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Regulatory elements involved in constitutive and phorbol ester-inducible expression of the plasminogen activator inhibitor type 2 gene promoter

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ABSTRACT

Gene transcription rates and mRNA levels of plasminogen activator inhibitor type 2 (PAI-2) are markedly induced by the tumor promoting agent phorbol 12-myristate 13-acetate (PMA) in human HT1080 fibrosarcoma cells. To identify promoter elements required for basal-, and phorbol ester-inducible expression, deletion mutants of the PAI-2 promoter fused to the chloramphenicol acetyl transferase (CAT) reporter gene, were transiently expressed in HT1080 cells. Constitutive CAT activity was expressed from constructs containing more than 215 bp of promoter sequence, whereas deletion to position −91 bp abolished CAT gene expression. Treatment of transfected cells with PMA resulted in a three- to ten-fold increase in CAT expression from all constructs except from the construct shortened to position −91. DNAse1 protection analysis of the promoter region between −215 and the transcription initiation site revealed numerous protected regions, including two AP1-like binding sites (AP1a and AP1b) and one CRE-like element. Site-directed mutagenesis of the AP1a site or of the CRE-like site resulted in the loss of basal CAT activity and abolished the PMA effect, whereas mutagenesis of AP1b only partially inhibited basal and PMA-mediated expression. Our results suggest that the PAI-2 promoter contains at least two elements required for basal gene transcription and PMA-mediated induction.

INTRODUCTION

Localized extracellular proteolysis mediated by the plasminogen activation system plays an essential role in inflammation, trophoblast implantation, tissue remodeling and development, as well as in tumor invasion and metastasis (1–3). Plasminogen activators (PA's) convert the proenzyme plasminogen into plasmin, a serine protease that activates procollagenase and, in cooperation with collagenase and elastase, contributes to the degradation of the extracellular matrix. PA activity is regulated by the plasminogen activator inhibitors (PAI's): PAI-1 and PAI-2. PAI-1 binds to the extracellular matrix and is a very efficient inhibitor of both tissue-type (t-PA) and urinary-type PA (u-PA), whereas PAI-2 rapidly inhibits u-PA and also reacts with two chain t-PA (4, 5). The binding of pro-u-PA and plasminogen to specific cell-surface receptors (6) and their subsequent activation (7) localizes proteolytic activity to the cell surface and contributes to the invasive potential of human monocytes (8), of keratinocytes (9) and of tumor cells (10, 11). Both PAI's react with receptor-bound u-PA and may play a role in the regulation of matrix degradation by invasive cells (12–15). The biosynthesis of both inhibitors is regulated by a variety of hormones, growth factors and cytokines. PAI-2 is induced by endotoxin in human and mouse macrophages (16, 17) and in human monocytes (18, 19), by the tumor-promoting phorbol 12-myristate 13-acetate (PMA) in monocyte-like (U-937) cells (4, 20, 21) and in human umbilical vein endothelial cells (22), by tumor necrosis factor alpha in human HT1080 fibrosarcoma cells (23) and endothelial cells (22). PAI-2 expression is furthermore induced by cholera toxin (an activator of adenylate cyclase) in cultured mouse macrophages (24) and by Dengue virus in cultured monocytes (25), whereas in HT1080 cells and mouse macrophages the biosynthesis of PAI-2 mRNA is suppressed by dexamethasone (24, 26). The transcriptional induction of PAI-2 by tumor necrosis factor or PMA, and also its inhibitory effect on receptor bound u-PA, suggest that PAI-2 is an important regulator of pericellular proteolysis in inflammatory processes.

We have previously isolated and sequenced 2kb of the PAI-2 gene promoter (27). To functionally characterize this promoter, deletion constructs fused to the CAT reporter gene were transiently expressed in human HT1080 fibrosarcoma cells. Measurements of CAT activities, as well as DNAse I protection analyses and site-specific mutagenesis, enabled us to identify three functional regulatory elements, two activator protein-1-like (AP1-like) and one motif resembling the c-AMP responsive element-like (CRE-like).

