The regulatory region of the human plasminogen activator inhibitor type-1 (PAI-1) gene

Andrea Riccio^{1,2*}, Leif Røge Lund³, Renata Sartorio¹⁺, Arturo Lania¹, Peter A. Andreasen^{3,4}, Keld Danø³ and Francesco Blasi^{1,2}

¹International Institute of Genetics and Biophysics, CNR, via Marconi, 10, 80125 Napoli, Italy,

²Institute of Microbiology, University of Copenhagen, Øster Farimagsgade 2A, 1353 Copenhagen K,

³Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, 2100 Copenhagen Ø and ⁴Institute of Biochemistry C, University of Copenhagen, Blegdamsvej 3C, 2200 Copenhagen, Denmark

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ABSTRACT

The human gene for plasminogen activator inhibitor type-1 (PAI-1) has been isolated and its promoter region characterized. PAI-1 regulation by glucocorticoids, transforming growth factor- β (TGF- β) and the phorbol ester PMA is shown to be exerted at the promoter level. A fragment spanning 805 nucleotides of the 5' flanking and 72 of the 5' untranslated region contain information enough to promote transcription and to respond to glucocorticoids when fused to a reporter gene and transfected into human fibrosarcoma cells. A moderately repetitive DNA sequence, containing a TATA box, a GRE consensus, a Z-DNA forming sequence and two imperfect direct repeats at the extremities, is present a few nucleotides 5' of the human PAI-1 gene transcription start site, raising the possibility that this gene could have been activated by DNA insertion during evolution.

INTRODUCTION

Plasminogen activators are serine proteases which convert plasminogen to plasmin by limited proteolysis. Plasmin, in turn, is a broad spectrum protease, which is capable of degrading fibrin as well as several extracellular matrix proteins. The plasminogen activators control this proteolytic cascade and are believed to play a central role in a variety of biological processes that require extracellular proteolysis, including thrombolysis, cell migration, tissue destruction and remodelling as well as neoplastic and non-neoplastic invasiveness (1,2). Two types of plasminogen activators are known in mammals: the urokinase-type (u-PA) and the tissue-type (t-PA), coded by different genes (3-5).

Plasminogen activator activity can be regulated at the level of biosynthesis, secretion, binding to cell surface receptors, and modulation of the enzymatic activity by specific and fast-acting inhibitors (for reviews, see refs. 1, 2, and 6).

Two types of PA-inhibitors, PAI-1 and PAI-2, have been identified in a variety of cell lines, tissues and biological fluids (see ref. 7 for a review). Both are glycoproteins of approximately M_r 50,000, are members of

the SERPIN (serine protease inhibitor) family, and their structures have been recently elucidated by cDNA cloning (8-14). PAI-1 is an arg-serpin, having an arginine residue at the reactive center (11). The human PAI-1 gene has been localized on the long arm of chromosome 7 (10, 15) and its intron-exon distribution recently determined (16).

PAI-1 production is influenced by a number of hormones and growth factors (7, 17-28). We have recently reported that transforming growth factor- β (TGF- β) and glucocorticoids cause an enhancement of PAI-1 activity and synthesis in human WI-38 lung fibroblasts and HT-1080 fibrosarcoma cells, and increase cellular PAI-1 mRNA levels (29, 30). To further study these mechanisms, we have now cloned the human PAI-1 gene, identified its promoter region, and found that TGF- β , glucocorticoids and the phorbol ester phorbol myristate acetate (PMA) enhance PAI-1 transcription rate acting at a unique transcription initiation site. We also report that a fragment spanning 805 nucleotides of the 5' flanking and 72 of the 5' untranslated region of the PAI-1 gene fused to the coding sequence of the E.coli CAT gene has promoter activity and responds to glucocorticoids when transfected into HT-1080 cells. Further analysis of the promoter region has shown the presence of a GRE consensus and a Z-DNA forming unit in a sequence which contains a low level homology to the Alu sequence, and is moderately repeated in the human genome. Therefore, the human PAI-1 gene may have acquired regulatory sequences by insertion during evolution.

MATERIALS AND METHODS

Materials

Deoxy- and dideoxytriphosphates were purchased from P.L. Biochemicals. ³²P-compounds were from Amersham Co. (UK). All restriction enzymes, T4 DNA ligase and S1 Nuclease were from Boehringer Mannheim GmbH (West Germany). Porcine transforming growth factor- β type 1 was from R & D Systems (Minneapolis, MN, USA). Dexamethasone and phorbol 12-myristate 13-acetate were from Sigma (St. Louis, MO, USA). All other reagents were of the highest purity grade.

Bacterial strains, plasmids and phage vectors

Escherichia coli K12, strain HB 101, was used for transformation. pEMBL8 (31) was used as plasmid vector for subcloning. pEMBL8cat (32), which contains the promoterless CAT gene, carried by the HindIII-BamHI fragment of pSV2cat (33), was used for the CAT-constructions. The human genomic library,

a gift of Dr. D. Toniolo, was an EcoRI partial digestion of white blood cell DNA cloned in phage EMBL4 (34).

