Structure and expression of the human Plasminogen Activator Inhibitor I gene

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STRUCTURE AND EXPRESSION OF THE HUMAN PLASMINOGEN ACTIVATOR INHIBITOR 1 GENE

Proefschrift

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STELLINGEN

1. Daar de Boer et al. geen rekening hebben gehouden met het effect van serum-depletie op PAI-1 expressie, is hun conclusie dat cytokines PAI-1 expressie in Hep G2 cellen kunnen induceren op zijn minst voorbarig.
   Dit proefschrift.
   De Boer et al. (1991) Thromb.Haemostas. 65, 181-185.

2. De door Westerhausen et al. gegeven verklaring voor het stimulerende effect van TGF-β op PAI-1 mRNA niveaus is strijdig met eerdere door deze groep gerapporteerde resultaten.
   Westerhausen et al. (1991) J.Biol.Chem. 266, 1092-1100.
   Fujii et al. (1989) Am.J.Cardiol. 63, 1505-1511.

3. De hogere selektiviteit voor fibrine van pro-urokinase ten opzichte van urokinase kan niet volledig verklaard worden door de hogere affiniteit van pro-urokinase voor plasminogeen gebonden aan fibrine.
   Pannell and Gurewich (1988) J.Clin.Invest. 81, 853-859.

4. Voor het verkrijgen van inzicht in de regulatie van t-PA en PAI-1 synthese bij de mens is het gebruik van gekweekte humane cellen veelal te prefereren boven het gebruik van proefdieren.

5. De suggestie van Thompson en Krisan dat de aanwezigheid van thiolase activiteit in de peroxisomen betekent dat daar ook de de cholesterol synthese plaatsvindt, is onjuist.
   Thomson and Krisan (1990) J.Biol.Chem. 265, 5731-5735.

6. Door te stellen dat het tau-eiwit een stabiliserende functie heeft in primaire cultures van neuronen waarbij nog geen gedefinieerde axonen gevormd zijn, verbinden Careres en Kosik aan anti-sense resultaten een non-sense conclusie.
   Kosik et al. (1989) Neuron 2, 1389-1397.
   Careres and Kosik (1990) Nature 343, 461-462.

7. Dat milieubeleid voor een groot deel gericht is op problemen die voorkomen hadden kunnen worden, betekent dat het milieu nog niet die prioriteit heeft die nodig is om het te beschermen.

8. Hoewel bij bridge een contract op 8 niveau nooit gemaakt kan worden, zou het uit taktische overwegingen toch te bieden moeten zijn.

9. Zolang radio "Fryslân" niet in heel Nederland ontvangen kan worden, horen de eerste klas kaatsuitslagen vermeld te worden in het NOS programma "Langs de Lijn".

Stellingen bij het proefschrift: Structure and expression of the human Plasminogen Activator Inhibitor-1 gene.

Piter J. Bosma, maart 1991

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CHAPTER 1

INTRODUCTION

1.1 The Plasminogen Activation System

Plasminogen activators (PAs) are serine proteases that convert the inactive proenzyme plasminogen into the broad spectrum protease plasmin, and thereby are able to generate systemic or localized proteolysis (see Fig. 1). Two types of active PAs are known: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). In addition to their primary role in the initiation of fibrinolysis (1,2), the PA system has been shown to be involved in a wide variety of important physiological and developmental processes, such as ovulation (3), cell migration (4), tumour invasion and metastasis (5), angiogenesis (6) and hormone processing (7). Obviously, the activity of such an important system should be carefully controlled, both in space and time: the emergence of premature and uncontrolled activity at an inappropriate site is potentially damaging. It is, therefore, not surprising that the activity of PAs is strictly regulated. Regulation of PA activity can be effectuated at several levels, including PA synthesis and secretion, interaction of PAs with co-factors or cell surface receptors, and inhibition by specific PA inhibitors (PAIs). In recent years, distinct PAIs have been identified: PAI-1 (8-10), PAI-2 (11) and protease nexin (12). Among these inhibitors, PAI-1 is unique in that it efficiently inhibits both u-PA and t-PA (13-15). It is the major PA-inhibitor in normal plasma (16,17) and a major component of the extracellular matrix of several cell types (18-22).