MATERIALS AND METHODS

Cell culture

Human HT1080 fibrosarcoma cells (American Type Culture Collection, Rockville, MD) were cultured to confluency according to standard techniques in 50 ml of Dulbecco's modified Eagle's Medium (DMEM) supplemented with 2mM glutamine (Biomerieux, Marcy, France) and 10% of heat-inactivated fetal calf serum (FCS, Gibco, Paisley, UK) at 37°C using 150 cm^2 culture flasks (Corning Glass Works, Corning, NY). Cells were serum starved overnight before the start of an experiment.

Northern blot analysis

HT1080 cells were incubated with either fresh serum-free DMEM alone or with medium supplemented with 25 ng/ml PMA (Sigma, St. Louis, Mo, USA) for 24 h. Cells were harvested and poly A^+ selected RNA prepared by affinity adsorption on oligo d(T) cellulose as previously described (28, 29). Electrophoresis in 0.95% agarose in the presence of 20% formaldehyde followed by Northern blot transfer was performed as described by Thomas (30), but using Gene Screen Plus membranes (New England Nuclear, Boston, MA). PAI-2 mRNA levels were determined by hybridization to a human PAI-2 cDNA probe (21) labeled with alpha [^{32}P] dATP, using the Random Primed DNA labeling kit (Boehringer Mannheim, Germany), to a specific activity of approximately 100 μCi/μg. Hybridization conditions and processing of filters were as previously described (29). Exposure times of filters to X-ray film (Kodak, Lausanne, Switzerland) were chosen to visually optimize the signal. As an internal control, filters were subsequently probed with a mouse β-actin cDNA probe (31), obtained from Dr. S. Petrucco (ISREC, Epalinges, Switzerland).

Run-on transcription assay

HT1080 cells were cultured for 24 h in fresh serum-free DMEM with or without 25 ng/ml PMA. Cell harvesting, nuclei isolation, elongation and labeling of initiated RNA transcripts, was performed using a modified (26) method of Greenberg and Ziff (32). Approximately 2×10^7 nuclei and 100μCi of alpha [^{32}P] UTP (specific activity of 3000 Ci/mmol) were used for each reaction. Filter strips containing 2μg of slotted p38 PAI-2 genomic DNA (27) or mouse β-actin cDNA, were hybridized with the labeled RNA transcripts for 36 h at 65°C, then washed, RNase treated, air dried and exposed to X-ray film for up to 9 days with an intensifying screen(32).

PAI-2 promoter deletion constructs

The deletion mutants used in this study were obtained either by polymerase chain reaction (PCR) methodology, using the genomic p52 PAI-2 clone (27) as a template, or by digestion of the PCR derived constructs with appropriate restriction enzymes. All constructs were verified by DNA sequencing (33).

The **−1063pUC**, **−428pUC**, **−215pUC** and **−91pUC** constructs were obtained by PCR, using the primers: T5′ CATA-TCTAGATCACATAGACATTACCAGA 3′; 5′ ATGCCCT-AAGCATCGCTTGG 3′; 5′ CAGGCATGTCTAGATATT-GAC 3′; and 5′ CCATATCTAGAGGACACAGATCAAAAG-ACAGA 3′, respectively, in combination with the primer 5′ ACATGGATCCTCTTGAGACCTGAGTTGCTGTCTG-ACGG 3′ (ending at position +43). The amplified fragments (−1063, −215, −91bp) were digested with *Xba* I and *Bam*H I (underlined on the primer sequences) and inserted between the *Xba* I and *Bam*H I sites of pUC18. *Xba* I linkers were added to the −428 bp amplified fragment and the product subcloned into the *Xba* I site of pUC18.

The **−904pUC** construct was derived from −1063 pUC by complete *EcoR* I and partial *Bgl* II digestions. The fragment, containing the first 904 bp up-stream the transcription initiation site, was cloned into the *Bam*H I-*Eco*R I sites of pUC18.

The **ΔAlupUC** construct was derived from −1063 pUC, by removing a 321 bp *Bgl* II-*Bgl* II fragment (−905 to −578), containing a complete Alu (proximal) repeated sequence.

The **−339pUC** construct was derived from −428 pUC by digestion with *Sal* I and *Nco* I, followed by Klenow 'fill in' and self religation.