Genomic library screening and nucleotide sequencing

The screening of the human genomic library and the Southern blot analysis were performed according to Maniatis et al. (35). Sequence analysis was carried out either by the chemical degradation method (36) or by a modification of the Sanger dideoxy technique on double-stranded DNA (37).

Cell cultures

Serum-free cultures of the human fibrosarcoma cell line HT-1080 and the embryonal human lung fibroblast cell line WI-38 were incubated for the indicated times, with or without the addition of 5 ng/ml TGF- β or 10^{-6} M dexamethasone, as previously described (29,30). The human monocyte cell line U937 (38) was cultured in suspension in RPMI 1640 with 10% FCS to a density of 5×10^5 cells/ml. At that time the phorbol ester PMA in DMSO solution at a final concentration of 100 ng/ml, or the same volume of DMSO, was added to the cells. The cells which were adherent to the culture plate 24 hours after PMA addition (usually 90%) were used for the experiment.

S1 nuclease mapping

S1 mapping was performed as already described (5). The probe for the localization of the transcription start site of PAI-1 gene was a double-stranded HindIII-EcoRI fragment, 877 bp long, EcoRI 5'end labeled, obtained from phage lambda PAI-1-9 (see Fig. 1). A 600 bp Fok I human β -actin probe, obtained from pHFBA-3'UT (39) was 5'end labeled and used as control (see Fig. 3). To define the transcription start site used by the PAI-1-CAT fusion genes in the transfected cells, a KpnI-EcoRI fragment, about 2 Kbp long, obtained from pLIUS2cat (see below) was 5'end labeled and used as a probe (see Fig. 5).

Construction of CAT-derivative vectors with PAI-1 inserts

pLIUS1cat was obtained by filling in a 877 bp HindIII-EcoRI fragment of the phage lambda PAI-1-9, comprising the transcription start site and 72 bp of the 5'-untranslated sequence of the PAI-1 gene, and cloning it in the SmaI site of pEMBL8cat. The same strategy was followed for cloning an about 9 Kbp EcoRI fragment of lambda PAI-1-9 in pEMBL8cat. The plasmid obtained was named pLIUS2cat.

DNA transfections and CAT assays

DNA-mediated transfection was carried out by the calcium phosphate precipitation technique (40). HT-1080 cells were cultured in DMEM with 10% FCS, steroid-depleted by treatment with 50 mg/ml activated charcoal for 4

hours at 4°C. One day prior to transfection, cells were plated on 10 cm dishes at a density of 5×10^5 cells/plate and the medium was changed 4 h before transfection. Each DNA- CaPO_4 precipitate, obtained with 5 pmoles of supercoiled plasmid DNA, was divided into two aliquots and added to two plates. 18 h later, the cells were washed, shocked for two minutes with 2 ml 10% DMSO, and refed with fresh medium, one plate with and one plate without the addition of 10^{-6} M dexamethasone. 48 hours after the addition of the DNA- CaPO_4 precipitate, the cells were harvested and the CAT activity was determined. CAT assays were performed as described by Gorman et al. (41), except that 1.25 mM acetyl coenzyme A and 0.2 μCi of [^{14}C]chloramphenicol were used per assay. The reaction was run for 30 min. To quantitate the conversion of labelled chloramphenicol to the acetylated derivatives, the radioactive spots were cut from the thin layer plates and counted in a liquid scintillation counter. A linear relationship between chloramphenicol conversion and amounts of extract was obtained up to 20% of substrate conversion. The protein concentration of the cell extract was determined by the Folin-Ciocalteu method (42).

RNA preparation

Total cellular RNA was isolated by the guanidinium isothiocyanate/CsCl method (35). RNA from transfected cells was prepared according to Gorman et al. (41).

Nuclear transcription assay

Preparation of nuclei, RNA elongation and isolation were performed as described by Greenberg and Ziff (43), except that the nuclei were frozen and stored at -80°C between preparation and analysis, and that the ^{32}P -labelled RNA was treated with 10 $\mu\text{g/ml}$ DNase (RNase free) (Worthington B.C., USA) for 30 minutes at 37°C . 5×10^7 nuclei were used for each assay. About 1×10^7 cpm were used for hybridization. The hybridization conditions were: 50% formamide, 5 x SSC, 5 x Denhardt's solution, 50 mM phosphate buffer, pH 7.0, 0.1% SDS, 100 $\mu\text{g/ml}$ yeast RNA and 10 $\mu\text{g/ml}$ pEMBL8 DNA, 37°C for three days. The following DNA probes were used: pA1, a plasmid carrying the human PAI-1 cDNA (11); pAT-PvuII, carrying the human fibronectin cDNA (kindly provided by Dr. G. Sebastio); pHF β A-3'UT, coding for the 3' untranslated region of human β -actin (39); a plasmid carrying a 540 bp SacI-KpnI fragment encompassing exon II of mouse β_2 -microglobulin gene (44); pUC18 vector (45).

