to carry out a careful analysis of a TIMP-3 promoter with respect to specific DNA elements. The results show that only a subset of these AP-1 sites appears to be functional for both sequence-specific protein binding and transcriptional activation, and that non-AP-1 sites are important as well.

MATERIALS AND METHODS

Cell culture

Mouse JB6 cells were cultured in Eagle's minimal essential medium (Whittaker, Walkersville, MD, U.S.A.) containing 5% (v/v) fetal calf serum. The cell doubling times for P[–] (Cl30.7b) and P⁺ (Cl22 and Cl41) cells are approx. 28 and 24 h respectively. NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum (BRL, Gaithersburg, MD, U.S.A.). For AP-1-mediated transcriptional assays, cells were seeded in 24-well plates 16 h prior to transfection. Approx. 4×10^4 cells per well were used to achieve optimal transfection efficiency.

DNase I footprinting analysis

mTIMP-3 promoter sequence probes of approx. 300 nt were prepared by the PCR and were 5'-end-labelled to high specific radioactivity on the top strand only. Approx. 1×10^5 c.p.m. of radiolabelled probe was incubated with or without 250 ng of purified c-Jun protein (Promega, Wisconsin, WI, U.S.A.) in 50 μ l of footprinting buffer (25 mM Tris/HCl, pH 8, 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 10% glycerol and 0.5 mM dithiothreitol) for 30 min on ice. A 50 μ l aliquot of a solution containing 5 mM CaCl₂ and 10 mM MgCl₂ was then added, and the samples were incubated at room temperature for 1 min. The samples were treated with 3 μ l of DNase I solution (50 units/ml) for 1 min at room temperature, after which the reaction was terminated by the addition of 90 μ l of stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS and 100 μ g/ml yeast tRNA). The samples were then extracted with phenol/chloroform and ethanol-precipitated. The digestion products were resolved on a 6% sequencing gel. Appropriate sequencing ladders were run in adjacent lanes.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed with nuclear protein extracts prepared from JB6 cells under the conditions described [25]. The simian virus 40 (SV40) AP-1-containing consensus sequence from the SV40 promoter (5'-CTAGTGATGAGTCAGCCGGATC-3') (Stratagene, La Jolla, CA, U.S.A.) and recombinant c-Jun protein (Promega) were used as positive controls. The mSV40 oligonucleotide (5'-CTAGTGACGAGTGAGCCGGATC-3') was identical with the SV40 oligonucleotide, except that the T in position 8 [position 1 of the PMA ('TPA')-responsive element (TRE)] was changed to a C, and the C in position 13 (position 6 of the TRE) was changed to a G. The corresponding changes were also made in the complementary oligonucleotide. The nuclear factor- κ B (NF- κ B) oligonucleotide (5'-CTTGGGACTTTCCT-ACGGCTAAA-3') was used as a competitor. In addition, six oligonucleotides that represent the six AP-1 or AP-1-like binding sites in the mTIMP-3 promoter were synthesized. The first (–1958 to –1950), second (–1348 to –1342), third (–1117 to

Table 1 Oligonucleotides containing the six AP-1 binding sites in the mTIMP-3 promoter

The binding sites are emboldened.

Binding site	Name	Oligonucleotide
First (–1958/–1950)	AP-1958	5'-GCCTCAAGT GA CT GA CTGCCGGC-3' 3'-CGGAGTTC ACT GA CT GA CGGCCG-5'
Second (–1348/–1342)	AP-1348	5'-AGGGATGCT GA GT CA GGGATGTA-3' 3'-TCCCTACG ACT CA GTCCCTACAT-5'
Third (–1117/–1111)	AP-1117	5'-CAGAAGCCT GA CT CA GGGAAGATC-3' 3'-GTCTTCGG ACT GA GTCTCTAG-5'
Fourth (–763/–754)	AP-763	5'-TTCTGGCAT GA TG TC ACATCTTT-3' 3'-AAGACCG TACT AC AGTGTAGAAA-5'
Fifth (–720/–714)	AP-720	5'-ACGCCCT TT AG TC ATTCTAACC-3' 3'-TGCGGGAG AAT CAG TAAAGATTGG-5'
Sixth (–617/–611)	AP-617	5'-AGGGTCTGT GA TA TA ATCCACA-3' 3'-TCCCGACAG ACT TAT TAAGGTGT-5'

