Synergistic Transcriptional Activation of the Tissue Inhibitor of Metalloproteinases-1 Promoter via Functional Interaction of AP-1 and Ets-1 Transcription Factors*

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The tissue inhibitor of metalloproteinases-1 (TIMP-1) is an inhibitor of the extracellular matrix-degrading metalloproteinases. We characterized response elements that control TIMP-1 gene expression. One contains a binding site that selectively binds c-Fos and c-Jun in vitro and confers a response to multiple AP-1 family members in vivo. Adjacent to this is a binding site for Ets domain proteins. Although c-Ets-1 alone did not activate transcription from this element, it enhanced transcription synergistically with AP-1 either in the context of the natural promoter or when the sequence was linked upstream of a heterologous promoter. Furthermore, a complex of c-Jun and c-Fos interacted with c-Ets-1 in vitro. These results suggest that AP-1 tethers c-Ets-1 to the TIMP-1 promoter via protein-protein interaction to achieve Ets-dependent transcriptional regulation. Collectively, our results indicate that TIMP-1 expression is controlled by several DNA response elements that respond to variations in the level and activity of AP-1 and Ets transcriptional regulatory proteins.

The extracellular matrix provides a controlled environment for cellular differentiation and tissue development. The integrity of the extracellular matrix is maintained through a balance between the amount and activity of matrix-degrading proteolytic enzymes and their associated activators and inhibitors (1–3). The expression of tissue inhibitor of metalloproteinases-1 (TIMP-1), an inhibitor of a key regulatory class of matrix-degrading proteinases, is under strict developmental and tissue-specific control. TIMP-1 expression is largely confined to adult bone and ovary and to tissues undergoing remodeling or inflammation. In cultured cells its expression is regulated by serum and growth factors and, in F9 cells, by differentiation (4, 5).

The basic structure of the TIMP-1 promoter has been described previously (6, 7). TIMP-1 possesses a TATA-less promoter and is composed of six exons. DNA sequences conferring transcriptional activation by viruses, serum, phorbol esters, and transforming growth factor-β have been localized to the 5’ region of the gene (6, 8). This regulation can be quite dramatic, with serum inducing endogenous TIMP-1 expression by as much as 3 orders of magnitude (6).2 Sequences including this region and approximately 2 kilobases more in the 5’ direction have also been shown to direct appropriate embryonic expression of the gene (9). Furthermore, TIMP-1 is negatively regulated by the extracellular matrix in primary mammary epithelial cells, although the sequences conferring regulation have not been identified.3

The TIMP-1 gene is induced in response to the activation of c-Fos (10), suggesting that in vivo the gene is regulated by AP-1 family members. In fact, inspection of sequences in the TIMP-1 regulatory region has revealed two potential AP-1 binding sites or TPA response elements; however, neither the element that selectively binds AP-1 in vitro nor the sequence that directs AP-1-dependent transcriptional enhancement in vivo has been identified (5, 8).

Several members of the AP-1 family have been shown to have positive (e.g. JunD) or negative (e.g. JunB) effects on transcriptional regulation (11, 12). Each AP-1 family member is homologous to one of the two prototypes of the family, c-Jun or c-Fos (13). The ratio of various Jun and Fos proteins expressed in cells may be one determinant of their transcriptional efficacy on target genes. Functionally, AP-1 has been shown to be necessary for collagenase induction by phorbol esters, oncogenes, and α5β1 integrins (14, 15) and for stromelysin induction by epidermal growth factor (16, 17).

The TIMP-1 promoter also has multiple binding sites for Ets transcription factors. One of these sites has been shown to bind Ets in vitro (5). Like AP-1 and TIMP-1, Ets proteins are serum-inducible. These proteins appear functionally diverse, participating in transcriptional regulation as well as DNA replication and growth control (18, 19). In Drosophila, Ets-related proteins are critical for cellular differentiation (20, 21). Ets proteins appear to bind DNA and to activate transcription as monomers or in complexes with other proteins. For example, Elk-1, an Ets-related protein, forms a ternary complex with serum response factor and is phosphorylated in response to growth factors (22, 23).