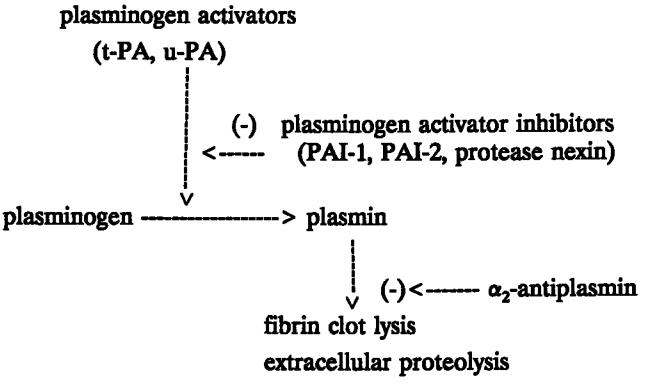

Figure 1. The plasminogen activation system.
Schematic representation of the major steps of the cascade that generates fibrinolytic or proteolytic activity through activation of plasminogen. Specific inhibition can occur at the level of plasminogen activation by PA inhibitors and at the level of plasmin by α_2-antiplasmin. Among these inhibitors PAI-1 and PAI-2 are primarily PA inhibitors, while protease nexin has a broader specificity towards serine proteases.

1.2 Characteristics of PAI-1

PAI-1 is a single chain glycoprotein with a molecular weight of about 50 kDa. The primary structure of human PAI-1 deduced from the nucleotide sequence of the PAI-1 cDNA has been confirmed by direct amino acid sequencing of its amino terminal end (23-26). Pre-PAI-1 consists of 402 amino acid residues. Removal of the signal peptide, the first 21 or 23 amino acid residues, renders the mature PAI-1 of 379-381 amino acids, with heterogeneity of the amino terminus (22,26). The mature PAI-1 contains three potential glycosylation sites and lacks cysteine residues.

PAI-1 has significant homology with members of the serine protease inhibitor (Serpin) family (23-26). Serpins evolved over a period of 500 million years from an ancestral serine proteinase inhibitor. Their common feature is a shared tertiary structure with a stressed loop near the C terminus and a common functional principal. Serpins inactivate their target protease by forming a 1:1 complex (for a review see: 27). They are important regulators in coagulation, inflammation and complement activation (28).

PAI-1 rapidly reacts with one- and two chain t-PA and two chain u-PA, with a second-order rate constant of 10^7 - 10^8 M^{-1} sec^{-1} (13-15,29), but not with single chain u-PA (30). In addition to its reaction with PAs, PAI-1 can interact with several other haemostatic components like factors XIa and XIIa (31), and activated protein C (32), albeit with much lower affinity. The 1:1 complexes between PAI-1 and t-PA or u-PA are at least partially resistant to dissociation by denaturing agents such as urea or sodium dodecyl sulphate, although these agents regenerate some activity of the bound enzyme (33). PAI-1 can be recovered from the complex in a form in which the reactive bond is cleaved, and a carboxyl-terminal fragment of about 4 kDa is released (34). Determination of amino-terminal amino acid sequences of PA-processed PAI-1 and sequence alignment with other Serpins (23) show that the reactive centre of PAI-1 is formed by the Arg346-Met347 bond (26,35). This implies that PAI-1 belongs to the subgroup of the Arg-Serpins, following the terminology of Carrell and Travis (36).

In vitro PAI-1, although synthesized in an active form, is rapidly converted into an inactive ("latent") variant (34,37,38). The inactive form can be reactivated by protein denaturants, such as sodium dodecyl sulphate, guanidine or urea (39,40), and by negatively charged phospholipids (41). The physiological relevance of such a re-activation, if any, remains to be determined. Recently it was shown that PAI-1 can bind to vitronectin, which results in a stabilization of the inhibitor (42,43). Interaction of PAI-1 with vitronectin occurs with high affinity in plasma, and in conditioned media and matrices of several PAI-1 producing cells (44,45). Interestingly, binding to vitronectin may endow PAI-1 with thrombin inhibitory properties (46).