Oligodeoxynucleotides synthesis

Oligodeoxynucleotides, used as hybridization probes or sequencing primers, were synthesized with the Applied Biosystem 381A DNA synthesizer.

The following oligonucleotide probes were used in the study. Probe A (see Fig. 1) is a 21mer (5'-TGGGTCTTCTTGACAGCGCTC-3') derived from nucleotides 27-47 of PAI-1 cDNA (11). Probe C is a 28mer (5'-GGGTGCTGGAGTGGGGAAGGGAGTCTT-3') derived from nucleotides 538-565 of PAI-1 cDNA (8). Probe D is a 39mer already described (11).

ELISA

Concentrations of PAI-1 in conditioned culture fluids were measured by an ELISA with a combination of monoclonal and polyclonal antibodies, as previously described (29), except that the monoclonal anti-PAI-1 IgG were from hybridoma clone 2 (46).

Identification of a repeated sequence in the promoter region of the human PAI-1 gene

The presence of DNA sequences repeated in the human genome was investigated by Southern blot analysis. A 877 bp Hind III-EcoRI fragment, containing 805 bp of 5' flanking and 72 bp of 5' untranslated sequences of the PAI-1 gene (see Fig. 1 and 2), was purified from plasmid pLIUS1cat (see above), digested with different restriction enzymes, electrophoresed, blotted onto nylon membranes and hybridized to ³²P-labeled human placental DNA. The hybridization was carried out at 65°C in 750 mM NaCl, 75 mM NaCitrate (5 x SSC), 0.5% Ficoll, 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, 10 µg of herring sperm DNA per ml, 0.1% sodium dodecyl sulphate (SDS). Filters were washed twice for 30 min at room temperature in 2 x SSC, 0.1% SDS, twice for 30 min at 50°C in 2 x SSC, 0.1% SDS, and twice for 30 min at 50°C in 0.1% SSC, 0.1% SDS.

RESULTS

Identification of a lambda clone carrying the human PAI-1 gene

A human DNA genomic library, constructed as an EcoRI partial digest in the lambda vector EMBL4 (34), was screened for the PAI-1 gene, using as a probe the 2100 bp EcoRI-BamHI restriction fragment derived from the previously isolated and sequenced PAI-1 cDNA clone A1 (11).

Two positive clones, lambda-PAI-1-8 and lambda-PAI-1-9, each containing inserts of about 14 kbp, were characterized by restriction analysis and Southern blots, using several cDNA fragments and synthetic oligonucleotides as probes (Fig. 1) and shown to overlap by about 1.5 Kb. Hybridization of PAI-1 cDNA probes to human placenta DNA gives the same restriction map as the positive lambda clones, showing that no rearrangement of genetic material has been created during the preparation of the library (not shown). In a

Figure 1. Restriction map of the lambda-PAI-1 clones. The inserts of the recombinant clones lambda-PAI-1-8 and lambda-PAI-1-9 with the position of the relevant restriction sites are indicated in the upper two lines of the figure. The PAI-1 cDNA clone A5 (11) and the derived hybridization probes (A-E) are in the lower two lines. A, C and D probes are oligodeoxy-nucleotides whose sequence was derived from either PAI-1 cDNA (8, 11) or PAI-1 amino acid sequences (8). Probes B and E are restriction fragments, obtained from PAI-1 cDNA clone A5. The horizontal lines A'-E' in the middle line of the figure indicate the genomic restriction fragments hybridizing to the different cDNA probes A-E. The different restriction sites are indicated as follows: Ba, BamHI; Bg, BglII; EI, EcoRI; HIII, HindIII; Kp, KpnI; Sa, SalI; Sc, SacI.

previous study (11), we have reported the existence of two PAI-1 mRNAs of 2.4 and 3.4 kbp, respectively, containing the same coding sequence and differing exclusively in the length of the 3' untranslated sequence. The analysis of the hybridization of the lambda-PAI-1 clone restriction fragments (Fig. 1, A'-E') to the different PAI-1 cDNA probes (Fig. 1, A-E) suggested that the longer PAI-1 cDNA clone, pA5 (11), is coded by about 12 Kbp of genomic DNA, entirely contained in the isolated recombinant lambda clones. The nucleotide sequence of about 1 Kbp at the 5' end (Fig. 2) and of about 2 Kbp at the 3' end (not shown) of the hybridizing genomic region confirmed this result.

Loskutoff et al. recently reported that the 3' untranslated region of the PAI-1 gene is not interrupted by introns (16). Our sequence data confirm this finding, indicating that the PAI-1 mRNA heterogeneity depends on different choice of two polyadenylation sites and not on alternative splicing.

