RAPID COMMUNICATION

Regulation of the Plasminogen Activator Inhibitor Type-2 Gene in Monocytes: Localization of an Upstream Transcriptional Silencer

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Transcriptional regulation of the plasminogen activator inhibitor type-2 (PAI-2) gene appears to be an important factor in the response of mononuclear phagocytes to inflammation. We have investigated here the molecular basis for PAI-2 synthesis in monocytic cells by reporter gene deletion analysis. A DNA fragment containing 5.1 kb of 5' flanking region through to the start of the second exon was fused to a chloramphenicol acetyl transferase (CAT) reporter gene, transfected into macrophage and nonmacrophage cells and tested for PAI-2 promoter-directed CAT activity in the presence and absence of phorbol ester. Deletion analysis showed the existence of three major transcription regulatory regions. (1) A positive regulatory region contained in the proximal promoter mediates basal transcription and 12-phorbol 13-myristate acetate inducibility. (2) A negative regulatory region, or silencer, present between -1977 and -1675, was found to repress PAI-2 promoter activity in an orientation- and position-independent manner, but not in a cell-specific manner. (3) A second positive regulatory element, located upstream between approximately -5100 and -3300, appears to overcome inhibition mediated by the silencer in a cell-specific manner, suggesting a mechanism for the regulation of this gene. We have localized the motif responsible for silencer activity to a 28-bp DNA sequence containing a unique 12-bp palindrome centered at an Xba I restriction enzyme site, CTCTCTAGAGAG, which is designated the PAI-2-upstream silencer element-1 (PAUSE-1). This element binds a specific PAUSE-1 binding factor as determined by mobility shift analysis. We conclude that PAI-2 gene transcription is regulated by both positive and negative control mechanisms that may be important for the regulation of other genes as well.

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MONONUCLEAR phagocytes are key regulators of inflammatory and immune responses. A protein that has the potential to significantly influence the biological response of the monocyte in an inflammatory situation is plasminogen activator inhibitor type-2 (PAI-2). PAI-2 is a member of the family of serine proteinase inhibitors or serpins that are a large, structurally conserved family of proteins that regulate processes as diverse as blood coagulation, fibrinolysis, inflammation, cancer development, and neurology. The increasing number of pathologic conditions that result from acquired deficiencies and genetic abnormalities in members of this family highlight their importance to many cellular functions and biological systems.

The characteristics of PAI-2 suggest that it may be a key biological regulator of several macrophage functions. PAI-2 is a potent inhibitor of urokinase-type plasminogen activator (uPA), and, as such, plays an essential role in regulating uPA-initiated proteolysis that occurs during migration of macrophages to various targets such as inflammatory sites. However, the role of PAI-2 does not appear to be limited by its ability to inhibit plasminogen activation, because our work and that of others shows that PAI-2 expression is associated with the regulation of cell death. Expression of PAI-2 inhibits tumor necrosis factor α (TNF)-induced apoptosis and prevents programmed cell death of human macrophages infected with Mycobacterium avium. The data suggest that PAI-2 may inactivate one of the serine proteinases implicated in a cell death pathway.

PAI-2 synthesis is rapid and strong in response to inflammatory stimuli including interleukin-1β, TNF, lipo polysaccharide, and native and modified low-density lipoprotein in monocytes. TNF is the major cytokine effector for the induction of septic shock and for most inflammatory and cell-mediated immune reactions. The synthesis of PAI-2 by monocytes after endotoxin or TNF stimulation may inhibit plasminogen activation, thus facilitating the deposition of fibrin. Furthermore, the synthesis of PAI-2 may inhibit or delay macrophage cell death due to the cytolytic effects of TNF in an inflammatory environment. These activities are particularly relevant in sepsis and in advanced myelomonocytic leukaemias (M4 and M5) in which PAI-2 synthesis is markedly elevated.

In vivo, the PAI-2 gene is expressed primarily in activated monocytes and macrophages, in placental trophoblasts, and in differentiated keratinocytes, although a greater number of cell-types may be induced to synthesise PAI-2 in vitro. The dual functionality of PAI-2 and its relatively restricted patterns of expression suggest a high degree of specificity for PAI-2 synthesis and the involvement of critical regulatory pathways and factors for controlled expression of this gene. It is not known whether the general absence of PAI-2 gene expression in many cells and tissues is the result of active or passive mechanisms affecting gene transcription.

We have previously investigated the mechanisms involved in the regulation of PAI-2 gene expression in monocytes. Nuclear run-on transcription assays show that the synthesis of PAI-2 mRNA in both U937 and HL60 monocytic cells is controlled at the transcriptional level and is independent of ongoing protein synthesis. The 5' proximal flanking region of the PAI-2 gene contains DNA sequence elements that are clearly involved in PAI-2 gene transcription.
tion, including a TATA box (TATAAA) located 31 bp upstream of the transcription initiation site (tis), three AP-1 sites, two AP-2 sites, and a CAMP-responsive element (CRE) located between -544 and -97. A retinoic acid response element has been identified further upstream at -1659.