It is widely assumed that AP-1 and c-Ets interact to regulate gene expression, because their binding sites are often juxta-

2 S. K. Logan and Z. Werb, unpublished observations.
3 R. S. Talhouk, M. J. Bissell, and Z. Werb, unpublished observations.
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posed in cellular promoters, including TIMP-1, and because the proteins act synergistically at a multimerized viral enhancer site (24). However, such effects have not been documented for cellular promoters, although related proteins such as serum response factor and Elk-1 have been shown to interact (25). In this study we used TIMP-1 promoter sequences to examine the transcriptional regulatory proteins that control TIMP-1 transcription and to characterize the interaction of AP-1 and Ets in the context of a cellular promoter.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Monolayers of F9 embryonic carcinoma cells were grown at 7% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, and Pen/strep (penicillin G at 100 units/ml and streptomycin SO4 at 100 mg/ml). Partially confluent cultures (50–80%) were transfected by the calcium phosphate procedure (26, 27). Fresh medium was added 1–4 h before the cells were transfected. The calcium phosphate-DNA precipitate was prepared in 125 mM CaCl2, HEPES-buffered saline (pH 6.92, 25 mM HEPES, 0.75 mM Na2HPO4, H2O, 140 mM NaCl). The amounts of plasmid transfected were within the range of the assay conditions used. In experiments showing an average of two samples, variation between samples was ±15%. Cells were incubated for 15 h with precipitate and then either assayed immediately or washed and assayed 24 h later. In experiments in Figs. 4 and 5, the cells were then shocked by the addition of 4% formaldehyde in Dulbecco's modified Eagle's medium at 37 °C for 2 min, washed twice with 37 °C phosphate-buffered saline (PBS), refed with fresh medium, and grown for an additional 24 h. In all other transfection experiments the cells were washed once with PBS and immediately scraped from the plates in 1 ml of PBS. The same trends were observed with the use of either transfection method; however, the number of counts/minute tended to be higher for both constitutive and inducible expression in the longer experiments.

For chorioallantoic aldehyde transferase (CAT) assays, the cells were washed with 5 ml of PBS, scraped from the plates in 1 ml of PBS, resuspended (250 mM Tris-HCl, pH 7.8), and lysed by four cycles of freeze/thaw (−70 °C/37 °C). Cell debris was pelleted by centrifugation for 5 min at 10,000 × g. CAT activity was assessed in a 1-h incubation with [14C]-labeled acetyl coenzyme A, followed by extraction of the labeled acetylated chorioallantoic with ethyl acetate (28). Scintillation fluid was added to the ethyl acetate supernatant and monitored for radioactivity. β-Galactosidase activity was monitored by adding 50 ml of cell extract to 450 ml β-galactosidase buffer (60 mM Na2HPO4, pH 8.0, 1 mM MgSO4, 10 mM KCl, 50 mM β-mercaptoethanol). The reaction was started by the addition of the substrate, 100 μl of 4 mg/ml O-nitrophenyl-β-D-galactosidase. The samples were then incubated at ambient temperature until the color began to develop, and spectrophotometry was performed at absorbance of 420 nm.

RESULTS

c-Jun and c-Fos Induce Expression of the TIMP-1 Promoter—Previous analysis of the mouse TIMP-1 promoter identified a sequence that was responsive to serum, phorbol esters, and transforming growth factor-β when placed upstream of a promoterless CAT gene (8). This sequence is located 5’ to the first exon and contains a TPA response element (TRE) similar to the one shown to bind AP-1 (c-Fos and c-Jun) in the collagenase and stromelysin gene promoters (Fig. 1) (34–36). (In this paper, we use the terms c-Fos/c-Jun interchangeably with AP-1.) To determine whether this TRE could be activated directly by AP-1 in vivo, we cotransfected expression vectors for c-Fos and c-Jun with reporter constructs in which regions of the TIMP-1 promoter were placed upstream of a CAT gene. We used F9 cells, which do not have demonstrable AP-1 activity (37, 38). Initially, we tested the effect of AP-1 activity on two TIMP-1 promoter sequences, −1320/−601 and −834/−601, that possess a TRE identified by sequence comparison with known transcription factor binding sites (Fig. 1). Cotransfected AP-1 transcriptionally activated both TIMP-1/CAT reporter structures to a similar extent but had virtually no effect on the sequence from −847 to −820. The small letters on the left side of the sequences show nucleotides compatible with a SalI-cleaved site, and those on the right show nucleotides compatible with a Xhol-cleaved site. All reporter constructs were prepared by using pBLCAT2 Bam−, a derivative of pBLCAT2 in which the polylinker BamHI site GGAATTC was changed to GC by standard methods; this removed an Ets motif previously reported by Trembly et al. (25).