Identification of the transcription start site and analysis of the promoter region

The region of the PAI-1 gene coding for the 5' end of the transcription unit was identified in the lambda PAI-1-9 DNA by Southern hybridization using a synthetic 21-mer complementary to nucleotides 27-47 of PAI-1 cDNA clone A1 (Fig. 1). The nucleotide sequence of about 800 bp of the genomic

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      -800              -780              -760
AAGCTTTTACCATGGTAACCCCTGGTCCCGTTCAGCCACCACCACCCACCCAGCACACC
HindIII              -720              -700
TCCAACCTCAGCCAGACAAGGTTGTTGACACAAGAGAGCCCTCAGGGGCACAGAGAGAGT
      -680              -660              -640
CTGGACACGTGGGGAGTCAGCCGTGTATCATCGGAGGCGGCCGGGCACATGGCAGGGATG
      -620              -600              -580
AGGGAAAGACCAAGAGTCCTCTGTTGGGCCCCAAGTCCTAGACAGACAAAACCTAGACAAT
      -560              -540              -520
CACGTGGCTGGCTGCATTGCCCTGTGGCTGTTGGGCTGGGCCAGGAGGGAGGGCGCT
      -500              -480              -460
CTTTCCTGGAGGTGGTCCAGAGCACCGGGTGGACAGCCCTGGGGGAAAACCTCCACGTTT
      -440              -420              -400
TGATGGAGGTTATCTTTGATAACTCCACAGTGACCTGGTTCGCCAAAGGAAAAGCAGGCA
      -380              -360              -----
ACGTGAGCTGTTTTTTTTTCTCCAAGCTGAACACTAGGGGTCTAGGCTTTTTGGGTCA
      -320              -300              -280
CCCGGCATGGCAGACAGTCAACCTGGCAGGACATCCGGGAGAGACAGACACAGGCAGAGG
      -260              -240              -220
GCAGAAAGGTCAAGGGAGGTTCTCAGGCCAAGGCTATTGGGGTTTGTCAATTGTTCTGT
      -200              -180              -160
AATGCTCTTACACACGTACACACACAGAGCAGCACACACACACACACATGCCTCAG
      -140              -120              -100
CAAGTCCCAGAGAGGGAGGTGTCGAGGGGACCCGCTGGCTGTTTCAGACGGACTCCCAGA
      -80              -60              -40
GCCAGTGAGTGGGTGGGGCTGGAACATGAGTTCATCTATTTCTGCCACATCTGGTATA
      -20              +1              20
AAAGGAGCAGTGGCCACAGAGGAGCAGCTGTGTTGGCTGCAGGGCCAAGAGCGCTG
-----              60              80
TCAAGAAGACCCACACGCCCCCTCCAGCAGCTGAATTCCTGCAGCTCCGGGCAGCCGCC
      100              120      EcoRI      140
GCCAGAGCAGGACGAACCGCCAATCGCAAGGCACCTCTGAGAACTTCAGgtaggagaaaa
      160
gcaaactccctcc

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Figure 2. DNA sequence of the 5' end and the 5'-flanking region of the human PAI-1 gene. The first transcribed nucleotide is indicated as +1. The TATA sequence is boxed. The poly purine-pyrimidine tracts and the GRE consensus sequence are underlined. The imperfect repeats flanking the Alu-like sequence are indicated by interrupted lines. The HindIII and EcoRI sites used to generate the S1 mapping probe are also indicated. The beginning of the first intron sequence is reported in lower case letters.

DNA located 5' of the 21-mer hybridization site was determined (Fig. 2). The transcription initiation point of the PAI-1 mRNA was identified by S1 nuclease protection. We hybridized a 5' end-labelled 877 bp Hind III-EcoRI DNA probe, obtained from phage lambda PAI-1-9 and containing the first 72 nucleotides of the PAI-1 cDNA (see Fig. 3C), to the total RNA extracted from dexamethasone-treated HT 1080 cells and treated the hybrid with S1 nuclease. The major protected band (lane 4 of Fig. 3A) aligns with the sequencing ladder of the PAI-1 probe at an adenine residue located 72 nucleotides 5' to

the endlabeled nucleotide in the EcoRI site. A protected band of the same size is also found when RNA extracted from other cell lines is subjected to S1 nuclease mapping (Fig. 3B). This finding, together with the fact that the extreme 5' end nucleotide of two independent PAI-1 cDNA clones, differing at the 3' end, coincides with the adenine nucleotide identified by the S1 mapping (11), strongly suggests that this residue (indicated as +1 in Fig. 2) is the natural 5' end of the PAI-1 mRNA.