Southwestern blotting data implicate additional regulatory elements upstream and downstream of the proximal promoter region that may control cell-type-specific gene expression and in particular, its synthesis in monocytic cells. In the present report, reporter gene deletion analysis is used to identify functional elements in the PAI-2 gene responsible for the differential synthesis of PAI-2 in (1) the monocyte-like U937 cell that constitutively expresses low to negligible levels of PAI-2, but in which PAI-2 is induced in response to the differentiating agent 12-phorbol 13-myristate acetate (PMA); and (2) the HeLa cell, a cervical carcinoma that does not synthesise PAI-2 either constitutively or in response to inducing agents. The results show the involvement of a silencer and an antisilencer or transactivating element in the regulatory mechanism. Localization of the silencer shows a novel palindromic sequence motif important for repression of transcription, which we have designated the PAI-2 upstream silencer element-1 (PAUSE-1).

MATERIALS AND METHODS

Cell culture. The human macrophage-like U937 cell line, the monocyte-like RC2A leukemic cell line, the epithelial-like HeLa cell line, and the fibrosarcoma cell HT1080 were obtained from the American Type Culture Collection (Rockville, MD). The mature human macrophage cell line, MonoMac6, was kindly provided by Dr H.W.L. Zeigler-Hitbrock (University of Munich, Munich, Germany). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamate and incubated in 5% CO2 and 95% air atmosphere at 37°C. Cell viability was determined by trypan blue dye exclusion. All cultures were checked routinely and determined to be mycoplasma-free. PMA was added at 40 ng/mL.

Deletion mutagenesis of the PAI-2 promoter. Standard techniques were used for all recombinant DNA manipulations. Isolation of the PAI-2 gene containing 5.1 kb of the 5′ flanking region through to the start of the second exon (pBS8.8pro) is described elsewhere. An 8.8-kb EcoRI/XbaI DNA fragment spanning the region -5100 to -66 of the PAI-2 gene was inserted into the blunt-ended Sal I site of the promoter-less chloramphenical acetyltransferase (CAT) vector, pCATBasic (Promega, Madison, WI), to yield the longest reporter construct (8.8 kb), which was designated pCATS'5'. Subsequent 5′ deletions were generated by inserting the 7.2-kb HindIII fragment of pBS8.8pro into the Cla I site of pGEM-7Zf (Promega), followed by excision with EcoRI/HindIII into the blunt-ended Sal I site of pCATBasic. A series of deletions from the 5′ end were generated after digestion with Kpn I and Sma I and treatment with exonuclease III/mung bean nuclease for various times. The resulting mutant constructs were characterized by DNA sequence analysis. The numbering system used for the PAI-2 promoter sequence is based on transcription initiating at C in position -1, as reported and independently confirmed (T.M. Antalis, unpublished data).

Transient transfections and CAT assays. Cells were transfected by electroporation using conditions optimized for each cell line. Generally, cells (5 × 106) in log phase were electroporated at 250 mV, 960 mF using a Bio-Rad Gene Pulser with Capacitance Extender (Bio-Rad, Hercules, CA) in the presence of 20 μg of plasmid DNA, unless otherwise specified. All plasmid DNA preparations used for electroporation were CsCl gradient purified. Plasmid DNAs included a series of PAI-2 promoter-CAT mutant constructs, a promoter-less CAT negative control, and positive controls consisting of the β-actin promoter (4.3-kb EcoRI-Alu I DNA fragment) fused to the CAT reporter gene (pBSACAT) and pCAT-Control (Promega), a construct that contains the CAT reporter gene driven by the SV40 promoter and enhancer. Electroporated cells were diluted into 5 mL of RPMI 1640 and 10% fetal calf serum and allowed to incubate for 20 to 24 hours in the presence or absence of 40 ng/mL PMA. Cells were harvested and cell extracts were assayed for CAT activity as described. CAT activity was monitored by quantitation of signals from thin-layer chromatographs using a Molecular Dynamics PhosphorImager and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). CAT activity was standardized to the protein concentration of the cell pool as determined by Bio-Rad protein assay using bovine γ-globulin as the protein standard. To compare CAT conversion values between several independent experiments and between different cell lines, the CAT activity in each experiment was normalized relative to the CAT activity of an identical cell pool transfected with a positive control plasmid (β-actin CAT or pCAT-Control).

Construction of additional PAI-2 promoter mutants. The PAI-2 5′ flanking region (−1977 to −1675) containing the silencer element was amplified by polymerase chain reaction (PCR) using the following synthesized oligonucleotide primers: ForwardSup: GATTTGAGATACTTGGAACTAATCAAGATATCGAGTTCAGGATGTGGTATAG; and ReverseSup: GGTCGAAGATATCGAGTTCAGGATGTGGTATAG. Each primer contains an EcoRV restriction enzyme site (underlined) for ease of subsequent genetic manipulations. After amplification, the 350-bp product was digested with EcoRV and cloned directly into the EcoRV site of pBluescriptKS (Promega) generating the plasmid designated pBSK + 350 and the DNA sequence verified. The PAI-2 upstream insertion mutants were constructed by excising the 350-bp silencer fragment with EcoRV and cloning into the EcoRV site of pCATS'5' at position -1437 from the transcription initiation site in two orientations (pCATS'5' + 1.7 + 350B [forward]and pCATS'5' + 1.7 + 350A [reverse]). The PAI-2 deletion mutant, pCATS'5'-1.9D540, was constructed using a two-step cloning procedure involving digestion of pCATS'5'-1.9 with HindIII and EcoRV to release a 540-bp gene fragment (−1977 to −1437).