–1111), fourth (–763 to –754), fifth (–720 to –714) and sixth (–617 to –611) binding sites were designated AP-1958, AP-1348, AP-1117, AP-763, AP-720 and AP-617 respectively (Table 1). The oligonucleotides used for EMSA are also shown in Table 1.

All oligonucleotides were annealed in equimolar amounts (50 ng/ μ l) and end-labelled with [γ -³²P]ATP (Amersham, Arlington Heights, IL, U.S.A.) using polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Unincorporated oligonucleotides were removed by using a Sephadex G-25 column (Boehringer Mannheim). Portions of 3 μ g of nuclear protein extracts from P⁺ cells were incubated with 3 μ g of anti-AP-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) and 1 μ g of poly(dI-dC) (Boehringer Mannheim) in binding reactions containing 40 mM Tris (pH 7.5), 120 mM KCl, 8% Ficoll, 4 mM EDTA and 1 mM dithiothreitol for 30 min at room temperature. This reaction was performed by using four different anti-Jun and anti-Fos antibodies (specific c-Jun antibody, pan Jun family antibody, specific c-Fos antibody and pan Fos family antibody). The epitopes recognized by the pan Jun and pan Fos antibodies correspond to part of the DNA binding domain of the appropriate protein, while the specific c-Jun and c-Fos antibodies recognize sequences outside the DNA binding domain. End-labelled double-stranded oligonucleotides (1×10^5 c.p.m.) corresponding to each of the six AP-1 binding sites were added to the nuclear protein/antibody mixture, and the incubation was continued for 30 min at room temperature. The reaction products were resolved by electrophoresis on a 6% polyacrylamide gel in Tris/borate/EDTA electrophoresis buffer (Digene Diagnostics, Inc., Beltsville, MD, U.S.A.), dried and exposed to Kodak X-Omat film.

mTIMP-3-promoter-luciferase reporter construction

Seven luciferase reporter constructs consisting of 5' deletion fragments of the mTIMP-3 promoter from approx. 0.5 to 2.9 kb were generated by PCR amplification with a 3.4 kb mTIMP-3 genomic clone (containing 2.9 kb of promoter) as the template [22]. The pGL2-Basic luciferase reporter construct (Promega) containing six AP-1 binding sites from the mTIMP-3 promoter was named 6AP, and successive deletions of the 5' end resulted in luciferase reporter constructs containing between five and zero AP-1 binding sites, which were designated 5AP, 4AP, 3AP, 2AP,

Table 2 PCR primers used for construction of mTIMP-3-luciferase reporters

Construct	Designation	Primer	
6AP (−2846/+58)	GTIP3.01	5'-TCTAGAAAAGTCCAAACAGAGG-3'	(−2846/−2825)
	5E.04E	5'-AGAAGATTCTTGGCGCTGGC-3'	(+58/+39)
5AP (−1445/+58)	GTIP3.09	5'-CAGCATTCTGCAGCTAAAC-3'	(−1445/−1426)
	5E.04E	5'-AGAAGATTCTTGGCGCTGGC-3'	(+58/+39)
4AP (−1147/+58)	GTIP3.11	5'-CACTGCACGTCTGTAACCTCT-3'	(−1147/−1128)
	5E.04E	5'-AGAAGATTCTTGGCGCTGGC-3'	(+58/+39)
3AP (−796/+58)	GTIP3.13	5'-GACCCAGGCCTTTGTGATTG-3'	(−796/−777)
	5E.04E	5'-AGAAGATTCTTGGCGCTGGC-3'	(+58/+39)
2AP (−746/+58)	GTIP3.13a	5'-ACTGTCCACAGAAAACAACG-3'	(−746/−726)
	5E.04E	5'-AGAAGATTCTTGGCGCTGGC-3'	(+58/+39)
1AP (−676/+58)	GTIP3.13b	5'-CATACTAGCACAAATCGCCCT-3'	(−676/−657)
	5E.04E	5'-AGAAGATTCTTGGCGCTGGC-3'	(+58/+39)
0AP (−491/+23)	Meth.05	5'-TGAGGGAAGCTGATGTCCAGCGGTTTCCT-3'	(−491/−463)
	Meth.02	5'-ACTGTGAGCGAGAGTCCAGGCTGCAGAGT-3'	(+23/−7)