1.3 Physiological Significance of PAI-1

The broad distribution of PAI-1 throughout tissues of the body and its presence in

platelets and plasma suggest an important role for PAI-1 in the regulation of PA activity. Until now, however, no direct evidence has been provided which underlines the anticipated importance of PAI-1 in PA regulation. Circumstantial evidence mainly comes from studies on the role of PAI-1 in plasma, and includes the following observations:

- high plasma PAI-1 levels are associated with deep vein thrombosis and cardiovascular disease (47-49).
- a good correlation is seen between plasma PAI-1 levels and a variety of risk factors for atherosclerotic and thrombotic disease, including insulin and triglycerides (50,51).
- high plasma PAI-1 levels are related with the recurrence of a second myocardial infarction (52).
- decreased functional PAI-1 activity in plasma is associated with a bleeding tendency (53,54).
- elevated PAI-1 levels in transgenic mice contribute to the development of venous occlusions (55).

However, these studies do not elucidate whether changes in PAI-1 levels are causally related to the observed clinical symptoms or are the result of a common denominator. To confirm a causal link between plasma PAI-1 levels and disease, intervention studies are required.

1.4 Regulation of PAI-1 in vivo

PAI-1 is present in plasma, platelets, and many other tissues of the body (55-57). Most studies on the regulation of PAI-1 in intact organisms have dealt only with its level in plasma, and a great many factors and conditions have been described that are associated with changes in plasma PAI-1 levels. PAI-1 in plasma is subject to diurnal variation (58,59), increases with age (60,61) and during pregnancy (62), and is enhanced in various disease states, including severe liver disease, hyperlipoproteinemia, coronary heart disease and acute myocardial infarction (47,48,63,71,72).

Also, PAI-1 is correlated to triglyceride and insulin levels (50,51,63,64), and PAI-1 plasma levels are higher in smokers than in non-smokers (65). Furthermore, PAI-1 is under hormonal control. After long term administration of stanozolol or the use of oral contraceptives, a decrease of plasma PAI-1 level is seen (66-69). In men, PAI-1 levels are inversely correlated with testosterone levels (70).

Finally, PAI-1 behaves as an acute phase reactant : a rapid increase of PA inhibitory activity is seen after major surgery, severe trauma, and myocardial infarction (71,72). Injection of endotoxin, IL-1 or TNF in humans or animals also strongly enhances the PAI-1 plasma level (73-76). Accordingly PAI-1 is also significantly enhanced in patients suffering from septicemia (73,77,78).

1.5 Regulation of PAI-1 in vitro

PAI-1 is produced by a variety of cell lines and primary cell cultures (for a review see: 79). It is the major secreted protein in cultured human and bovine endothelial cells (34,80), in the human fibrosarcoma cell line HT-1080 (81), and in a variety of human mesothelial cell lines (22). Expression of PAI-1 in cultured cells is modulated by a great variety of compounds including hormones,growth factors, cytokines, endotoxin, and phorbol esters (for a review see: 82).

The origin of plasma PAI-1 is not known with certainty, but endothelial cells, hepatocytes and smooth muscle cells are likely candidates. Table 1 gives a general overview of the regulation of PAI-1 expression in these cells and in the human fibrosarcoma cell line HT-1080, frequently used in PAI-1 regulatory studies. The expression of PAI-1 is regulated in a cell specific way. For example, in endothelial cells but not in hepatocytes PAI-1 synthesis is increased by acute phase mediators like TNF-α and IL-1, which also enhance PAI-1 level in plasma. On the other hand, the effect of insulin on PAI-1 in plasma can be mimicked in cultured human hepatocytes but not in endothelial cells.