SV40CAT + 350d was constructed by direct cloning of the Sal I/BamHI fragment from pBSK + 350 into pCAT-Promoter (Promega) digested with Sal I/BamHI, resulting in the insertion of the 350-bp silencer downstream of the CAT reporter gene. SV40CAT + 350u was constructed by inserting the 350-bp silencer fragment into the blunt-ended Bgl II site upstream of the SV40 promoter in pCAT-Promoter (Promega). SV40CAT + 350u was constructed by inserting the 350-bp silencer fragment into the blunt-ended Bgl II site upstream of the SV40 promoter in SV40CAT + 350d.

The constructs containing mutations in the 12-bp Xba I palindromic sequence were constructed as follows: pCATS'5'-1.9XbaI, was generated by linearizing pCATS'5'-1.9 with Xba I, endfilling to destroy the restriction enzyme site, and religating the resultant blunt ends with T4 DNA ligase. pCATS'5' + 1.7 + 350CATTAG was generated using a PCR-based overlap extension strategy to mutate the central bases in the Xba I palindromic, TCTAGA, to the sequence CATTAG, as described below. The following synthesized 28-mer CATTAG oligonucleotides were used: CATTAG: GGTATAAGGGCTCCATTAGAGATTTCAG12; and CATTAGR: GCCAAAACCTCC AACCGAGGCCTTATGAC, ForwardSup and CATTAGR primers and XbaForward and CATTAGR primers, respectively, were combined to generate DNA subfragments containing overlapping mutations within the 350-bp silencer element at the Xba I site. These DNA fragments were then combined and ForwardSup and ReverseSup were used to generate a 350-bp DNA fragment containing......
the desired mutation. This product was digested with EcoRV and cloned into the EcoRV site of pCAT5'-1.7 and the DNA sequence was verified.

Constructs containing subfragments of the 350-bp silencer fragment (pCAT5'-1.7 + 165, pCAT5'-1.7 + 175, pCAT5'-1.7 + 85, pCAT5'-1.7 + 90, pCAT5'-1.7 + 111) were generated by digestion of pBK5 + 350 with EcoRV, XbaI, SpeI, and/or Sau3A as required. Endfilling and cloning the released subfragments into the EcoRV site of pCAT5'-1.7. Sequence and orientation were verified by DNA sequence analysis.

pCAT5'-1.7 + PAUSE-1 was generated by annealing synthesized single-stranded oligonucleotides containing the 12-bp XbaI palindromic region (PAUSE-1) in equimolar amounts, endfilling using the Klenow fragment of DNA polymerase, and cloning into the EcoRV site of pCAT5'-1.7. The oligonucleotides used were as follows; XbaMain: 'GCTATAGGGCCTCCTAGAGAGGCTTTAACC'; and XbaPri: 'GCTAAAACCTCTCTAGAG'.

RNase protection assay. U937 cells were transfected with plasmid DNA constructs described in the text and incubated in the presence of PMA (40 ng/mL) for 6 hours, at which time cells were lysed and RNA was isolated using the method of Chomczynski and Sacchi.29 Identical transfections run in parallel were assayed for CAT activity. After treatment with DNasel (RQI; Promega), 10 μg of RNA was resuspended in 10 μL of hybridization buffer (80% [vol/vol] formamide, 40 mmol/L PIPES, pH 6.4, 400 mmol/L NaCl, 1 mmol/L EDTA, and 10 mg (tRNA) containing the 32P-labeled probe. Hybridization was performed at 55°C for 16 hours, after which the samples were digested with RNase A and T1 at 37°C for 60 minutes. Reactions were treated with proteinase K and 0.5% sodium dodecyl sulfate at 37°C for 30 minutes, extracted with phenol/chloroform, and loaded on a 6% Tris-borate/EDTA (TBE) acrylamide/urea gel.

For the probe, a 650-bp PCR-amplified DNA fragment containing the CAT cDNA sequence was subcloned into the HindIII site of pBluescript KS. The antisense RNA probe (873 nt) was generated by linearization of the plasmid with KpnI and in vitro transcription using T7 polymerase in the presence of 32P-UTP. A protected fragment of 753 bp would be predicted to be generated after RNase digestion of mRNA from PAI-2 promoter expression constructs.