1AP and 0AP respectively. The primers used for PCR amplification for the seven luciferase reporter constructs are shown in Table 2. For 0AP, the PCR fragment was blunt-ended and ligated into pGL2-Basic vector that had been predigested with *Sma*I. For the other six constructs (6AP to 1AP), the PCR fragment was ligated into pCRII (Invitrogen, San Diego, CA, U.S.A.) and digested with *Kpn*I and *Xho*I. After purification, the insert was ligated into pGL2-Basic that had been predigested with *Kpn*I and *Xho*I. Restriction enzyme digestion and DNA sequencing were used to confirm the correct orientation and the absence of PCR-generated mutations in the AP-1 sites.

Transient transfection and transcriptional transactivation assays

JB6 and NIH 3T3 cells were transiently transfected with one of the seven luciferase reporter constructs consisting of the 5' deletion fragment of mTIMP-3 using the calcium phosphate method as described previously [5]. Reporter plasmid DNA at 3.75 µg/well for P[−] and P⁺ cells and at 1.25 µg/well for NIH 3T3 cells was used to achieve optimal transfection efficiency. For a known AP-1-regulated positive control, the JB6 cells and NIH 3T3 cells were transiently transfected with the construct (GCN)₄-luciferase, consisting of four AP-1 sites upstream of the albumin minimal promoter [5]. For a negative control, the empty luciferase vector (pGL2-Basic) was transiently transfected. After a 36 h incubation, the cells were lysed with 0.1 ml of lysis buffer (Promega) for 15 min at room temperature. Luciferase activity was assessed as light emission measured in a Monolight luminometer (Analytic Luminescence Laboratory, San Diego, CA, U.S.A.) and normalized to co-transfected β-galactosidase activity.

Site-directed mutagenesis

This was performed using a site-directed mutagenesis kit (Stratagene). The oligonucleotide primers containing the desired mutation were prepared. These primers, each complementary to opposite strands of the vector, were annealed and extended during temperature cycling by means of *Pfu* DNA polymerase. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks was generated. Following temperature cycling, the product was treated with *Dpn*I endonuclease, which is specific for methylated and hemimethylated

DNA and was used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The vector DNA incorporating the desired mutation was then transformed into *E. coli*.

RESULTS

Purified recombinant c-Jun protein binds to all six AP-1 or AP-1-like binding sites in the mTIMP-3 promoter

DNase I footprinting analysis was performed to examine whether the putative AP-1 sites were able to be bound by an AP-1 complex. For this purpose we used purified c-Jun transcription factor, as the low levels of AP-1 components in nuclear extracts prevented us from obtaining clear footprints (results not shown). As shown in Figure 1, some degree of DNase I protection was observed for all six putative AP-1 sites. Given this observation, we decided to test further the ability of each individual AP-1 site to bind AP-1 in EMSAs using nuclear extracts.