Having established the 5' and 3' ends of PAI-1 gene, we can conclude that the human PAI-1 gene covers a region of 12 Kbp. Since the region covered by phage lambda-PAI-1-9 extends about 12 Kbp upstream from the transcription start site, it is likely that the entire regulatory region is contained within this clone.

The sequence of the PAI-1 gene shows a colinearity with the cDNA sequence from the transcription start site up to a position +144 and then diverges with a consensus sequence for a "donor" splicing site (Fig. 2).

Figure 3. Identification of the transcription start site of the PAI-1 gene by S1 nuclease mapping. Total cytoplasmic RNA (30 µg) was hybridized to PAI-1 and/or β-actin probes (see Materials and Methods) and the hybrids were treated with 150 units of S1 nuclease for 1 h at 37°C.

Panel A: lane 1: PAI-1 probe without RNA and S1 nuclease digestion; lane 2: β-actin probe without RNA and S1 nuclease digestion; lane 3: both probes and total RNA from *D. melanogaster*; lanes 4 and 5: RNA from HT-1080 cells treated with 10^{-6} dexamethasone for 24 hours and PAI-1 and β-actin probe, respectively; lane 6: both probes and RNA from control HT-1080 cells; lanes 7 and 8: both probes and dexamethasone-treated HT 1080 RNA from two different experiments. The nucleotide ladder of Maxam and Gilbert reactions on probe 1 is used as a size marker. Fragments generated by the chemical degradation sequencing method run 1 1/2 nucleotides faster than the one generated by the S1 nuclease enzyme, so a correction of -1 1/2 nucleotides should be made in the alignment of S1 bands with the sequencing ladder.

Panel B: lanes 1 and 3: both probes and RNA from control WI-38 cells from two different experiments; lanes 2 and 4: both probes and RNA from TGF-β treated (5 ng/ml for 8 hours) WI-38 from two different experiments; lane 5: both probes and RNA from control HT-1080 cells; lane 6: both probes and RNA from TGF-β treated (5 ng/ml for 8 hours) HT-1080 cells; lane 7: both probes and RNA from control U937 cells; lane 8: both probes and RNA from PMA-treated (100 ng/ml for 24 hours) U937 cells; lanes 9 and 10: RNA from TGF-β treated HT-1080 cells and PAI-1 and β-actin probe, respectively; lane 11: both probes and *D. melanogaster* total RNA; lane M: pBR322 DNA digested with Hae III.

Panel C: the 877 bp restriction fragment used as probe for the PAI-1 gene transcription start site detection (probe 1) and the 72-nucleotide-long protected fragment are shown. A star indicates the labelled 5'-end of the probe and an arrow the initiation of transcription. A partial nucleotide sequence of the transcription start site region is shown below. The DNA sequence of the S1-protected portion of the PAI-1 probe is underlined.

Thus, the first intron of the human PAI-1 gene interrupts the 5' flanking sequence of PAI-1 transcript. The interruption occurs one nucleotide before the methionine initiation codon, as also reported by Loskutoff et al. (16).

The sequence of a fragment of the PAI-1 gene, spanning 806 bp of the 5' flanking region, the transcription start site and the transcribed region up to the first exon-intron junction, is shown in Figure 2. The analysis of the sequence shows the following features.

Starting from the transcription initiation site (+1 in the figure) and proceeding in the 5' direction, a typical TATA box (TATAAAA) is encountered at -30. No typical CAAT box sequence is present around -70 to -80. The sequence between -17 and -402 matches 43% of the residues of an atypical Alu sequence described in the African green monkey genome (47) and 39% of the residues of the Alu consensus (48) (allowing 15 single-base 3 two-base and 1 three-base loops). The same sequence also shares some other features of Alu sequences: two imperfect direct repeats (AAAGGAAAAGCAG at the 5' end and AAAGGAGCAG at the 3' end) are present at the two sides; the region -152 to -198 contains two contiguous stretches of alternating purine-pyrimidine residues, potentially forming Z-DNA structures (49); a sequence of 10 continuous thymidine residues is located at -378. The most conserved hexanucleotide (TGT(T/C)CT) of the glucocorticoid regulatory element (GRE)(50) is present at -299 in the anti-sense orientation (AGGACA). No typical Sp-1 binding sites (GGGCGG) are found, although a GC-rich sequence is present at -650.

Because of the similarity to the Alu sequences, the promoter region of the human PAI-1 gene has been analyzed for the presence of sequences repeated in the human genome. Labeled total genomic DNA was found to hybridize to a restriction fragment containing nucleotides -806 to -192 (lacking the Z-DNA stretch) of the PAI-1 gene 5' flanking region, under moderately stringent conditions (see Methods).

The PAI-1 transcription start site in TGF- β , dexamethasone and PMA-treated cells

We have recently reported that TGF- β and glucocorticoid treatments affect positively the PAI-1 mRNA level in WI-38 and HT-1080 cells, respectively (29, 30). A treatment with the phorbol ester PMA of several cell lines, including U937 monocytes, causes an increase of PAI-1 protein (Lund et al., manuscript in preparation).