Gel mobility shift assay. Hela and U937 cell nuclear protein extracts were prepared as described.30 HeLa cell nuclear extract was also commercially obtained (Promega). Binding reactions were performed at room temperature for 20 minutes with approximately 5 × 105 cpm of radiolabeled DNA, 4 μg poly(dI-dC), and 3 μg of nuclear protein in binding buffer (10 mmol/L Tris-HCl, pH 8.0, 0.1 mmol/L EDTA, 10 mmol/L MgCl2, 50 mmol/L NaCl, 200 μg/mL bovine serum albumin, 2% glycerol, 1 mmol/L diithiothreitol). Double-stranded excised DNA fragments were radiolabeled by endfilling with the Klenow fragment of DNA polymerase in the presence of 32P-dCTP. Synthesized single-stranded oligonucleotides (XbaMain and XbaPri, as described above) containing the 12-bp XbaI palindromic region (PAUSE-1) were annealed in equimolar amounts and radiolabeled by endfilling using the Klenow fragment of DNA polymerase, and cloning into the EcoRV site of pCAT5'-1.7. The oligonucleotides used were as follows; XbaMain: 'GCTATAGGGCCTCCTAGAGAGGCTTTAACC'; and XbaPri: 'GCTAAAACCTCTCTAGAG'.

RESULTS

The 5'-flanking region of the PAI-2 gene contains cell-type specific regulatory elements. To analyze sequences that may be important for transcriptional regulation of PAI-2 gene expression, the 5'-flanking region of the PAI-2 gene was isolated and 5.1 kb of DNA 5' to the PAI-2 transcription initiation site through to the start of the second exon was ligated to the bacterial CAT reporter gene in the plasmid designated pCAT5'-5.1 (diagrammed in Fig 2A). The promoter activity of pCAT5'-5.1 was compared after transient transfection into a range of cells, the monocyte-like U937 and RC2A cells, the macrophage-like MonoMac6 (MM6) cell, the epithelial HeLa cell, and the HT1080 fibrosarcoma cell, as shown in Fig 1. To control for differences in transfection efficiency between cell lines, CAT activity was normalized to the activity of a reference plasmid transfected into an identical cell pool. Transfection of pCAT5'-5.1 into the monocytic cell lines (U937, MM6, and RC2A) and the fibrosarcoma HT1080 cells resulted in a low basal level of CAT activity. This basal activity was induced from 2.6-fold to 5.5-fold in response to PMA in U937, MM6, and HT1080 cells and greater than 10-fold in RC2A cells in response to PMA. Similarly, basal levels of PAI-2 promoter-directed CAT activity were detected in HeLa cells, but CAT activity was not increased in response to PMA. We have previously shown by Northern blot analysis that, although PAI-2 mRNA is not constitutively synthesized in substantial amounts by cells of the monocytic lineage, PAI-2 mRNA is
transiently induced in response to PMA in monocytic cells within 4 hours.\textsuperscript{32} A similar PAI-2 mRNA induction profile is observed in HT1080 cells in response to PMA, with maximal levels found at approximately 8 hours, and PAI-2 mRNA is not detected in HeLa cells either in the presence or absence of PMA (data not shown). Thus, the pattern of CAT reporter gene activity observed for the PAI-2 promoter-CAT constructs mirrors the pattern of steady-state mRNA expression in these cells. These data show that the 8.8-kb 5'-flanking region of the PAI-2 gene contained within pCAT-5'-5.1 is likely to contain regulatory elements responsible for the observed cell-specific expression and PMA inducibility of the PAI-2 gene.

Deletion analysis of 5.1-kb flanking sequence of the PAI-2 gene. To identify specific sequence elements that may be involved in regulating PAI-2 gene expression, a series of CAT constructs with serial deletions within the PAI-2 5'-flanking region were generated by unidirectional exonuclease III digestion. As shown in Fig 2A, the deletions range from -5.1 kb 5' to the transcription initiation site of the PAI-2 promoter to constructs that terminate within the first intron and are missing the transcription initiation site. Each construct was assayed for CAT activity in U937 cells in the presence and absence of PMA, and the data from these experiments are summarized in Fig 2B. The results showed that basal levels of CAT expression were obtained in untreated U937 cells throughout the range of PAL2 deletion constructs. After treatment with PMA, a stimulation of CAT activity was observed with the -0.9- to -5.1-kb constructs, indicating that PMA-responsive elements were present in all constructs and were thus likely to be located within the proximal region of the promoter 3' to the -907-bp end point of pCAT-5'-0.9. The promoter-less constructs pCAT-5' + 0.1 and pCAT-5' + 0.5 showed that sequences upstream of the transcription initiation site were required for induced CAT activity and that the CAT activity observed was unlikely due to possible cryptic promoter sequences contained within the first intron. The full-length pCAT-5'-5.1 construct showed
maximal PMA-inducible CAT activity. However, the constructs containing 5'-flanking sequences between approximately -1.7 and -3.3 showed marked repression of CAT activity, with PMA-inducible CAT activity again restored in pCAT5'--1.7 and in the subsequent shorter constructs, pCAT5'--1.3 and pCAT5'--0.9. These data suggest that a negative regulatory element exists upstream of pCAT5'--1.7 (position -1675) in the PAI-2 5'-flanking sequence.