Differential protein binding activities of the six AP-1 or AP-1-like sites in the mTIMP-3 promoter

EMSAs were used to investigate the protein binding activities of the six AP-1-like sites evaluated above. For these assays, nuclear protein extracts were prepared from JB6 P⁺ cells expressing endogenous mTIMP-3. As shown in Figures 2(A) and 2(B), all six AP-1 sites showed some protein binding, with the AP-1958, AP-763 and AP-720 oligonucleotide binding sites showing the greatest binding activity when P⁺ cell nuclear protein extracts were used. Only the AP-720 complex showed a retardation length identical to that of the SV40 AP-1 control, suggesting a similar protein composition of the two complexes. When the EMSA was carried out using pure c-Jun protein yielding DNA-bound Jun/Jun homodimer, only AP-720 showed strong binding activity (Figure 2C). Pure c-Jun protein did not bind to the AP-763 oligonucleotide or to AP-1348 or AP-1117, which contain classical AP-1 consensus sequences (Figure 2C and Table 1). When another AP-763 oligonucleotide (5'-ATGTTCTTCTG-GCATGATGTCAC-3') which contained the AP-1-like sequence (underlines) at the 3' end rather than in the centre was used, no protein binding was observed (results not shown), demonstrating a possible anchoring function of the sequences flanking AP-763.

Figure 1 DNase I footprinting analysis demonstrates that all six putative AP-1 sites in the mTIMP-3 promoter can bind c-Jun protein *in vitro*

DNase I digestion products spanning the six putative AP-1 binding sites in the absence (—) or presence (++) of purified c-Jun protein were examined. The six putative AP-1 binding sites are indicated by brackets. The four probes used to cover the AP-1 sites spanned nucleotides (left to right on Figure) —2161 to —1797, —1445 to —1128, —1147 to —777 and —796 to —472. The positions of binding sites AP-1117 and AP-763 were obtained by long exposure of the gels to allow accurate reading of the DNA sequence ladders near the primers (results not shown).

Together, these results indicate that AP-720 has the strongest binding activity for c-Jun protein and that the non-consensus AP-1958, AP-763 and AP-720 sites showed the strongest binding for nuclear proteins.

Given that the major binding in EMSAs was seen with oligonucleotides bearing non-classical AP-1 sites, we investigated whether these sites function as AP-1 sites. Efficient competition by a sequence such as the TRE (TGAGTCA)-containing SV40 sequence known to be regulated by classical Jun/Fos complexes, but not by a mutated SV40 or unrelated sequence, would suggest the possibility of AP-1-like function. This analysis, shown in Figure 3, utilized non-radioactive competitor oligonucleotides derived from the SV40 AP-1 sequence. As shown in Figure 3 (upper panel), binding of nuclear extract protein to the AP-1117, AP-720 and AP-617 oligonucleotides was fully competed by a 100-fold excess of the classical SV40 AP-1 oligonucleotide (lanes 8, 12 and 14); binding to AP-1958 (lane 4) and AP-763 (lane 10) was substantially affected, while binding to AP-1348 (lane 6) was only slightly affected. The similar retardation lengths and the complete competition show that the protein complexes bound to the SV40 AP-1 site and to AP-720, but not to other sites such as AP-763, are likely to be similar if not identical.

Figure 2 AP-1958, AP-763 and AP-720 show greatest binding to JB6 P⁺ cell nuclear protein in EMSAs using oligonucleotides corresponding to the six AP-1 binding sites in the mTIMP-3 promoter

AP-1958, AP-1348, AP-1117, AP-763, AP-720 and AP-617 indicate the first, second, third, fourth, fifth and sixth AP-1 binding sites respectively in the mTIMP-3 promoter. The SV40 construct containing an AP-1 consensus sequence (Stratagene) was used as a control. Portions of 3 µg of nuclear protein from P⁺ [CI41 (A) and CI22 (B)] cells were incubated with 1×10^5 c.p.m. of radiolabelled oligonucleotides. (C) A sample of 2.5 µg of c-Jun protein (Promega) was used as a control. Only AP-720 showed the same retardation length as the SV40 control. These experiments were performed under identical conditions for each protein and used the same oligonucleotides to control for variation in preparation.