In order to study the molecular mechanism of PAI-1 gene regulation, we analyzed by S1 mapping the transcription start point during regulation by

glucocorticoids, TGF- β and PMA, using the PAI-1 probe described above. A human β -actin probe, which gives a protected band of 45 nucleotides, was used as a control. The S1 analysis (Fig. 3A and 3B) shows the same major protected PAI-1 band with RNA extracted from control and dexamethasone-treated HT-1080 cells (Fig. 3A, lanes 6-8), control and TGF- β -treated WI-38 cells (Fig. 3B, lanes 1-4), control and TGF- β -treated HT-1080 cells (Fig. 3B, lanes 5-6), and control and PMA-treated U937 cells (Fig. 3B, lanes 7 and 8). It is concluded that the same transcription start site is maintained in the PAI-1 gene before and after treatment of these cell lines with these three different compounds. In addition, in agreement with the data obtained from Northern blots and ELISA determinations (29,30, Lund et al., manuscript in preparation), the scanning of the S1 autoradiogram showed that dexamethasone, PMA and TGF- β increase the intensity of the protected PAI-1 band, 8-fold, more than 20-fold and 6 or 2-fold (in the two tested cell lines), respectively. The same treatments in neither case affect the intensity of the β -actin band more than 40% of the controls. These data have been reproducibly obtained several times.

Regulation of transcriptional activity of the PAI-1 gene

In order to determine whether the increase in PAI-1 mRNA levels, following the treatment with dexamethasone, TGF- β or PMA, is due to an enhanced rate of transcription, we measured the transcriptional activity of the PAI-1 gene using a nuclear transcription (run-on) assay.

Nuclei were isolated from control and dexamethasone-, TGF- β - or PMA-treated HT-1080, WI-38 and U937 cells and the nuclear RNA, elongated in the presence of [32 P]-UTP, was extracted and hybridized to several cloned DNA probes (PAI-1, fibronectin, β -actin, β_2 -microglobulin, pUC18) immobilized on nitrocellulose. Autoradiographs of the hybridized and washed filters are shown in Fig. 4. A 7-fold enhancement in the PAI-1 gene transcription rate was obtained after the addition of dexamethasone to HT-1080 cells, while TGF- β caused a 2.5-fold increase in the transcriptional activity both in WI-38 and HT-1080 cells. PAI-1 transcriptional activity is also increased in the U-937 cells after a PMA treatment. In agreement with published measurements of the protein levels (51, 52), the human fibronectin gene also shows an increased transcriptional activity after treatment of HT-1080 cells with dexamethasone and of WI-38 cells with TGF- β . Dexamethasone, TGF- β and PMA do not modify the transcription rate of β -actin and β_2 -microglobulin genes in the tested cells, except probably a slight β -actin increase in PMA-treated U937 cells. This however does not decrease

Figure 4: Transcriptional activity of the human PAI-1 gene following stimulation with different factors. Nitrocellulose filters with linear, cloned DNAs were hybridized to ³²P-labelled run-on transcripts from nuclei isolated from control or stimulated cells, as indicated in the figure. Filters were exposed for 7 days. Abbreviations for DNA probes are as follows: fbn, human fibronectin; β-actin; human β-actin (39); β₂-mic; mouse β₂-microglobulin (44).

the significance and specificity of the PMA effect on PAI-1 gene transcription, since transcription of both β₂-microglobulin and fibronectin is not affected by PMA in U937 cells. No detectable or very low hybridization signals were given by the pUC18 probe, used as a negative control. Also the nuclear run-on data are representative of several other experiments with essentially the same results.

5' flanking region of the PAI-1 gene drives transcription of and confers glucocorticoid regulation to a heterologous reporter gene

The results obtained from the S1 mapping and nuclear run-on experiments indicate that at least three positive regulators of PAI-1 gene act by enhancing the rate of synthesis of a primary transcript starting from the same transcription initiation site.

To test whether sequences located in the 5'-flanking region of the human PAI-1 gene could promote transcription when fused to the coding sequence of a heterologous gene, we have fused either 805 bp or about 9 Kb of the PAI-1 gene 5'-flanking region to the CAT coding sequence of pEMBL8cat (plasmids pLIUS1cat and pLIUS2cat, respectively; see Materials and Methods). These plasmids, which also contain the first 72 transcribed nucleotides of the PAI-1 gene, were transfected into HT-1080 cells by the calcium-phosphate technique. The extent and the start point of transcription of the hybrid CAT genes were determined by S1 mapping on total RNA from transfected cells. The results, shown in Fig. 5, demonstrate that pLIUS1cat and pLIUS2cat were