Promoter segments of all PAI-2 pUC constructs were isolated by digestion with *Hind* III and *Bam*H I, gel purified and subcloned into the promoterless **pBLCAT3** plasmid (Luckow and Schütz,1987) upstream the Chloramphenicol Acetyl Transferase (CAT) reporter gene.

The **−1839CAT** construct containing two Alu repeated sequences was made in two steps: first, a 3 kb *Hind* III-*Afl* II insert was excised from the p52 genomic plasmid and cloned into the *Hind* III-*Afl* II sites of −1063 pUC, generating a −3300pUC construct. Second, a 1,9 Kb *Xba* I-*Bam*H I insert was isolated from this intermediate construct and transferred into pBLCAT3.

As shown in Figure 2, each construct contains the first 43 bp of exon 1 directly fused to the CAT reporter gene.

DNA transfections

The day before transfection 0.5×10^6 HT1080 cells were plated on 60 mm^2 dishes (Falcon, Becton Dickinson, New Jersey) in 5 ml DMEM containing 10% FCS and 2mM glutamine, and grown overnight. Cells were transfected with 10 μg of DNA (PAI-2 promoter CAT constructs) by the calcium phosphate precipitation method (34, 35). As positive and negative controls, 10 μg of pRSVCAT (36) and pBLCAT3 (37) were used, respectively. A glycerol shock (2 minutes) was performed 4 hours later and cells were maintained overnight in fresh medium. The next day, transfected cells were washed and incubated overnight either in serum-free medium (controls), or in serum-free medium supplemented with 25 ng/ml PMA. After 24 h incubation, cells were harvested, washed in PBS, resuspended in 100 μl of Tris buffer, and lysed by three freeze-thaw cycles. After centrifugation, the supernatants containing the cytoplasmic extracts were collected and kept at −20°C or immediately used for the analysis of CAT expression (35).

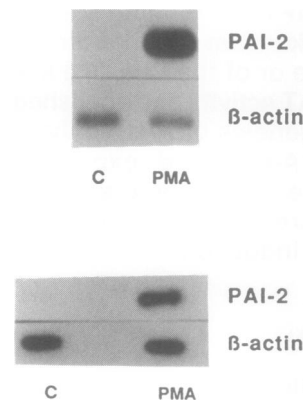

Figure 1. Effect of PMA on PAI-2 expression in HT1080 cells. Top panel: Northern blot analysis of mRNA levels. Cells were incubated either in fresh medium alone (C) or supplemented with PMA (25 ng/ml) for 24 h, lysed and 4 μg of poly A^+ selected RNA were applied to each lane. Relative changes in PAI-2 and β-actin mRNAs were determined by hybridization to specific cDNA probes. Exposure times were chosen to visually optimize the signals. Bottom panel: Effect of PMA on PAI-2 gene transcription. Nuclei were prepared from HT1080 cells incubated in fresh medium alone (C) or supplemented with PMA (25 ng/ml). Initiated RNA transcripts were elongated and labeled in the presence of alpha [^{32}P]UTP and hybridized to immobilized genomic p38 PAI-2 DNA and mouse β-actin cDNA.

CAT assay

Five μl of the transfected cells lysate was incubated with Acetyl Coenzyme A (Fluka) and ^{14}C-Chloramphenicol (Amersham) for 90 minutes at 37°C. The products were extracted with ethyl acetate and separated by thin layer chromatography on silica gel (Polygram Sil gel, Macherey-Nagel, Düren, FRG). The percentage of conversion of [^{14}C]-chloramphenicol to its two acetylated products was determined by counting radioactive regions in liquid scintillation vials (36).