Mutation of the SV40 AP-1 sequence at positions 1 (T) and 6 (C) of the TRE as described in [26] rendered the competition less efficient, but did not abolish it (Figure 3, lower panel). This suggests a lower binding affinity of the AP-720 binding complex than of the SV40 binding complex for the SV40 site. TRE positions 2–5 and 7, in addition to 1 and 6, appear to contribute to the protein binding activity of AP-720. Possible contributions to binding of SV40 sequences flanking the TRE cannot be excluded. Taken together with the observation that the unrelated NF-κB sequence did not compete (Figure 3, lower panel), these results suggest that the protein complex(es) binding to AP-720 tolerate some (but not complete) departure from the consensus AP-1 sequence for sequence-specific binding to mTIMP-3 DNA.

To determine whether the DNA binding complexes contained Jun and Fos family proteins, as expected for AP-1 complexes, EMSAs were performed with four different anti-Jun or anti-Fos antibodies (Figure 4). AP-763, which showed the strongest binding activity for nuclear protein, and AP-720, which showed in addition binding activity for purified recombinant c-Jun protein, were used for this experiment. Binding of nuclear protein to AP-763 was neither blocked nor supershifted by anti-Jun or anti-Fos antibodies, suggesting that the protein complex bound to AP-763 did not contain Jun or Fos family proteins (Figure 4A). In the case of AP-720, binding was completely blocked by pan Jun or pan Fos family antibodies, and showed a small amount of supershifting with the pan Fos family antibody (Figure 4B). The DNA binding domains of pan Fos may bind outside the points of DNA–protein contact in these regions, resulting in the lack of complete disruption of the complex following antibody binding. This might explain the supershifted bands seen in addition to the blocked shift obtained with antibody against the DNA binding site. The slight blocking or supershifting observed with the specific c-Jun or c-Fos antibodies (Figure 4B) and SV40 AP-1 or AP-720 oligonucleotide may reflect a low

Figure 3 AP-720 oligonucleotide, but not AP-763, shows complete competition with an SV40 oligonucleotide containing TGAGTCA in a competition EMSA experiment

Upper panel: the non-radioactive SV40 AP-1 oligonucleotide was used as a competitor. Portions of 3 μ g of nuclear extract from P⁺ (Ci22) cells were incubated with one of six oligonucleotides corresponding to the six AP-1 binding sites in the mTIMP-3 promoter in the presence of a 100-fold molar excess of competitor oligonucleotide (lanes 2, 4, 6, 8, 10, 12 and 14) or in the presence of no competitor oligonucleotide (lanes 1, 3, 5, 7, 9, 11 and 13). Lanes 2, 8, 12, and 14 show complete competition, lanes 4 and 10 show substantial competition, and lane 6 shows little competition. Lower panel: portions of 2 μ g of nuclear extract from Ci41 cells were incubated with SV40 or AP-720 in the presence of a 100-fold molar excess of non-radioactive SV40 (SV), mSV40 (mSV) or NF- κ B (NF) oligonucleotide, or in the presence of no competitor oligonucleotide (0).

content of c-Jun and c-Fos proteins in the JB6 cell DNA-bound complex relative to other Jun and Fos family proteins. Identical results were obtained even when the amount of antibody was raised to as high as 6 μ g per reaction (results not shown). These results indicate that: (i) SV40 AP-1 and AP-720 show almost identical patterns of supershifting and blocking with anti-AP-1 antibodies, even though the AP-1 sequence of AP-720 is not identical to the AP-1 consensus sequence found on the SV40 promoter (Table 1); and (ii) the protein complexes bound to SV40 AP-1 and AP-720 appear to be similar if not identical, and are likely to contain multiple species of Jun/Fos heterodimers.