Comparison of PAI-2 promoter activity in PAI-2--producing and --nonproducing cells. To assess the activity of the PAI-2 promoter in a different cell type that does not normally synthesise PAI-2, the deletion constructs were transiently transfected into HeLa cells and analyzed for CAT activity (Fig 2C). The overall pattern of CAT activity observed for the series of deletion constructs was similar to that observed in U937 cells, with two important exceptions. Firstly, in contrast to U937 cells, the CAT activity observed for each of the PAI-2 promoter deletion constructs was not significantly regulated by PMA in HeLa cells. The lack of PMA responsiveness in HeLa cells was consistent nonetheless with the absence of PAI-2 mRNA induction in these cells in response to PMA. Constitutive CAT activity in pCAT5'--1.7 and in constructs extending downstream of pCAT5'--1.7 was observed. However, constructs containing 5'-flanking sequences directly upstream of -1675 showed repression of CAT activity similar to that observed in U937 cells. Secondly, whereas pCAT constructs between and including -1.7 and -3.3 showed repression of CAT activity similar to that observed in U937 cells, the full-length pCAT5'--5.1 construct remained repressed in HeLa cells. This result suggests that the positive regulatory element present between approximately -3.3 and -5.1 shown in U937 cells is inactive in HeLa cells.

In summary, the deletion mutant data indicate that a negative regulatory element exists upstream of position -1675 (Δ−1977/−1675) that is functional both in the PAI-2--producing and --nonproducing cells examined. In the PAI-2--producing monocytic U937 cell, a positive cis-acting element upstream of approximately -3.3 kb selectively overcomes this inhibition. The negative regulatory element either is capable of overcoming or functions independently of transcriptional activation mediated through PMA, because it represses both constitutively- and PMA-induced reporter gene activities.

Addition of the negative regulatory element to a transcriptionally active deletion mutant suppresses transcription. To further characterize the negative regulatory element identified by deletion analysis, a 350-bp DNA fragment containing the region Δ−1977/−1675 was isolated by PCR amplification using flanking oligonucleotide primers and inserted into the transcriptionally active deletion mutant, pCAT5'--1.7, at the EcoRV site located at position -1437, as shown in Fig 3A. The resultant forward orientation (pCAT5'--1.7 + 350B) and reverse orientation (pCAT5'--1.7 + 350A) constructs were transiently transfected into both U937 and HeLa cells along with control constructs. As shown in Fig 3B, both of the insertion constructs showed reduced CAT activity (2.5- to 6-fold) in both cell types relative to the parental plasmid in the presence and absence of PMA. Thus, the negative regulatory element functions in a different position and in both orientations, properties characteristic of a transcriptional silencer.

Deletion of the negative regulatory element from a transcriptionally repressed deletion mutant restores PAI-2 promoter-directed CAT activity. A region extending from -1977 to -1437 containing the negative regulatory element was excised from the normally repressed deletion mutant pCAT5'--1.9, as diagrammed in Fig 3A. The resultant construct, pCAT5'--1.9Δ540, was transiently transfected into both U937 cells and HeLa cells along with control constructs. As shown in Fig 3B, the results demonstrated a substantial increase in CAT activity over the repressed parental plasmid, pCAT5'--1.9, but the CAT activities obtained with pCAT5'--1.7 were not attained. These data show that deletion of the silencer element is sufficient to restore PAI-2 promoter-directed gene activity, but sequences downstream of pCAT5'--1.7, specifically Δ−1675/−1437, may also contribute to the regulation of PAI-2 promoter activity.

RNAse protection analysis of PAI-2 promoter activity in PMA-stimulated U937 cells. To determine whether the repression of CAT activity was the direct result of reduced PAI-2 promoter-directed transcriptional activity, RNAse protection experiments were performed using RNA isolated from PMA-stimulated U937 cells transiently transfected with the constructs pCAT5'--1.9, pCAT5'--1.9Δ540, pCAT5'--1.7, and pCAT5'--1.7 + 350B. tRNA was included as a negative control. The 873-bp riboprobe used in these experiments was designed to span the CAT reporter gene sequence and include additional vector sequences that would not be predicted to be present in the mRNA transcripts generated by the PAI-2 promoter/CAT constructs. As shown in Fig 3C, one major protected product was detected corresponding to the predicted size of 753 bp, with no other specific reproducible bands identified. The intensities of the protected products were consistent with the CAT activities assayed in parallel transfections for the respective constructs (data not shown), with pCAT5'--1.7 showing the maximum levels of CAT mRNA transcription. These results show that the silencer directly represses CAT reporter gene transcription consistent with the decreases monitored in CAT reporter gene activity.