DNase I protection assay

Nuclear protein extracts were prepared from HT1080 cells cultured in serum-free medium. Isolated nuclei (26) were mechanically broken and proteins were precipitated with ammonium sulfate at 50% saturation prior to overnight dialysis (38). For DNase I protection analysis, a **−259pUC** construct was made by Exo III/Mung Bean nuclease digestion of the −428pUC construct. The *Hind* III—*Bam*H I insert (3 μg) of −259pUC was end-labeled (*) either at the *Hind* III site (*−259/+43) or at the *Bam*H I site (−259/+43*) using the Klenow fragment of DNA polymerase I (Boehringer Mannheim, Mannheim, FRG) and alpha [^{32}P]-dGTP and alpha [^{32}P]-dATP. The labeled fragments were isolated on a 0.9% low melting agarose gel, phenol extracted and resuspended in 10mM Tris-1mM EDTA at 10,000 cpm/μl. The DNase I protection assay was performed as described by Galas and Schmitz (39), using empirically determined concentrations of DNase I for limited DNA digestion, and ~10 μg nuclear protein extracts per sample. The reaction was terminated by addition of STOP buffer (40) and immediately frozen in liquid nitrogen. Digested DNA samples were processed as described previously (40) and ~3000 cpm of each sample were loaded on a 6% sequencing gel. Sequencing reactions (33) of both strands were made simultaneously and run in parallel.

Construction of site-specific mutants

To generate specific base mutations in the promoter, the insert of the −1063 pUC construct was transferred into the M13mp18 vector (New England Biolabs, Schwalbach, FRG). Selective base substitutions of the AP1-like sites and the CRE-like element were made using the Mutagene® protocol (Bio-Rad, Richmond CA, USA) and synthetic oligomers (Fig.5). Mutants were selected as described by Kunkel (41), using a *dut*$^-$, *ung*$^-$ Escherichia Coli strain, and cloned into pBLCAT3. The new chimaeric genes were confirmed by DNA sequence (42). The mutant constructs were transiently expressed in HT1080 cells, using the wild-type −1063CAT construct as a control. Transfected cells were incubated with serum-free medium alone or with 25 ng/ml PMA, and harvested after 24 h. Cellular extracts were assessed for CAT expression as described above.

RESULTS

Induction of PAI-2 mRNA and gene transcription by PMA

To investigate the possible modulation of PAI-2 by PMA, human HT1080 fibrosarcoma cells were incubated in either fresh medium alone or supplemented with PMA (25 ng/ml) for 24 h. PMA treatment resulted in an induction of mRNA expression (Fig.1, top panel) and gene transcription (Fig.1, bottom panel). Induction was selective as no changes in β-actin mRNA levels or β-actin gene transcription rate were observed in the presence of PMA over the same time period.

Transfection experiments with deletion mutants

To localize the promoter regions involved in basal and PMA-induced transcription, deletion mutants of the PAI-2 promoter (Fig.2) fused to the CAT reporter gene, were transiently expressed in HT1080 cells. Basal CAT activities were constitutively expressed by all deletion mutants containing 215 bp or more of promoter sequence (Fig.3), whereas constructs

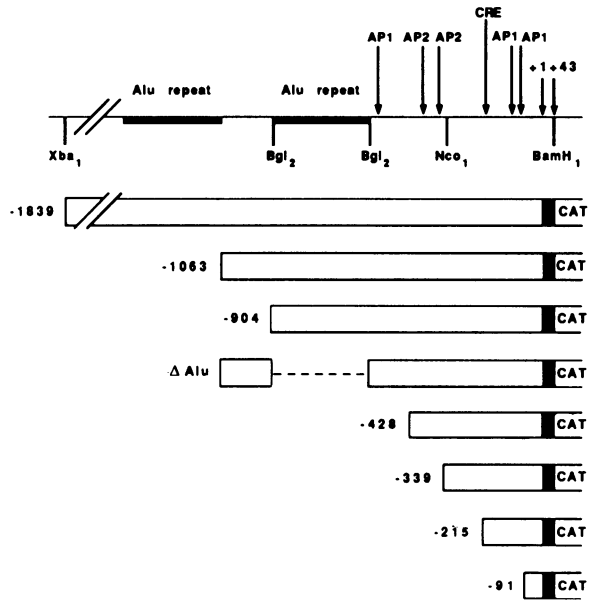

Figure 2. Schematic diagram of the PAI-2 promoter deleted regions fused to the CAT reporter gene. The upper line represents the restriction sites used for cloning, the positions of the putative protein-binding sites (AP1, AP2, CRE, or regulatory elements) and the two AluI inverted repeats. The first 43 bp of exon 1 (■), including the transcription initiation site (+1), are directly fused to the CAT reporter gene. Numbers to the left of each construct indicate promoter lengths in bp (□) upstream the transcription site. The ΔAlu construct was obtained by deletion (dashed line) of the proximal Alu repeated sequence from the −1063 construct.