Table 1. Regulation of PAI-1 expression in cultured cells

Regulator		Cell type	Effect	References
Acute phase reactants	LPS	endothelial cells	+	73-75,84-86
		smooth muscle cells	+	107
	IL-1	endothelial cells	+	74,75,87
	TNF-α	endothelial cells	+	75,86,88
		HT-1080	+	89,90
		human hepatocytes	0	75
		rat hepatocytes	0	75
Growth factors	TGF-β	endothelial cells	+	91
		HT-1080	+	81,92
		smooth muscle cells	+	107,108
		Hep G2	+	109
	bFGF	endothelial cells	+	91
	EGF	Hep G2	+	56
Hormones	Dexamethasone	HT-1080	+	92-96
		HTC	+	97
		human hepatocytes	0	110
		rat hepatocytes	+	98
	Insulin	Hep G2	+	99
		human hepatocytes	+	this thesis
PKC	phorbol ester	endothelial cells	+	100, this thesis
		Hep G2	+	this thesis
		HT-1080	+	101
cAMP	Forskolin	endothelial cells	-	100, this thesis
	CPT-cAMP	rat hepatocytes	+	98
Miscellaneous	Thrombin	endothelial cells	+	102-104
	HTG-VLDL	endothelial cells	+	105
	Heparin	endothelial cells	+	106

+ increase; - decrease; 0 no effect.

Cultured human endothelial cells and hepatocytes as in vitro model systems for the study of the regulation of PAI-1 synthesis, represent useful alternatives for in vivo studies in experimental animals (83). The use of cultured cells gives the possibility to study gene regulation in a relatively simple system, allowing detailed studies on the intracellular mechanisms of the various regulatory compounds.

1.6 Aim of this study

The goal of this thesis is to learn more about the regulation of PAI-1 gene expression in general, and about the regulation of PAI-1 in cultured human endothelial cells and hepatocytes in particular. Many effects on PAI-1 expression may result from regulation at the level of gene transcription. To be able to study the transcriptional regulation and to identify putative regulatory elements the PAI-1 gene and flanking DNAs were isolated and sequenced (see Chapter 2). In Chapter 3, the PAI-1 5'-flanking DNA was further characterized and the role of promoter regions in the regulation of basal PAI-1 gene expression was studied. The effect of insulin on PAI-1 in plasma can be mimicked in the human hepatoma cell line, Hep G2 (99). To confirm Hep G2 as a model for PAI-1 regulatory studies and to elucidate the regulatory mechanism, we investigated the effect of insulin on PAI-1 synthesis in primary cultures of human hepatocytes and Hep G2 cells (Chapter 4). We found that serum also enhanced PAI-1 expression in Hep G2. Serum effects can often be mimicked by activators of protein kinase C (PKC). We therefore compared in Chapter 5 the induction of PAI-1 synthesis by serum and the stable PKC activator, PMA, in Hep G2 cells. We determined the effect of both stimulators on PAI-1 mRNA levels and gene transcription rates. In addition, we studied their effect on the expression of genes coding for transcription factors possibly involved in PAI-1 regulation. In humans two different PAI-1 mRNA species of 3.2 kb and 2.4 kb exist (23). Both messengers contain the entire coding region and differ only in the length of their 3'-untranslated region. In Chapter 6 we explored whether the existence of two mRNA species could have implications for the regulation of PAI-1 expression. To that purpose we determined whether the two mRNAs differed with respect to important parameters such as stability and translational efficiency. In Chapter 7, we studied the effect of protein kinase C activation on PAI-1 expression in cultured human endothelial cells in order to investigate whether the expression of PAI-1 is cell-specifically regulated. In the final chapter (Chapter 8) the general conclusions that can be drawn from this work are discussed.