The mEts oligonucleotide was designed by changing 5 bp of the Ets consensus binding site in the TIMP promoter shown to bind bacterially expressed c-Ets (5). This site is also identical to the Ets binding site of the polyclonal virus enhancer that was used in the initial studies demonstrating interaction between c-Ets and AP-1 (24, 31).

The wild-type AP-1 oligonucleotide was annealed in equimolar amounts and end-labeled with T4 polynucleotide kinase and [γ-32P]ATP, followed by desalting through Sephadex G-25 spin columns. The following oligonucleotide was used for gel shift experiments: 5′-TGATGGATATGGCCGAGGAGCCGTT-3′. This oligonucleotide is identical to the TIMP-1 sequence from −847 to −820 and includes both the AP-1 and Ets binding sites. The larger DNAs used in mobility shift assays were HindIII–BamHI-generated fragments from pTCAT5′−858 and pTCAT5′−834, yielding fragments of 257 or 233 bp. These sequences were phosphorylated with calf intestinal phosphatase, isolated from the gel, and labeled with T4 polynucleotide kinase and [γ-32P]ATP, followed by desalting through Sephadex G-50 spin columns. Binding reactions were performed in 20 mM Tris, pH 7.9, 50 μM MgCl2, 1 μg/ml Poly(dI-dC), 10% glycerol, 0.2 mM dITP, 10% dNTPs, 0.02 μg of poly(dI-dC), and 1 mM dithiothreitol. Probes and the indicated amounts of bacterially expressed and purified c-Fos and c-Jun (gifts of Geoff Parson and Keith Yamamoto, UCSF) were added to the reactions on ice and brought to ambient temperature for 10–15 min before loading on a 6% nondenaturing polyacrylamide gel. The F9 cell nuclear extracts were prepared according to the method of Zimarino and Wu (32).

Common precipitation Analysis—Labeled c-Ets was prepared in vitro by transcription and translation in rabbit reticulocyte lysates (Promega) in the presence of [35S]methionine (DuPont NEN; 1190 Ci/mmol). The resulting c-Ets protein was then mixed with purified c-Fos and c-Jun and incubated for 30 min at ambient temperature. The reactions were diluted 10-fold in buffer A (100 mM NaCl, 50 μM HEPES, pH 7.5, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40) and preceeded by the addition of protein A-Sepharose (100 mg/ml) to 10% for 30 min at 4 °C with gentle rotation. Ethidium bromide (50 μg/ml) was also added to buffer A to show that AP-1 and c-Ets proteins were binding to one another rather than to contaminating DNA in the protein preparations (33). After removal of the Sepharose, affinity purified c-jun specific monoclonal antibody (c-jun/1AP-1 (N); Santa Cruz Biotechnology, Inc), and another aliquot of protein A-Sepharose were added before the slurry was incubated at 4 °C for 3 h with rotation. The beads were centrifuged and washed 4 times in 20 volumes of buffer A and one time in fresh tubes in buffer A without Nonidet P-40. Proteins were eluted from the beads with sample buffer and analyzed on SDS-polyacrylamide gels.
plasmid that lacked these sequences.

By sequence inspection of the TIMP-1 promoter, we identified another potential TRE located at approximately -21600. To determine whether this putative AP-1 binding site also participated in AP-1-mediated transcriptional enhancement in vivo, we compared the response of a variety of TIMP-1 promoter constructs to AP-1 by cotransfection into F9 embryonic carcinoma cells. Whereas AP-1 activated (>10-fold) a TIMP-1/CAT reporter construct containing TIMP-1 sequences between -21008/-601, the promoter region containing the putative TRE

**FIG. 1. Activation of TIMP-1 gene transcription by c-Fos and c-Jun.** The drawing at the top represents a portion of the TIMP-1 gene. Exons 1 and 2 are represented by open rectangles, and all positions are numbered relative to the translation initiation start site indicated by the arrow. The position of the TIMP-1 TRE is indicated by the black rectangle. The regions diagrammed below the map were cloned into a promoterless CAT vector, pBLCAT3, as described under “Experimental Procedures.” The two DNA reporter structures, -1320/-601 and -858/-601 (2 μg), or pBLCAT3 with no inserted TIMP-1 sequence (vector; 2 μg) were transiently transfected into F9 cells with 0.5 μg of Rous sarcoma virus c-jun and 0.5 μg of Rous sarcoma virus c-Fos (shaded bar) or without either protein (open bar). A β-galactosidase expression vector (0.25 μg of Rous sarcoma virus LacZ; Ref. 47) was cotransfected as an internal control, and CAT activity was normalized to β-galactosidase activity. Each data point represents an average of three experiments. The bars indicate S.D. ■, with AP-1; □, without AP-1.