The effect of the negative regulatory region (Δ−1977/−1675) on a heterologous promoter. To determine whether the PAI-2 negative regulatory region can exert its effect on a heterologous promoter, a single copy of the 350-bp DNA fragment was inserted into the enhancer-less pCAT-Pro- moter (pSV40CAT) in two positions, a position upstream of the SV40 promoter (pSV40CAT + 350u) and a position downstream of the CAT reporter gene (pSV40CAT + 350d), as diagrammed in Fig 4A. An additional construct containing two copies of the 350-bp fragment, one in each position, was also generated (pSV40CAT + 350dd). All three constructs showed a marked (>80-fold) repression of SV40-directed CAT activity after transfection into PMA-stimulated U937 cells, as can be seen by the 35S-chloramphenicol acetylation pattern shown in Fig 4B. Transfection of these constructs into HeLa cells and assay of CAT activity also showed strong repression of SV40-directed CAT activity (>10-fold), and
Fig 3. The PAI-2 silencer functions in a position-independent manner and in both orientations. (A) Diagram of PAI-2 silencer insertion and deletion mutant constructs. pCAT5'-1.7 + 350A and pCAT5'-1.7 + 350B are mutant constructs derived from insertion of the 350-bp silencer region into pCAT5'-1.7 at a unique EcoRV site in two orientations. pCAT5'-1.9d540 denotes pCAT5'-1.9, wherein a 540-bp fragment containing the silencer region has been deleted. (B) CAT reporter gene analysis of insertion and deletion constructs in HeLa cells in the absence (O) and presence (•) of PMA and in U937 cells in the absence (■) and presence (□) of PMA. Each value represents the mean and standard error from at least five independent experiments. (C) RNase protection analysis of RNA isolated from cells transfected with the indicated insertion and deletion constructs. Each lane contained 10 μg of total RNA, annealed simultaneously with the 873 nt CAT riboprobe. The 753 nt protected fragment is as indicated. Lane 1, CAT probe alone; lane 2, pCAT5'-1.9; lane 3, pCAT5'-1.7d540; lane 4, pCAT5'-1.7; lane 5, pCAT5'-1.7 + 350; lane 6, mRNA negative control. The intensities of the signals were quantitated by densitometry and the fold-conversion from 873 nt to 753 nt for each construct is given below each lane.

these results are summarized in Fig 4C. Thus, the PAI-2 negative regulatory element can repress the basal activity of a strong heterologous constitutive promoter and functions as a silencer in a position-independent manner.

The PAI-2 silencer region (Δ−1977 to −1675) contains potential transcription factor binding motifs. DNA sequence analysis of the termination positions of deletion mutants pCAT5'-1.9 and pCAT5'-1.7 showed a 303-bp sequence that is shown in Fig 5. Comparative analyses of this sequence with the Genbank transcription factor database showed no specific sequence identity with known silencer elements. However, the sequence was found to contain motifs corresponding to potential transcription factor binding sites, including an AP1-like element at position −1894, two AT-rich regions at positions −1805 and −1738, a motif homologous to the SV4O core enhancer at −1701, and a glucocorticoid receptor binding site at position −1701. In addition, a 12-bp palindromic sequence centered around the Xba I restriction enzyme site at position −1838 was identified.

Analysis of subfragments of the 350-bp region for silencer activity. As a first step towards identifying functional sequence motifs within the PAI-2 silencer and to determine whether the entire 350-bp region is required for silencer activity or whether subfragments spanning parts of this region may be sufficient for transcriptional repression, the several partial subfragments spanning the silencer region were individually introduced into the EcoRV site of the transcriptionally active pCAT5'-1.7 construct, generating the constructs pCAT5'-1.7 + 165, pCAT5'-1.7 + 175, pCAT5'-1.7 + 85, pCAT5'-1.7 + 90, and pCAT5'-1.7 + 111. The positions of the subfragments contained in each construct with respect to the 350-bp region are given in Fig 6A. The constructs were transiently transfected into both U937 and HeLa cells along with control constructs and the extracts tested for CAT activity relative to pCAT5'-1.7. None of the constructs containing subfragments inhibited transcription (Fig 6B). These results suggest that (1) regions spanning at least two subfragments are required for repression and/or (2) a region...
between two subfragments (disrupted by restriction enzyme digestion) may be required for silencing activity.

Identification of the PAI-2 upstream silencer element (PAUSE-1). A candidate intersubfragment region for silencer activity was present between the two largest fragments, 165 bp and 175 bp, because the smaller subfragments are contained within these two fragments. This region contains an Xba I restriction enzyme site within a 12-bp palindromic sequence that could potentially provide a binding site for a trans-acting transcription factor. To investigate whether this motif may be involved in silencing activity, two reporter plasmids were constructed containing different mutations within the 12-bp palindrome. The specific nucleotide changes are shown in Fig 5. pCATSV40+350d contained an insertion of two nucleotides interrupting the proximal AG palindromic sequence. In pCATSV40+350u, the 6 nucleotides comprising the Xba I site in pCATSV40+350d were scrambled, giving the sequence CATTAG and abolishing the central core of the palindromic sequence. As shown in Fig 6B, transfection of these constructs into both HeLa and U937 cells showed enhanced CAT activity relative to the CAT activity obtained with the repressed wild-type 350-bp fragment. These results suggest that PAI-2 silencer activity may involve the 12-bp palindromic motif centered at the Xba I site.