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CHAPTER 2

HUMAN PLASMINOGEN ACTIVATOR INHIBITOR-1 GENE

Promoter and Structural Gene Nucleotide Sequences

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SUMMARY

We have determined the nucleotide sequence of the human plasminogen activator inhibitor-1 (PAI-1) gene and significant stretches of DNA which extend into its 5'- and 3'-flanking DNA regions; a total sequence of 15,867 base pairs (bp) is presented. The sequenced 5'-flanking DNA (1,520 bp) contains the essential eukaryotic cis-type proximal regulatory elements CCAAT and TATAA; the more distal 5'-flanking DNA region, as well as some introns, contain sequence elements which share identities with known eukaryotic enhancer elements. A major finding is the identification of a large region of shared nucleotides (comprising of about 520 bp) between the 5'-flanking DNAs of PAI-1 and tissue-type plasminogen activator genes. The length of the PAI-1 5'-untranslated region was found to be 145 bp as determined by nuclease analysis. The remaining PAI-1 structural gene consists of amino acid coding regions (containing a total of 1,206 bp, coding for the 23 amino acids of the signal peptide and 379 amino acids of the mature PAI-1 protein), 8 intron regions (a total of 8,978 bp), and a long 3'-untranslated region of about 1,800 bp which contains several polyadenylation sites. Two types of repetitive DNA elements are located within the PAI-1 structural gene and flanking DNAs: we have found 12 Alu elements and 5 repeats of a long poly (Pur) element. These Alu-Pur elements may represent a subset of the more abundant Alu family of repetitive sequence elements.

INTRODUCTION

Plasminogen activators (PAs) are serine proteases which convert the proenzyme plasminogen into another serine protease, plasmin, by specific cleavage of a single peptide bond. Plasmin, a powerful broad spectrum protease, is involved in a variety of physiological and pathological processes like fibrinolysis, tissue remodeling, tumor growth, and metastasis (Reich, 1978; Danø et al., 1985; Emeis et al., 1985). Thus, precise regulation of PA activity may play a crucial role in the control of these processes. Two distinct types of PA have been identified, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). t-PA has been primarily associated with fibrinolytic function, while u-PA is believed to also have a regulatory role in other forms of extracellular proteolysis. As a specific inhibitor of both t-PA and u-PA, plasminogen activator inhibitor-1 (PAI-1) is a protein of great interest because of its potential role in the control of plasminogen activation.

PAI-1 is a glycoprotein present in plasma and in the α-granules of blood platelets and is synthesized by a variety of cells in vitro, including endothelial cells, hepatocytes, smooth muscle cells, and several tumor cell lines (Erickson et al., 1985; Knudsen et al.,

1987; Sprengers and Kluft, 1987). Molecular cloning of a human PAI-1 cDNA has been reported independently by several groups (Ny et al., 1986; Pannekoek et al., 1986; Ginsburg et al., 1986, Wun and Kretzmer, 1987). The deduced amino acid sequence of PAI-1 reveals a signal peptide of 23 amino acid residues and a mature protein containing 379 amino acid residues with three potential sites of N-linked glycosylation. Comparison of the sequence of PAI-1 with other proteins indicates that PAI-1 is a member of the serine protease inhibitor (Serpin) family (Carrell and Boswell, 1986).

The expression of PAI-1 is enhanced many-fold in several situations. In plasma, PAI-1 behaves as an acute phase reactant: a rapid increase in PA inhibitory activity is seen after major surgery, severe trauma, and myocardial infarction (Juhan-Vague et al., 1985; Kluft et al., 1985a). PAI-1 activity in plasma is also induced by agents such as bacterial wall lipopolysaccharide (endotoxin), interleukin-1, and tumor necrosis factor (Colucci et al., 1985; Emeis and Kooistra, 1986; Van Hinsbergh et al., 1987a). Studies with cultured cells also suggest that stimulation of PAI-1 production can be induced by a number of cellular stimuli including thrombin (Gelehrter and Sznycer-Laszuk, 1986; Van Hinsbergh et al., 1987b), endotoxin (Colucci et al., 1985; Emeis and Kooistra, 1986), inflammatory mediators (Emeis and Kooistra, 1986; Van Hinsbergh et al., 1987b), and glucocorticoids (Gelehrter et al., 1983; Baumann and Eldredge, 1982; Andreasen et al., 1987).

Recent reports suggest that PAI-1 may have functions other than intravascular control of PA activity. Several groups have recently presented evidence that the extracellular matrix produced by cells in tissue culture may contain PAI-1 (Pöllänen et al., 1987; Rheinwald et al., 1987; Knudsen et al., 1987). (It should be noted that the amino acid sequence of mesosecrin (Rheinwald et al., 1987) is identical to that of PAI-1.) In view of the well established role of PA in matrix remodeling and degradation, the presence of PAI-1 in the matrix may provide an important component in the regulation of a number of extravascular physiological and pathological processes.