**FIG. 2. AP-1 activates a proximal TRE but not a distal TRE in the TIMP-1 promoter.** The Ets binding sites are represented by open ovals. The position of the distal TRE is represented by a shaded rectangle. See the legend to Fig. 1 for an explanation of other symbols. Regions diagrammed below the map were cloned into either pBLCAT3 (CAT) or pBLCAT2 (tkCAT). These structures (2 μg) were transiently transfected into F9 cells in the presence (■) or the absence (□) of 0.5 μg of AP-1 as described in the legend to Fig. 1. Each data point represents an average of two samples from the same experiment; the values were normalized to β-galactosidase activity as described in the legend to Fig. 1. The experiment is representative of several independent experiments.
The protein-DNA complex was resolved from unbound DNA by nondenaturing gel electrophoresis and is indicated by the unlabeled TIMP-1 oligonucleotides were added in molar excess of labeled oligonucleotides as follows: lane 3, arrow indicates DNA not complexed with protein. The autoradiogram was exposed for 48 h at ambient temperature.

Lane 1, labeled oligonucleotides alone; lane 2, labeled oligonucleotides with added c-Jun and c-Fos proteins; lanes 3–5, same as lane 2, except that unlabeled TIMP-1 oligonucleotides were added in molar excess of labeled oligonucleotides as follows: lane 3, 100×; lane 4, 1000×; lane 5, 10,000×. The protein-DNA complex was resolved from unbound DNA by nondenaturing gel electrophoresis and is indicated by the upper arrow. The lower arrow indicates DNA not complexed with protein. The autoradiogram was exposed for 48 h at ambient temperature.

Fig. 3. A 28-bp TIMP-1 TRE transcriptionally responds to AP-1 in vitro and binds purified c-Jun and c-Fos protein in vitro. A, see the legend to Figs. 1 and 2 for an explanation of the symbols. Oligonucleotides containing the TIMP-1 TRE were placed upstream of a tkCAT gene (pBLCAT2) as single (wt × 1) or triple (wt × 3) copies. These plasmids were transiently transfected into F9 cells in the presence or the absence of 0.5 μg of AP-1 as described in the legend to Fig. 1. Each data point represents an average of two samples from the same experiment; the values were normalized to β-galactosidase activity as described in the legend to Fig. 1. This experiment is representative of three independent experiments. B, a TIMP-1 oligonucleotide containing the TIMP-1 TRE at −847/−841 binds purified c-Fos and c-Jun in vitro. 32P-labeled double-stranded oligonucleotides containing the TIMP-1 sequence 5′-TGGATGAGTAATGCGTCCAGGAAGCCTG-3′ were incubated with 100 ng of c-Jun and 100 ng of c-Fos proteins expressed and purified from E. coli (lanes 2–5). The underlined sequence is the AP-1 binding site, or TRE. Lane 1, labeled oligonucleotides alone; lane 2, labeled oligonucleotides with added c-Jun and c-Fos proteins; lanes 3–5, same as lane 2, except that unlabeled TIMP-1 oligonucleotides were added in molar excess of labeled oligonucleotides as follows: lane 3, 100×; lane 4, 1000×; lane 5, 10,000×. The protein-DNA complex was resolved from unbound DNA by nondenaturing gel electrophoresis and is indicated by the upper arrow. The lower arrow indicates DNA not complexed with protein. The autoradiogram was exposed for 48 h at ambient temperature.

Between −3450/−1008 was not activated (Fig. 2). Furthermore, this sequence was not by itself transcriptionally responsive to AP-1 when placed upstream of either a promoterless CAT gene or a thymidine kinase promoter-driven CAT gene (Fig. 2). Therefore, our functional studies indicate that a TRE located between −858/−601 is a target for AP-1-dependent transcriptional activation, but the TRE at −1600 is not.