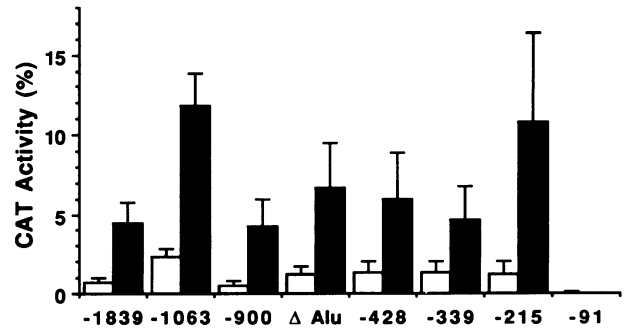

Figure 3. CAT activity of PAI-2 promoter deletion mutants. Deletion mutants (Fig.2) were transiently expressed in HT1080 fibrosarcoma cells, in presence (■) or absence (□) of 25 ng/ml PMA. CAT activities were expressed as percentage conversion of [^{14}C]-chloramphenicol into its acetylated forms. The bar diagram represents the mean values (± standard error) of 5 to 15 separate transfection experiments, each performed in duplicate. As a negative control, the promoterless pBLCAT3 construct was included in each experiment and its mean background value (~0.04%) was subtracted from the respective mean values of the deletion mutant transfections.

shortened to position −91 produced no detectable basal CAT activity.

The difference in CAT activity between the −215 and the −91 constructs was significant ($p < 0.05$; Wilcoxon signed-rank test).

Treatment for 24 h with PMA, produced a 3−10 fold increase in CAT activity from deletion mutants containing 215 bp or more of promoter sequence, whereas the construct shortened to −91 did not respond to phorbol ester treatment. These results indicate that essential elements for basal expression and phorbol ester-mediated induction lie between −215 and −91.

The PAI-2 promoter contains two Alu repeat sequences (Fig.2), mobile DNA elements that might influence gene expression (43, 44). As basal and PMA induced CAT activity produced by the ΔAluCAT construct was similar to that of the −1063CAT construct (one Alu repeat) and the −1839CAT construct (two Alu repeats), we may conclude that the Alu sequences in the PAI-2 promoter do not significally influence PAI-2 gene expression.

DNase I protection assay

To identify possible protein binding sites between positions −215 and −91, as well as potentially important cis-acting elements downstream of −91, a DNase I protection (footprint) assay was performed using the −259/+43 construct (Fig.4). Footprint analysis revealed several protected regions, most of them present on both DNA strands:

1) −37 to −30 and −24 to −20 (AAGCTGTATAAAACC-AGT) which surrounds the TATAA box (underlined);

2) −67 to −42 (GAGAAAAAAAAATGCCATGTGGG-AGG) contains part of an SP1-like site (underlined) (45) and a poly A sequence;

3) −80 to −72 (CAAAAGAC);

4) −110 to −91 (−94 on the other strand) containing an AP1-like element (−103 to −97: TGAATCA), further referred as AP1a. An other AP1-like element, AP1b, (−116 to −109: CTGAGTAA), is only partially covered (2 bases);

5) −129 to −141 (GTATTTCTATTCT);

6) −194 to −178 containing a CRE-like element (−189 to −182: TGACCTCA);

7) −217 to −207 (GATATTGACAA).

No significant differences in the pattern of protection were observed when DNase I protection was performed using extracts prepared from PMA-treated cells (data not shown).

Transfection of site-specific mutants

As footprint and sequence analysis confirmed the presence of three potential cis-acting elements,—two AP1-like sites (AP1a and AP1b), and one CRE-like site—, which are known to