Deletion analysis of the transcriptional activity of the mTIMP-3 promoter identifies maximal activity for the 2AP construct (–746 to +58)

The 2.9 kb mTIMP-3 promoter contains six AP-1 or AP-1-like elements as well as other sites, all of previously undetermined functional significance. To investigate the functional significance of these AP-1 or AP-1-like elements, as well as that of other regulatory sequences, we constructed luciferase reporters driven by 5' deletion fragments of the mTIMP-3 promoter containing from zero to six AP-1 or AP-1-like sites and binding sites for c-Myc, NF- κ B, p53 and other binding proteins. This series of deletion constructs contained from 500 bp to 2.9 kb of the 5'

Figure 4 SV40 AP-1 and AP-720 oligonucleotides show identical patterns of supershifting and blocking with anti-AP-1 antibodies

The protein binding activity of the SV40 AP-1 control sequence was compared with those of the AP-763 oligonucleotide (A) and the AP-720 oligonucleotide (B) by using several anti-Jun or anti-Fos antibodies. As described in the Materials and methods section, nuclear protein from P⁺ (Ci22) cells was incubated with specific c-Jun antibody (c-J), pan Jun family antibody (J), specific c-Fos antibody (c-F) and pan Fos family antibody (F). The same protein was also incubated with the AP-763, AP-720 and SV40 AP-1 oligonucleotides in the absence of antibody (0).

Figure 5 Analysis of 5' deletion constructs shows greatest transcriptional promoter activity for the 2AP construct (–746/+58) containing two AP-1-like sites (AP-720 and AP-617)

JB6 P[–] cells were transiently transfected with one of the seven luciferase reporter constructs, along with positive (4 $\times$ AP-1; pos.) and negative (pGL2-Basic vector; vec.) controls, using a calcium phosphate method as described in the Materials and methods section. Transcriptional activity was measured with a Monolight luminometer (Analytic Luminescence Laboratory) and normalized to β -galactosidase activity. Each measurement represents the mean $\pm$ S.E.M. of three independent experiments. The six AP-1 or AP-1-like sites are indicated by the solid boxes (AP-1958, AP-1348, AP-1117, AP-763, AP-720 and AP-617). RLU, relative light units.

mTIMP-3 sequence, as shown in Figure 5. These constructs were transiently transfected into P[–] JB6 cells, which are known to show endogenous mTIMP-3 expression, and cell lysates were

Table 3 mTIMP-3 promoter luciferase activity

These assays used the same luciferase reporter constructs as with the JB6 P[−] cells in Figure 5. 4 × AP-1 and pGL2-Basic vector represent positive and negative controls respectively.

Construct	10 ^{−3} × Activity (relative light units)	
	P ⁺ cells	NIH 3T3 cells
4 × AP-1	39.9 ± 4.6	21.6 ± 1.1
6AP	194.7 ± 27.2	30.9 ± 4.0
5AP	143.8 ± 17.1	40.4 ± 2.6
4AP	237.2 ± 35.7	45.0 ± 3.9
3AP	254.6 ± 35.1	49.8 ± 3.9
2AP	371.6 ± 75.9	70.7 ± 6.0
1AP	69.6 ± 15.3	18.3 ± 2.4
0AP	60.8 ± 8.6	21.6 ± 1.8
pGL2-Basic vector	0.2 ± 0.02	0.3 ± 0.02

assayed for luciferase activation. The 2AP construct (−746 to +58), containing both the AP-720 and AP-617 binding sites, showed the greatest promoter activity in P[−] JB6 cells (Figure 5), suggesting that AP-720 alone or together with AP-617 is important for activating expression of this gene. Increasing the length of the 5′ sequence upstream of the AP-720 binding site resulted in decreased promoter activity, suggesting the presence of negative regulatory elements within this region.

The transcriptional activity of the mTIMP-3 promoter was the same as or greater than the transcriptional activity of the positive control (4 × AP-1), which contains AP-1 but not other transcription factor binding sequences (Figure 5). In addition, the 0AP construct (−491 to +23) showed activity nearly equal to that of the positive control reporter, suggesting that non-AP-1 sites downstream of nucleotide −491 contribute to the activity of the mTIMP-3 promoter. Determinants of maximal activity appear to be located between positions −491 and −746. The transcriptional activity of 2AP (−746), which contains two AP-1 binding sites, was higher than that of 6AP (−2846), which contains six AP-1 binding sites (Figure 5), suggesting that: (i) not all AP-1 or AP-1-like sites contribute to activation of the expression of this gene, and (ii) there are probably negative element(s) in the mTIMP-3 promoter. Transcription factor binding sites for c-Myc and NF-κB have also been identified by sequence inspection of the mTIMP-3 promoter [22]. These binding sites are, however, contained in the 6AP (−2846) construct and are 5′ to the sequences contained in 2AP (the most active of the deletion constructs), suggesting that these sites may not contribute significantly to basal transcriptional activity.