To determine whether the TRE located within the −858/−601 fragment was the DNA binding site that enabled transcriptional activation by AP-1, we next examined the response to AP-1 of an oligonucleotide containing this TRE upstream of a heterologous promoter (tkCAT). Like many other transcription factor binding sites, a single TIMP-1 TRE was not transcriptionally active. However, when three tandem repeats of this site were placed upstream of tkCAT, significant AP-1-dependent transcriptional enhancement was achieved (Fig. 3A). In addition, this TRE bound bacterially expressed and purified c-Jun and c-Fos in vitro, suggesting that the TRE located between −858/−834 is a site of AP-1 action on this promoter (Fig. 3B).

To test for additional AP-1 binding sites, we used a TIMP-1 reporter construct, −834/−601, in which the TRE had been deleted. Surprisingly, this construct was still AP-1-inducible in F9 cells, albeit to a lesser extent than the −858/−601 construct, suggesting that additional AP-1 binding sites are present in the −834/−601 sequence (Fig. 4).

Constitutive Expression of the TIMP-1 Promoter Is Modulated by Cell-specific Factors—To examine AP-1-mediated TIMP-1 expression in cells containing endogenous AP-1, we compared the results of transfections done in AP-1-deficient F9 cells with those done in 3T3 fibroblasts, which contain endogenous AP-1 family proteins. In contrast to F9 cells, the −858/−601 TIMP-1 promoter construct was actively transcribed in 3T3 cells in the absence of exogenous AP-1 (Fig. 4). These results suggest that the endogenous Jun and Fos proteins present in 3T3 cells are sufficient for TIMP-1 transcriptional activation. This is further supported by the observation that exogenous AP-1 did not substantially increase transcription of −858/−601 in 3T3 cells (Fig. 4). These results are similar to those observed for the stromelysin promoter, in which AP-1 functions in basal level transcription (39). Interestingly, deletion of the TRE between −858 and −834 resulted in a promoter that no longer responded to endogenous AP-1 in 3T3 cells. As in F9 cells, AP-1-dependent transcriptional activation from the −834/−601 promoter construct was achieved only when high levels of AP-1 were provided exogenously (Fig. 4). These data suggest that when the concentration of AP-1 is relatively low, as in 3T3 cells in the absence of cotransfected AP-1, the canonical high affinity AP-1 binding site between −858 and −834 is occupied, resulting in AP-1-dependent transcriptional en-
synergistic interaction between AP-1 and c-Ets-1 — The TIMP-1 AP-1 binding site near −858 is adjacent to a c-Ets-1 binding site (Fig. 6A), as it is in promoters of many other genes, including the metalloproteinases collagenase and stromelysin. This sequence has been previously shown to bind c-Ets-1 in vitro, although no functional relevance was demonstrated (5).

To determine whether Ets proteins function in the control of TIMP-1 expression, we examined activation of the −858/−601 TIMP-1/CAT reporter structure in the presence of cotransfected expression vectors for c-Ets-1 and AP-1, separately and in combination. c-Ets-1 alone did not activate the −858/−601 sequence of the TIMP-1 promoter (Fig. 6B). Furthermore, under conditions in which large amounts of AP-1 were present in cells and AP-1-mediated transcriptional activity was fully operative, c-Ets-1 had no effect on the AP-1-dependent transcriptional response (data not shown). However, under conditions of limiting AP-1 (i.e. 0.05 μg), c-Ets exhibited synergistic activation of the promoter fragment (Fig. 6B). The synergy was dose-dependent, because an Ets-dependent increase in transcriptional activation from the promoter was observed in the presence of AP-1 by increasing the amount of c-Ets-1 in the cell (Fig. 6B). Therefore, although c-Ets-1 alone does not induce
transcriptional activation, it is capable of functionally interacting with AP-1 and potentiating TIMP-1 transcriptional enhancement.

To determine whether the synergistic transcriptional enhancement observed between c-Ets and AP-1 could be conferred upon a heterologous promoter, an oligonucleotide containing the AP-1 and Ets binding sites was placed upstream of tkCAT. After transfection into F9 cells, the results were similar to those obtained with the larger TIMP-1 promoter fragment. Neither AP-1 nor c-Ets-1 alone had much effect on the reporter plasmid's transcriptional activation potential at the concentrations used (Fig. 7). However, when even at high concentrations AP-1 alone was able to activate at a multimerized but not a single binding site as shown above.) Moreover, together they showed nearly a 10-fold induction over that of either protein alone (Fig. 7). This synergistic activation was sequence dependent for AP-1, because mutation of this binding site abolished transcriptional activation (Fig. 7). Although Ets has been shown to bind to this Ets site in vitro (5), mutation of the binding site (see “Experimental Procedures”) had little effect on transcriptional synergy with AP-1 in vivo (Fig. 7). These results indicate that the interaction between AP-1 and Ets may not require site-specific DNA binding by c-Ets-1 but rather may use protein-protein interactions between AP-1 and c-Ets-1 to achieve synergistic transcriptional regulation.