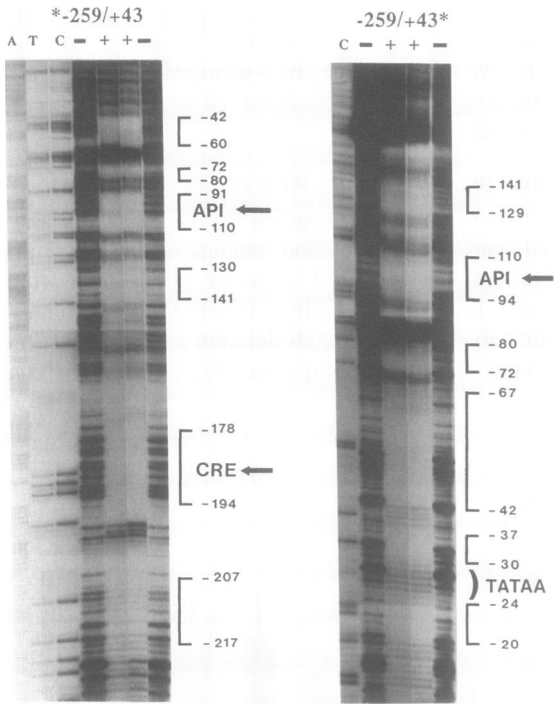

Figure 4. DNase I footprint analysis of the human PAI-2 promoter. Inserts of the −259pUC construct were labeled either at the *Hind* III site (−*259/+43, left panel) or at the *Bam*H I site (−259/+43*, right panel). The labeled DNA samples were incubated in presence (+) or absence (−) of HT1080 nuclear protein extracts, partially digested with DNase I and applied to a 6% denaturing gel, beside Maxam−Gilbert sequencing reactions (left panel: lanes A, T, C ; right panel: lane C). The autoradiography revealed at least seven different protected regions (boxed with numbers). The arrows indicate a −91 (−94) to −110 footprint, corresponding to the AP1-like sites (AP1a and part of AP1b), and a −194 to −178 region, containing the CRE-like element. The TATA box, itself partially protected, is surrounded by two protected regions (−37 to −30 and −24 to −20).

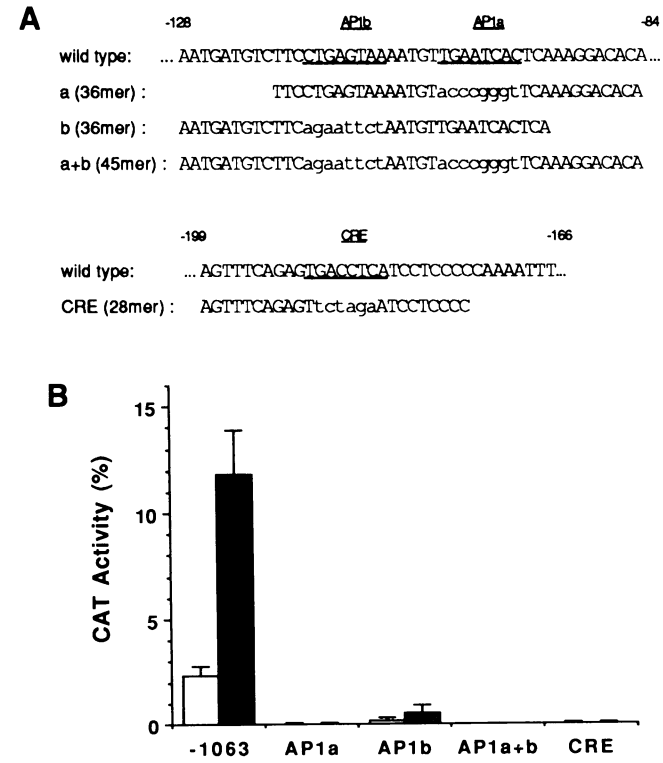

Figure 5. CAT activity of site-specific mutants in presence of PMA. **A.** Synthetic oligomers used for site-specific mutation. The oligomers a, b, a+b and CRE were used to prepare the mutant CAT constructs AP1a, AP1b, AP1 a+b, and CRE. The template (wild-type) promoter sequence is shown; the mutated sequences are underlined and given in lower case. **B.** CAT activity of the site-specific mutants in non-treated and PMA-treated HT1080 cells. The four mutants were transiently expressed in HT1080 cells in presence (■) or absence (□) of 25 ng/ml of PMA. CAT activities for each construct are represented as a mean (± standard error) of five to ten transfections performed in duplicate. Mean values from the promoterless $pBLCAT_3$ construct were subtracted from mean values of the corresponding experiments.

modulate gene expression in other systems, these sites were mutated using oligomers (Fig. 5, panel A). The site-specific mutants, derived from the wild type −1063 construct, were transiently expressed into HT1080 cells and their CAT activities compared to that of the wild type. The CRE, AP1a and AP1a+b mutants produced undectable basal CAT activities, whereas basal expression generated from the AP1b construct was reduced but still detectable (Fig. 5, panel B). After PMA stimulation, only the AP1b mutant, partially protected in the footprint analysis (Fig. 4), showed an increase in CAT activity.