To determine directly whether the 12-bp palindromic motif was responsible for the silencer activity, a 28-bp fragment containing the 12-bp palindromic sequence was inserted into the EcoRV site in pCATS5'-1.7 at position -1437, resulting in pCATS5'-1.7 + PAUSE-1. The PAUSE-1 sequence is shown in Fig 7. pCATS5'-1.7 + PAUSE-1 was transiently transfected into both U937 and HeLa cells along with control constructs. As shown in Fig 6C, the presence of the inserted 28-bp DNA fragment represses PAI-2 promoter-directed CAT activity to a similar extent as pCATS5'-1.7 + 350B. These results show that the 28-bp motif mediates transcriptional silencing of PAI-2 promoter activity, and we have designated this element the PAI-2 upstream silencer element-1, or PAUSE-1.
PAUSE-1 is a target for a DNA binding factor. Because the functional reporter gene experiments show that the PAUSE-1 sequence is involved in PAI-2 silencing activity, we investigated whether this sequence could bind nuclear DNA binding factors. Gel mobility shift assays using a synthetic double-stranded oligonucleotide containing PAUSE-1 showed one shifted complex, as shown in Fig 7, indicating the presence of a DNA binding factor present in HeLa cell nuclear extracts. The specificity of this interaction was confirmed by the addition of 1,000-fold excess cold PAUSE-1, which effectively competed with the radiolabeled PAUSE-1 for binding, as shown in Fig 7, indicating that the central 6 bases of the palindrome are likely to be important for factor binding. Two double-stranded oligonucleotides, one containing the Xba* mutation and the other containing a disruption of the proximal AG dinucleotide repeat, also did not compete with PAUSE-1 for binding, indicating that an intact palindrome, particularly the purine dinucleotide motif, is important for factor binding. These results show that PAUSE-1 is a target for a trans-acting factor that is likely to be involved in repression of PAI-2 promoter activity.

DISCUSSION

Silencing of gene expression plays an important role in the regulation of a number of eukaryotic genes. In this study, we investigated 8.8 kb of PAI-2 5' flanking sequence upstream of the first translated exon for functional regulatory elements important in the control of PAI-2 gene expression. This 8.8-kb sequence was sufficient to drive high expression of the CAT reporter gene in PAI-2-expressing, PMA-induced U937 monocyteic cells and did not show appreciable activity in PAI-2–nonexpressing HeLa cells, suggesting that it contains the essential determinants for the observed restricted expression of PAI-2. Functional deletion studies showed the existence of a transcriptional silencer and two positive regulatory regions involved in the transcriptional regulation of the PAI-2 gene.

The first positive regulatory region was identified downstream of position −907 in the proximal promoter and contains elements important for PMA responsiveness of the PAI-2 promoter in U937 cells. Spanning the region −894 to −585 is an Alu repeat sequence. Although Alu repeat sequences may influence gene expression, the removal of this Alu repeat sequence has been shown in other cells to have no significant effect on the regulation of PAI-2 promoter activity. The primary determinants important for responsiveness to PMA in HT1080 fibrosarcoma cells lie downstream of coordinate −585 and our data suggest that this is likely to be the case also for monocytic U937 cells. Specific elements likely to be involved are the CRE element at position −189 and an API-like site at position −103 that have been shown in HT1080 fibrosarcoma cells to be important for both basal and PMA-induced PAI-2 gene expression. However, in contrast, our data suggest that, in HeLa cells, these elements are controlled differently, because constructs containing sequences downstream of −1675 are not regulated by PMA but are constitutively active (see Fig 2).

Upstream of position −894 in the PAI-2 5' flanking sequence exists a second Alu repeat sequence between −1387 and −1076 that does not appear to influence PAI-2 promoter activity (Fig 2). However, further upstream, the region between position −1977 and −1675 defines a negative regulatory element that represses PAI-2 promoter activity in a
Fig 6. The PAUSE-1 site mediates repression of PAI-2 promoter activity. (A) Diagram showing the position and size of each of the subfragments generated within the silencer region. (B) CAT reporter gene analysis of subfragments and mutants of the 350-bp silencer region in HeLa cells and in U937 cells in the absence and presence of PMA. The mutations contained in pCAT5' + 350CATATTAG and pCAT5' + 350Xba* are shown in Fig 3. (C) CAT reporter gene analysis of pCAT5'-1.7 + PAUSE-1, wherein a 28-bp sequence is inserted in the EcoRV site of pCAT5'-1.7. In both (B) and (C), CAT activity is expressed as the percentage of CAT conversion normalized to the protein concentration of each sample. Controls include identical cell pools transfected with the promoter-less pCATBasic and pSV40CAT. Error bars represent the standard error derived from three to five separate experiments.

cell-type-independent manner. Insertion of a DNA fragment containing Δ-1977/-1675 at an upstream position in a transcriptionally active PAI-2 promoter deletion mutant was shown to repress CAT activity in an orientation-independent manner; conversely, deletion of a 540-bp region containing the silencer restored CAT activity.

The PAI-2 negative regulatory region showed strong repression of the SV40 promoter in both U937 and HeLa cells. The repression was observed when the 350-bp region was placed in both upstream and downstream positions relative to the SV40 promoter. The level of inhibition observed with silencers in strong heterologous promoters can be dependent on the number of copies present, but we observe strong repression with a single copy of the 350-bp sequence. This region behaves as a silencer, an autonomous cis-acting negative regulatory element whose action is promoter, position, and orientation independent.