Physical Interaction Between AP-1 and c-Ets-1—To examine the possibility that c-Ets-1 might associate with components of the AP-1 complex, we tested whether the proteins in solution could physically interact. For these experiments, c-Ets-1 was synthesized in reticulocyte lysates in the presence of [35S]methionine, and c-Fos and c-Jun proteins were expressed and purified from Escherichia coli. Jun and Fos proteins in combination were then mixed with labeled c-Ets-1, the reactions were immunoprecipitated with a c-Jun-specific monoclonal antibody, and the labeled proteins were subjected to SDS gel electrophoresis. Under these conditions, c-Ets-1 was coprecipitated with a c-Jun-c-Fos complex (Fig. 8, lane 3). Control reactions with no added c-Jun or c-Fos yielded little detectable coprecipitation of c-Ets-1 (Fig. 8, lane 2). Thus, Jun-Fos heterodimers appear to form a specific complex with c-Ets-1. These results indicate that c-Ets-1 interacts with Jun-Fos heterodimers in vitro. Together with the fact that specific DNA binding by c-Ets-1 is not required for synergy with AP-1, our results suggest that AP-1 might tether c-Ets-1 to the TIMP-1 promoter to achieve Ets-dependent transcriptional enhancement in vivo.

DISCUSSION

The TIMP-1 gene is precisely regulated in vivo, with high expression restricted to adult bone and ovary (9, 40, 41). TIMP-1 is also induced in development (4, 9) and in areas of inflammation and during tissue remodeling (3, 5) when AP-1 expression is high (40, 42). To elucidate some of the regulation of the TIMP-1 gene, we used F9 teratocarcinoma cells, which do not contain demonstrable AP-1 when they are undifferentiated. Upon differentiation to parietal endoderm, F9 cells up-regulate their endogenous TIMP-1 expression (4) and AP-1 activity (37, 38). In these experiments we used TIMP-1 promoter structures
in conjunction with the transcriptional regulatory proteins c-Ets-1 and AP-1 to investigate their roles in TIMP-1 transcriptional regulation. We functionally characterized three response elements necessary for TIMP-1 transcriptional enhancement (Fig. 9A). The first element consists of a high affinity canonical AP-1 site located at −858/−834 of the TIMP-1 promoter. Although this site was highly responsive to c-Jun and c-Fos heterodimers in vivo and bound purified AP-1 in vitro, it also responded to other AP-1 family members, including c-Jun homodimers, c-Fos, and JunD, as well as c-Fos and undetermined partners resident in F9 cells. Next to this canonical AP-1 site lies an Ets binding site that is entirely dependent on the activity of AP-1 at the adjacent binding site for Ets-dependent gene activation. Moreover, this Ets/AP-1 element in the form of an oligonucleotide transferred the AP-1-dependent Ets response to a heterologous promoter.

A third response element, between −834/−601 of the TIMP-1 promoter, also responds to AP-1 and was revealed when the canonical AP-1 site between −858/−834 was deleted. Although c-Fos and c-Jun heterodimers appear to be capable of binding and activating transcription through this site in the TIMP-1 promoter, other AP-1 family members are not and activate transcription exclusively through the high affinity TRE between −858/−834. These results suggest that AP-1 family members bind to different sites in the TIMP-1 promoter with different affinities, resulting in distinct patterns of TIMP-1 expression.

FIG. 6. Activation of TIMP-1 by AP-1 and c-Ets-1. A, sequence arrangement of the AP-1 and Ets DNA binding sites in the TIMP-1 promoter. B, synergistic transcriptional activation by c-Ets-1 and AP-1. The TIMP-1 promoter structure −858/−601 was cotransfected with or without c-Ets-1 expression vector (3 or 6 μg) in the presence (●) or the absence (○) of 0.05 μg of c-Jun and 0.05 μg of c-Fos. The values were normalized to β-galactosidase activity as described in the legend to Fig. 1. This experiment is representative of three independent experiments.