DISCUSSION

We have studied the transcriptional regulation of the human PAI-2 gene in human HT1080 fibrosarcoma cells. Treatment of these cells for 24 hours with 25 ng/ml PMA caused an increase in PAI-2 mRNA. Results obtained from the nuclear 'run-on' transcription assay indicate that at least part of the increase in mRNA is due to an increase in PAI-2 gene transcription. This transcriptional response of PAI-2 to PMA prompted an analysis of the PAI-2 gene promoter. Transient expression of PAI-2 promoter deletion constructs in HT1080 cells indicated that constitutive- and PMA-inducible expression requires DNA element(s) between position −215 and −91. DNAse 1 protection analyses of a broader region indicated that at least 7 protein binding sites are present between position −259 and +43. Four protein binding sites were detected between position −215 and −91, three of which bear sequence homology with known cis-acting elements: one which differs from the CRE consensus sequence (TGACGTCA) at the central position (underlined) (TGACCTCA: position −189 to −182), and two which deviate from the AP1 consensus sequence (TGAC/GTCA) by single base substitutions (underlined) (AP1a: TGAATCA: position −103 to −97; and AP1b: TGAGTAA: position −115 to −109). Site directed mutagenesis of the CRE-like element or the AP-1a site resulted in a loss of basal and PMA-inducible expression. The CRE is a mediator of cAMP-dependent responses and acts as a binding site for the CRE binding poteins (CREB's) (46), whereas AP1 elements convey PMA responsiveness (47) and act as binding sites for a family of transcription factors which include c-*fos* and c-*jun*. Interestingly, the CRE-like element (TGACATCA) in the promoter of the c-*jun* proto-oncogene acts as a high affinity AP1-binding site (48). The same sequence is found in the human t-PA gene and is required for PMA-induced transcription (40). The CRE-like element (TGACCTCA) in the PAI-2 promoter differs from the CRE consensus sequence (TGACGTCA) at the same position as the CRE's in the c-*jun* and t-PA genes. These observations imply that the AP1a and CRE-like sites mediate, at least in part, the PMA response. However, at present we cannot exclude the possibility that other cryptic elements contribute to the phorbol ester response of the PAI-2 gene.

Mutagenesis of the AP1b element was less effective in that basal expression and PMA-inducible expression was only partially reduced. This is interesting in light of our observation that the protected region between −110 and −91, which completely covers the AP1a site, only partially protects (2bp) the AP1b site. Although mutation of the AP1b site led to a reduced constitutive activity, we cannot discount the possibility that this is due to the modification of important sequences flanking the AP1a element. Similarly, other elements downstream of position −215 could also contribute to this response. The protected regions flanking either side of the TATA box are likely to play some role. Further investigations are needed to determine a possible contribution of these particular sites and the other protected regions in the regulation of the PAI-2 promoter.

The PAI-2 promoter contains two Alu inverted repeated sequences between −1390 and −1100 (distal) and between −870 and −580 (proximal). A recent report demonstrated that some Alu sequences may contain a negative regulatory element (44). To investigate the presence of such an element, we compared promoter constructs containing either no Alu sequence (ΔAlu), one Alu sequence (−1063CAT), or two Alu repeats (−1839CAT). The similar CAT activities generated from each of these constructs suggest that the Alu sequences in the PAI-2 promoter do not contain such a repressor element.

In this study, we have performed a functional analysis of the PAI-2 promoter and have identified a CRE-like element and an AP1-like binding site which are essential for constitutive expression, and which may also be required for phorbol ester inducibility. The PAI-2 gene is also transcriptionally responsive to the inflammatory cytokine, tumor necrosis factor (TNF) in HT1080 cells (23). Experiments are being undertaken to determine the possible role of these elements during TNF-mediated expression.

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