We next extended the observations made in P[−] cells (Figure 5) to JB6 P⁺ cells and to NIH 3T3 cells, both of which also express endogenous mTIMP-3. Once again, 2AP showed the greatest luciferase activity in JB6 P⁺ and NIH 3T3 cells (Table 3), suggesting the importance of the AP-720 site in mTIMP-3 transcriptional activity. Comparison of the transcriptional activities of these reporter constructs showed a similar rank order among the two JB6 variants and NIH 3T3 cells (Figure 5 and Table 3). Taken together, these results show that the maximal transcriptional activity of the mTIMP-3 promoter, as measured by transient transfection, is associated with the 2AP reporter construct (−746 to +58) containing two AP-1-like sites (AP-720 and AP-617) in both JB6 and NIH 3T3 cells.

To ascertain whether AP-720, AP-617 and/or sequences flanking these elements are responsible for the transcriptional activity of the 2AP mTIMP-3 reporter shown in Figure 5, several

Table 4 mTIMP-3 promoter mutants: AP-1 sites

The AP-1 sites in AP-720 and AP-617 are underlined, and mutated nucleotides are emboldened. Dashes indicate deletions.

Name of mutant	AP-720 -TCTTAGTCATT-	AP-617 -GTTGAATAATT-
m720-1	-TCCTAGT A AATT-	
m720-2	-TCCTAGG C AATT-	
m720-3	-TCTT-----TT-	
m720-4	-TTT T AGTCATT-	
m617/m720-1	-TCTT-----TT-	-GTTG-----TT-
m617/m720-2	-TTT T AGTCATT-	-GTTG-----TT-

Table 5 Activity of mTIMP-3 promoter mutants

The constructs are described in Table 4 and Figure 5. The pGL2-Basic vector is a negative control.

Construct	10 ^{−3} × Activity (relative light units)
2AP	129.9 ± 7.1
m720-1	61.8 ± 2.8
m720-2	61.2 ± 2.3
m720-3	74.3 ± 2.4
m720-4	69.9 ± 3.0
m617/m720-1	51.5 ± 4.9
m617/m720-2	54.1 ± 2.7
1AP	55.4 ± 3.6
0AP	53.2 ± 2.8
pGL2-Basic vector	2.8 ± 0.4

constructs with point mutations in the AP-720 and/or AP-617 sites were prepared (Table 4). Constructs were designed such that no new transcription factor binding sites were generated in the mutated plasmids. The T (position 1 of the TRE) and C (position 6 of the TRE) have been demonstrated to be essential for inducibility by PMA and for protein binding activity [26]. Mutation of the AP-720 sequence decreased mTIMP-3 promoter 2AP activity in P⁺ cells (Table 5). This suggested that positions 2–5 and 7, as well as positions 1 and 6, of the TRE sequence and the flanking nucleotide immediately 5′ in the AP-720 oligonucleotide contribute to the transcriptional activity of mTIMP-3. The observations that the construct mutated in both AP-720 and AP-617 showed activity similar to that of the construct mutated in AP-720 only, and that these mutant activities were similar to the activity of unmutated 1AP, suggest only a small contribution of AP-617 to the activity of the mTIMP-3 promoter. The relatively high activity of the 0AP construct (also high in Figure 5 and Table 3) underscores the conclusion that AP-1-independent events also regulate the mTIMP-3 promoter. It is, nevertheless, clear from these results that the activity increment that distinguishes the 2AP from the 1AP mTIMP-3 reporter construct can be attributed to the AP-1 site at −720 rather than to non-AP-1 flanking sequences.