PAI-2 silencer activity is associated with a 303-bp region spanning positions Δ-1977/-1675 as defined by deletion studies. This region was found to contain binding sites for several DNA binding proteins as determined by mobility shift analyses (data not shown). However, one motif was shown to confer silencer activity when inserted into a transcriptionally active mutant. This motif contains a perfect 12-bp palindrome centered around an Xba I site at position -1832, termed PAUSE-1. The three repeated pyrimidine dinucleotide (CT) and the three repeated purine dinucleotide (AG) motifs within the CTCTCTAGAGAG palindrome are striking. The first half of the PAI-2 silencer contains a region that shows 90% homology to the consensus silencer sequence 5'-ANCCTCTCC/T-3' identified in cis-acting negative regulatory elements of other genes, whose members include human collagen type II, chicken lysozyme, rat osteocalcin, human β-interferon, and polyoma virus. Sequence comparison of the PAI-2 silencer with some of these negative regulatory elements is shown in Table 1. Scram-
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Table 1. Sequence Similarity Between the PAL2 Silencer Motif and Silencer Sequences Associated With Other Genes

| PAI-2 (-1840) | CCACAGTGAGGCTCTCTGAGAGTTTCTAGCA |
| Lysozyme (-1090) | AGCACCTACACCTCTCTCTAGAGTTTTAGC |
| Growth hormone (-366) | AGGGGCAAGGCTCTCTAGAGGTATTTT |
| Polyoma virus (5225) | AGGAAGCAAAAAGCCTCTCCACCCCTT |
| β-Interferon (-46) | TGAGATGGTCCTCTCTCTATTCAGAGGAATTTCCC |
| Insulin (-112) | GGCTCACCACCCCTCTGAGACATGGTCCC |
| Insulin (-271) | GAGGGCCTTTACCTCTCTCTAGAGGAGTACAGG |
| Factor IX (-1680) | TATTTCTCCACATCCTCTCTCTCCACAGCTCCTGGCTTCTCATGAG |

DNA sequences identical to the PAL2 pyrimidine dinucleotide repeat homologous to the consensus silencer identified by Baniahmad et al. are shown in boldface. Purine motifs and T repeats are underlined. The numbers in parentheses denote nucleotide positions.

It is of note that this region contains DNA-protein binding sites previously targeted by Southwestern blot mapping. Silencers identified in eukaryotic genes are proposed to be involved in silencing gene expression in nonexpressing tissues. The results presented here provide the first demonstration that PAI-2 gene transcription is repressed in nonexpressing cells. They further provide the basis for a mechanism, which is diagrammed in Fig 8, that may explain the observed regulation of PAI-2 gene expression. Under conditions in which transcription is repressed, trans-acting factors constitutively bound to the silencer may interact with positive factors associated with the proximal promoter to repress gene transcription and PMA responsiveness in both PAI-2-producing and -nonproducing cells. However, under conditions in which transcription occurs, as in the case of PAI-2-producing cells, the binding of positive trans-acting factors to the upstream antisilencer may overcome the inhibitory

Fig 8. Hypothetical model and locations of the major functional elements in the 5' flanking region of the PAI-2 gene. The numbers indicate the first nucleotide positions of the element sequences and the Δ indicates the minimum defined sequence determined to date containing the element. The proximal promoter region of the PAI-2 gene (-585/-1) allows for on/off switching of the gene in response to external inducing agents such as PMA in U937 cells or allows constitutive expression, as in the case of HeLa cells. In cells in which the PAI-2 gene is maintained in a repressed state, the upstream silencer is active. In the case of PAI-2-expressing cells, there may be competition between an active upstream antisilencer and silencer binding factors to allow de-repression of transcriptional activity. In the case of PAI-2-nonproducing cells, the upstream antisilencer is inactive or absent, thereby maintaining the PAI-2 gene in a repressed state.
effect of the negative factors bound to the silencer. This regulation could occur through several mechanisms, but may involve interactions between trans-acting factors associated with either the silencer or the proximal promoter region and the antisilencer element. In PAI-2−nonproducing cells, these trans-acting factors would either not be present or be inactive, thus maintaining a repressed state. Thus, the PAI-2 silencer may be part of an efficient repression mechanism operative to restrict PAI-2 expression to specific cells. Although the proposed scheme fits with the available data, an assessment of these interactions will ultimately require the identification and characterization of the specific DNA binding factors that interact with these sequences.

This study clearly establishes that both positive and negative regulatory elements contribute to the control of PAI-2 gene transcription in monocytes. The identification of a transcriptional silencer in the PAI-2 gene may have important implications for inflammatory processes in which PAI-2—and uPA-mediated proteolysis play important roles. Future work will need to define other elements necessary for regulated gene expression and to characterize the cooperation between the PAUSE-1 silencer and upstream elements involved in the control of transcription in monocytes. Definition of this regulatory mechanism may aid in the management of inflammatory diseases with modification of PAI-2 transcription through pharmacologic intervention and may also allow investigation of possible abnormal PAI-2 gene regulation in disorders involving monocytes, such as myelomonocytic leukemias.

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