FIG. 7. Transcriptional activation of a 34-bp oligonucleotide containing the TIMP-1 AP-1 and c-Ets binding sites upstream of tkCAT. Oligonucleotides containing an Ets binding site (open oval) and an AP-1 binding site (black rectangle) (WT), a wild-type Ets site and a mutant AP-1 site (mAP-1), or a wild-type AP-1 site and a mutant Ets site (mEts) were cotransfected with or without c-Ets expression vector (4 μg) in the presence (●) or the absence (○) of 0.05 μg of c-Fos and 0.05 μg of c-Jun. Each data point represents an average of three samples from the same experiment; the values were normalized to protein concentration. This experiment is representative of three independent experiments.

FIG. 8. In vitro binding of AP-1 and c-Ets-1. [35S]Methionine-labeled c-Ets-1 protein was produced by in vitro transcription and translation in reticulocyte lysates (Promega). The labeled product was mixed with unlabeled bacterially expressed and purified c-Jun and c-Fos and incubated at ambient temperature for 30 min. The mixture was precleared with an excess of protein A/G-agarose beads before the addition of fresh beads and antibody against the amino terminus of c-Jun. The beads were washed extensively before the proteins were subjected to gel electrophoresis on a 10% SDS-polyacrylamide gel. Lane 1, [35S]methionine-labeled c-Ets-1 product from in vitro translation; lane 2, labeled c-Ets-1 with no added c-Jun or c-Fos (this lane represents the nonspecific association of labeled c-Ets-1 with the c-Jun antibody-protein A/G-agarose resin or interaction of c-Ets-1 with endogenous AP-1); lane 3, labeled c-Ets-1 with c-Jun and c-Fos. The arrows indicate the c-Ets-1 protein translation products. This experiment is representative of three independent experiments.
Both Ets and AP-1 families of transcription factors play important roles in differentiation and development (19, 20, 24, 40, 42). Through cotransfection experiments with AP-1 and cEts expression vectors, as well as through communoprecipitation experiments with AP-1 and Ets proteins, we have demonstrated that these transcriptional regulatory proteins functionally and physically interact. This mode of TIMP-1 regulation may be analogous to the complex formed at the serum response element by the interaction of the ubiquitous transcription factor, the serum response factor with the ternary complex factor, an Ets domain protein that cannot bind the serum response element itself (43). The ternary complex factor is recruited to the serum response element after phosphorylation in response to activation of the mitogen-activating protein kinase pathway by growth factor stimulation. Like ternary complex factor, phosphorylation of cEts-1 in response to growth factors results in the Ets-AP-1 complex formation and transcriptional regulation.

The role of Ets in transcriptional regulation is similar to that of proteins such as adenovirus E1a protein. Both E1a and Ets possess internal negative regulatory domains (44–46). This inhibition is relieved by interaction with other transcription factors. Similarly, our results indicate that Ets activates TIMP-1 only in the presence of AP-1. It is likely that such interactions cause a conformational change in the Ets protein, enabling it to interact with components of the general transcriptional machinery and affect transcription. In fact, there is evidence that Ets may play a role in the formation of an initiation complex at minimal core promoters lacking the TATA sequence, such as TIMP-1 (reviewed in Ref. 18). This mode of action may be similar to the ability of E1a to regulate transcription of multiple genes that lack a common promoter element (46).

On the basis of our results, we propose a simple model that accounts for both basal and induced TIMP-1 expression (Fig. 9B). According to this scheme, TIMP-1 expression would not be evident in the absence of AP-1; hence, TIMP-1 would be inactive in cells that lack AP-1 activity. Our results also indicate that the Ets-dependent activation of TIMP-1 is entirely dependent on the activity of AP-1 at the adjacent binding site; thus, cEts-1 alone would not be sufficient for TIMP-1 gene expression. The model further envisions that in the presence of low levels of Jun and Fos proteins, a functional AP-1-TIMP-1 complex is formed, resulting in low level basal expression; under these conditions, TIMP-1 can be induced by Ets proteins, which functionally interact synergistically with AP-1, thus leading to increased TIMP-1 expression. Finally, at high AP-1 concentrations, multiple AP-1 sites are occupied and cooperate to induce TIMP-1 transcription; Ets protein, under these conditions, would not substantially increase TIMP-1 expression. We conclude that TIMP-1 gene expression is controlled by both Ets and AP-1 family members, suggesting that multiple signal transduction pathways coordinate and regulate TIMP-1 expression.

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