DISCUSSION

We have recently described the full-length mTIMP-3 cDNA sequence and its transcriptional promoter sequence [22]. Sequence analysis revealed a number of important potential tran-

scription factor binding sites. Within this region are six sites for the transcription factor AP-1, two sites for NF- κ B, one site for c-Myc and one site for p53 [22]. This large number of potential transcription factor binding sites may indicate a complex mechanism of transcriptional regulation for mTIMP-3. In the present study, regulation of mTIMP-3 expression by AP-1 was investigated by using DNase I footprinting analysis, EMSA and transcriptional activation assays. The results show that, of the six AP-1 binding sites in the mTIMP-3 promoter, AP-720, a non-consensus AP-1 binding site (5'-TTAGTCA-3', nt -720 to -714), is important for transcriptional activation and shows the strongest binding activity for c-Jun protein. This result is similar to that for mTIMP-1, in which a non-consensus binding site (5'-TGAGTAA-3') functions as a strong binding site for Jun/Fos heterodimeric and Jun/Jun homodimeric AP-1 proteins [10].

The explanation for the non-consensus site (AP-720) appearing to be more important for transcriptional activation than the consensus binding sites (AP-1348 and AP-1117) is at present unclear. One possibility may involve the protein-protein interactions that influence DNA binding. Other proteins may be involved in the DNA binding complex that contains Jun and Fos proteins, and this complex may bind with higher affinity to non-consensus than to consensus AP-1 sites. However, the observation that the non-consensus AP-720 site binds purified c-Jun more efficiently than the other AP-1 sites suggest that flanking DNA sequences outside of the consensus AP-1 site may have a strong influence on transcription factor binding. Another possibility is that the distance between the various sites and the transcriptional start site may influence transcriptional activity. In addition, the distal AP-1 sites may have a more significant role in the context of the natural chromatin configuration of the gene, whereby DNA bending and chromatin structure may bring these sites in closer proximity to the basal transcriptional machinery.

Like mTIMP-1, which has a transcriptional regulatory element within intron 1 [27], it is also possible that mTIMP-3 has regulatory element(s) downstream of the transcriptional start site. Although AP-1 transcription factor binding site(s) are critical for transcriptional activation in both mTIMP-1 and mTIMP-3, mTIMP-3 exhibits different functions and has a different pattern of expression [16,20–24], suggesting differences in the mode of regulation. In this respect, hTIMP-3 is a TATA-less gene and has one major transcription start site [18], while mTIMP-3 has three TATA boxes and multiple transcriptional start sites [22].

AP-1 binding sites appear to contribute to the basal transcriptional activity of mTIMP-3, as suggested by the observation that mTIMP-3 promoter activity was generally lower in the reporter construct without AP-1 binding sites than in those constructs that contained them (compare 0AP and 2AP). It is clear, however, that significant basal activity occurs independently of AP-1 sites. The similar pattern of activity among the neoplastic and preneoplastic JB6 variants [22] also confirms that the lack of expression of mTIMP-3 in neoplastic JB6 cells is not due to a lack of activated transcription factors.

In summary, we conclude from these observations that AP-1 binding sites in the promoter of the mTIMP-3 gene show differential binding and transcriptional activities, suggesting a role for only a subset of AP-1 sites, principally the -720 site, in

the positive regulation of mTIMP-3 expression. The function of these sites does not correlate directly with the extent of similarity to the published AP-1 consensus sequence. It is noteworthy, however, that the site associated with both highest activity and high specific binding appears to be occupied exclusively by complexes containing Jun and Fos family proteins. The control of expression of mTIMP-3 is likely to be complex and to involve multiple non-AP-1 regulatory elements as well. Further investigations are under way to elucidate the role of these non-AP-1 elements, especially those immediately 5' or 3' to the transcriptional start site.

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