Figure 5: S1 nuclease analysis of the transcription site of PAI-1-CAT fusion genes. Top: PAI-1-cat specific probe (see below) was hybridized with $\approx 30 \mu\text{g}$ of total cytoplasmic RNA from HT-1080 cells transiently transfected with pLIUS1cat (lane 1), pLIUS2cat (lane 2), or pEMBL8cat (lane 3), or from non-transfected cells (lane 4). Molecular weight markers: lane M, ϕx phage DNA digested with HaeIII; lane M', pBR322 plasmid DNA digested with HaeIII. Bottom: diagram of probe used for the mapping of PAI-1-cat mRNA. The length of the S1-protected portion (349 nucleotides) of the PAI-1-CAT probe is indicated.

both transcribed in the transfected HT-1080 cells and that the transcription start point is unique and coincides with that expected on the basis of data with the endogenous PAI-1 gene (Fig. 5, lanes 1 and 2). No protected bands were visible in the lanes corresponding to pEMBL8cat-transfected and untransfected cells (lanes 3 and 4 of Fig. 5).

We tested whether the responsiveness to glucocorticoids was retained by a hybrid PAI-1-CAT gene. HT-1080 cells were transfected with pLIUS1cat or the plasmids pRSVcat and pEMBL8cat, as positive and negative controls, respectively. The cells were treated for 30 hours with 10^{-6} M dexamethasone, harvested 48 hours after the addition of DNA precipitate, and the CAT-activity of the cell lysates measured. The dexamethasone-susceptibility of the expression of the fusion gene is shown qualitatively in Fig. 6. A quantita-

Figure 6: Glucocorticoid regulation of PAI-1-CAT fusion genes in transiently transfected HT-1080 cells. ^{14}C -chloramphenicol acetylation during a 30 min incubation with amounts of extracts corresponding to 25 μg total protein of cells transfected with the indicated CAT-vectors was analyzed by thin layer chromatography and autoradiography. The cells were or were not treated with 10^{-6} M dexamethasone for 30 hours. 0.2 μCi of ^{14}C -chloramphenicol was applied to the blank lane and the same amount of ^{14}C -chloramphenicol acetylated by 0.025 units of CAT enzyme was applied to the CAT-enzyme lane.

tive analysis of the data from three independent experiments is shown in Table I. The percentage of chloramphenicol acetylation and the ELISA-quantitation of the PAI-1 protein level in the medium are indicated. The expression of the PAI-1-CAT fusion gene was increased approximately 3-fold by dexamethasone in HT-1080 cells. No change in pRSV-CAT expression could be detected after dexamethasone treatment and, independent of dexamethasone-treatment, almost no CAT-activity was found in cells transfected with the pEMBL8-CAT vector. Conversely, the level of PAI-1 present in the medium of the cells transfected with any of the three CAT-vectors was enhanced 4-5 fold after 30 hours of dexamethasone treatment, in agreement with previous results (30).

Table 1
Glucocorticoid regulation of PAI-1-CAT fusion genes.

Plasmid	Fold induction	
	PAI-1 protein	CAT activity
pEMBL8-CAT	4.39+/-0.06 (4.45,4.33,4.40)	1.01+/-0.09 (0.93,1.00,1.11)
pRSV-CAT	3.00+/-0.84 (2.16,3.84,3.00)	1.03+/-0.04 (1.08,1.00,1.02)
pLIUS1-CAT	3.43+/-1.13 (2.50,4.70,3.10)	2.84+/-0.47 (2.44,3.36,2.73)

HT-1080 cells were transiently transfected with pLIUS1-cat (see Materials and Methods) and cultured with and without dexamethasone for 30 hours.

The concentration of PAI-1 in the conditioned medium was determined by ELISA. The fold increase in PAI-1 concentration by dexamethasone treatment was calculated after normalizing the PAI-1 concentration against the protein content of the extract.

For determination of the CAT-activity in cell extracts, a dose-response experiment with increasing amounts of extract was used to assure linearity in the assay. The fold increase of CAT activity by dexamethasone treatment was calculated as the ratio between the slope of the lines relating % acetylation of chloramphenicol and amount of protein in the assay, in non-treated and treated cell extracts, respectively.

Numbers refer to the mean +/- SD from three independent experiments. Values from each experiment are reported in brackets.