To learn more about how the expression of the PAI-1 gene is regulated, we need to know the nucleotide sequence of the complete gene and flanking DNAs so that potential enhancer-like sequence elements can be identified. Toward this goal we have isolated the PAI-1 gene from a human genomic cosmid library and determined its complete nucleotide sequence. Our sequence reveals the same exon/intron structure for the PAI-1 gene as seen in the partial characterization of the PAI-1 gene reported by Loskutoff et al. (1987). Our complete sequence also reveals the identity and location of possible cis-acting regulatory elements in the 5'-flanking DNA region and the location of repetitive DNA elements. In addition, a major finding is that the PAI-1 and t-PA 5'-flanking DNA regions contain sequences which share extensive identity. Although the identification of possible regulatory elements by sequence identity is speculative, the 5'-flanking sequence and its subclone will supply the necessary material and information needed to design biological experiments to test the authenticity of such potential regulatory elements. Such

experiments may also elucidate the identity of other cis-type regulatory elements which are not apparent at this time. (Part of this work has been presented at the XIth International Congress of Thrombosis and Haemostasis (Bosma et al., 1987).)

EXPERIMENTAL PROCEDURES *)

Materials

Restriction enzymes were purchased from Boehringer Mannheim GmbH, Promega Biotec, and New England Biolabs. Agarose ultrapure DNA grade was from Bio-Rad. DNA polymerase I, S1 nuclease, and bovine alkaline phosphatase were obtained from Boehringer Mannheim, and polynucleotide kinase was obtained from U.S. Biochemical Corp. T4 ligase was obtained from Collaborative Research, and mung bean nuclease was obtained from Pharmacia LKB Biotechnology Inc. Radioactive nucleotide [γ-^{32}P]ATP (2000-3000 Ci/mM; 1 Ci = 3.7 x 10^{10} Bq) was obtained from ICN Pharmaceuticals and [α-^{32}P]CTP (2000-3000 Ci/mM) and GeneScreen filter membranes were from Du Pont-New England Nuclear. Chemicals used for DNA sequencing were obtained from vendors recommended by Maxam and Gilbert (1980). X-ray roll film (Kodak XAR-351) and DNA sequencing gel stands and safety cabinets were obtained from Fotodyne, Inc. Intensifying screens (Quanta III: 35 cm x 1 m) were obtained from Du Pont.

Screening of human cosmid library and subcloning

A human genomic cosmid library was kindly provided by Dr. J. Hoeymakers (Erasmus University, Rotterdam). This library original contained 1.6 x 10^6 independent recombinant clones and was prepared by cloning partial MboI-digested human placenta DNA (size fractionated for fragments in the range of 40-50 kb) into the BamHI-cut cosmid vector pTCF (Grosveld et al., 1982). One equivalent of this cosmid library was screened with a ^{32}P-labeled (nick-translated) 1400-bp PAI-1 CDNA probe (van den Berg et al., 1987) for the presence of PAI-1 containing cosmid clones according to the procedures described by Maniatis et al. (1982). Cloned DNA inserts used for making ^{32}P-labeled probes were isolated from ultrapure DNA grade agarose gels by electroelution. Five cosmid clones which contain PAI-1 sequence were isolated, and cosmid DNAs were purified as described by Maniatis et al. (1982). The location of the PAI-1 gene in each cosmid clone was identified by restriction enzyme site analysis and blot hybridization. Two of these cosmid clones, designated PAI-Cos1 and PAI-Cos2, were subsequently mapped in detail. Both cosmid clones contain the complete PAI-1 gene (see Fig. 1).

*) Letter codes for amino acids and nucleotide sequence ambiguities are in accordance with those proposed by IUB Nomenclature Committee (1985) Eur. J. Biochem. 150, 1-5.