DISCUSSION

Regulation of most eukaryotic genes is the result of the action of several factors which use different molecular mechanisms. We have studied the regulation of the PAI-1 gene by three factors which are known to act through independent pathways. Steroid hormones affect the transcriptional rate (50, 53), as well as RNA stability (54). The regulatory effect can be either direct or indirect, depending on the requirement of protein synthesis (55). A specific soluble receptor binding to specific DNA sequences directs transcriptional response to glucocorticoid hormones in mouse mammary tumor virus, Moloney murine sarcoma virus, and in many eukaryotic genes, like human metallothionin IIA, chicken lysozyme, human growth hormone (for reviews, see refs. 50 and 53). The glucocorticoid-regulatory elements (GRE), demonstrated with gene transfer experiments, have been found to correspond to binding sites for the glucocorticoid receptor and to be characterized by

a well-conserved consensus sequence with enhancer-like properties. PMA, a potent tumor promoter having a wide spectrum of effects from mitogenicity to differentiation (56), acts through its high affinity receptor protein kinase C (57), the stimulation of which probably starts a cascade of post-translational events which leads to biochemical and genetic (i.e. transcriptional) events. Recently, a PMA-responsive element (TRE), characterized by a conserved 9 bp motif (58-60), which binds the transcription factor AP-1, has been identified in the promoter regions of several PMA-inducible genes (59, 60). The mechanism of action of TGF- β is much less understood. However, it is known that it acts via a specific membrane receptor (61-63). Some of its effects may be indirect, due to the induction of the c-sis proto-oncogene (64). It has been recently reported that the TGF- β -induced enhancement of promoter activity for the $\alpha_2(I)$ collagen gene is mediated by a binding site for nuclear factor I (65).

The transcriptional effect of glucocorticoids on the human PAI-1 gene appears to be direct. In fact, blocking of protein synthesis with cycloheximide in HT-1080 cells does not block the increase of PAI-1 mRNA (30). In WI-38 cells, cycloheximide itself is an inducer of PAI-1 mRNA (29). The effect of protein synthesis inhibition on TGF- β induction, therefore, could not be tested in this cell line. We have not investigated yet if cycloheximide affects PMA induction of PAI-1 mRNA. In this paper, we show that dexamethasone, TGF- β and PMA act at the transcriptional level, as demonstrated by nuclear run-on transcription assays and that their effects do not depend on the activation of cryptic promoters, since the 5'-end of PAI-1 mRNA is not changed after treatment with either dexamethasone, PMA or TGF- β . However, we are not able to exclude that a stabilization of PAI-1 mRNA may also contribute to the inducing effect of these agents. We also show that 802 bp of 5'-flanking plus 72 bp of 5' untranslated sequence of the PAI-1 gene are able to direct a correct transcription when fused to an heterologous coding sequence. The same fragment contains information enough to confer glucocorticoid responsiveness to a fusion gene, in gene transfer experiments.

The sequenced 807 nucleotides of the PAI-1 promoter were analyzed for the presence of general consensus sequences for regulatory sites. A sequence homologous to the 15 nucleotides proposed by Beato (50) for the GRE and containing the most conserved hexanucleotide TGT(T/C)CT is present once at position -299 from the transcription initiation site (Fig. 2). The orientation of the hexanucleotide motif in the PAI-1 5'-flanking sequence is the

opposite of that found in the MMTV-LTR, but that is compatible with the enhancer properties of GREs (50). Whether this site corresponds to an authentic GRE remains to be demonstrated. Preliminary results show that a PAI-1-CAT fusion gene carrying 343 nucleotides of 5'-flanking sequence retains the responsiveness to glucocorticoid hormones (not shown). The consensus sequences for nuclear factor I, and for transcription factor AP-1, proposed by Jones et al. (66) and by Angel et al. (60), respectively, are not present in the 807 bp of the PAI-1 promoter sequenced so far, although several similar sequences could be identified.

An Alu-like sequence can be identified between positions -402 and -17. Its structure resembles that of an unusual Alu sequence, described in the African green monkey genome (47), in which the first monomer ends with 18 alternating purine-pyrimidines, instead of the A-rich consensus sequence. Imperfect direct repeats flank this element (Fig. 2), as has been reported for most of the Alu sequences. A sequence alignment reveals a 50% degree of similarity, excluding the poly purine-pyrimidine tract, both with the human Alu consensus sequence (48) and with the unusual Alu element described by Saffer (47). It has been reported that a stretch of dT-dG alternating sequence is highly repeated in the eukaryotic genomes (5×10^{-4} copies in humans) (67). No significant homology is found when the $(dT-dG)_n$ flanking sequences of the PAI-1 gene are compared with some of the other genes containing the poly purine-pyrimidine tract (67). Southern analysis data show that a repetitive sequence is present in the region -806, -192, which do not contain the potential Z-DNA sequence. Thus, a repetitive Alu-like sequence, containing a TATA box, a GRE consensus, a Z-DNA forming sequence and two imperfect direct repeats at the extremities, is part of the human PAI-1 promoter. Whether or not it contains all of the elements required for correct PAI-1 expression is not yet known. However, the possibility of gene activation, via the insertion of an Alu-like sequence, is extremely attractive. The elucidation of the structure of the PAI-1 promoter region in species different from homo sapiens may provide an answer to this question.

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*To whom correspondence should be addressed

+On leave from Department of Pediatrics, 2nd Medical School, University of Napoli, via Pansini 5, 80131 Napoli, Italy

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