Restriction enzyme fragments of PAI-Cos2 were subcloned into pUC9 to facilitate DNA sequencing and later gene engineering experiments. This was accomplished by subcloning a 7.0-kb EcoRI fragment (which contained the promoter region) and three other EcoRI fragments (1.8, 6.9, and 5.2 kb) which contained the major portion of the PAI-1 structural gene (see Fig. 2). These non-overlapping clones are referred to as pPAI-E7.0, pPAI-E1.8, pPAI-E6.9, and pPAI-E5.2, respectively.

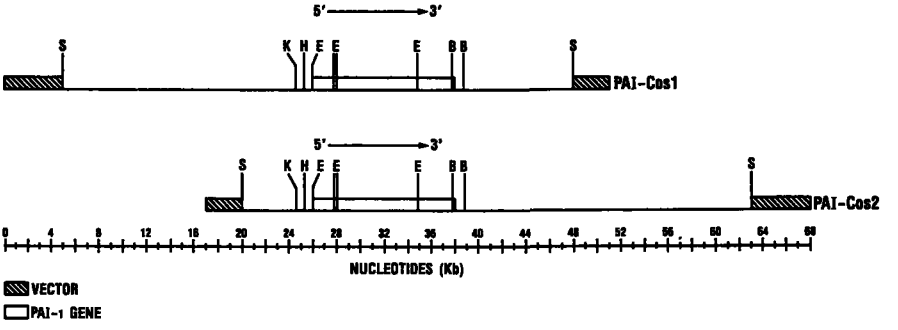


Figure 1. The physical location of the PAI-1 gene and overlapping regions of cosmids PAI-Cos1 and -Cos2. The restriction enzyme maps and blot hybridization with the PAI-1 cDNA clone were used to locate the PAI-1 gene in each of the cosmid clones; enzyme sites present in the vector, pTCF (Grosveld et al., 1982) are not shown. The restriction enzymes used are indicated as follows: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; S, SalI.

Nucleotide sequencing

Chemical sequencing was performed essentially as described by Maxam and Gilbert (1980). Appropriate restriction enzyme digests were prepared using either PAI-1 subcloned plasmid DNAs (10-20 μg) or the parent cosmid PAI-Cos2 (30 μg). The 5'-phosphate groups were removed by treatment with bovine alkaline phosphatase, without removing the restriction enzyme; the pH of the restriction enzyme buffer was adjusted to 8.4 by the addition of 0.1 volume of 1 M Tris-HCl (pH 8.4). Then, 5-10 units of phosphatase were added, and the samples were incubated at 55°C for 30 min. Restriction enzymes and phosphatase were removed by two extractions with a mixture of phenol/chloroform/isoamyl alcohol (1:1:0.04, by volume) followed by one extraction with chloroform/isoamyl alcohol (1:0.04). Endlabeling with [γ-^{32}P]ATP was done using conditions described by Slightom et al. (1980), and end-labeled fragments were isolated on glycerol (10-20%) polyacrylamide (5-7.5%)gels as described by Maxam and Gilbert (1980). Because long DNA sequencing reads were expected in the range of 600 bp, the specific chemical reaction times (at 20°C) were reduced as follows: 1 μl of dimethyl

sulfate for the G reaction, incubate for 30 s; 30 μl of 95% formic acid for the A reaction, incubate for 2.5 min; and 30 μl of 95% hydrazine for the C + T and C reactions, incubate for 2.5 min (Chang and Slightom, 1984). Chemical reaction times were reduced even further (one-half from that described above) for reactions involving DNA fragments larger than 1.5 kb to ensure samples were not overreacted and thus could be read out to at least 600 bp. DNA sequence reactions were subjected to electrophoresis on gels measuring 20 cm wide, 104 cm long and with a wedge spacer thickness which varies from 0.4 mm (bottom) to 0.2 mm (top) (obtained from C.B.S. Scientific Co.). Because the wedge is not continuous through the complete gel, these gels are referred to as "bell-bottom". Additional information concerning the design, pouring, and electrophoresis running times has been described previously by Slightom et al. (1987). Generally, using a 4% polyacrylamide bell-bottom gel, nucleotide sequence reads extend out to about 500

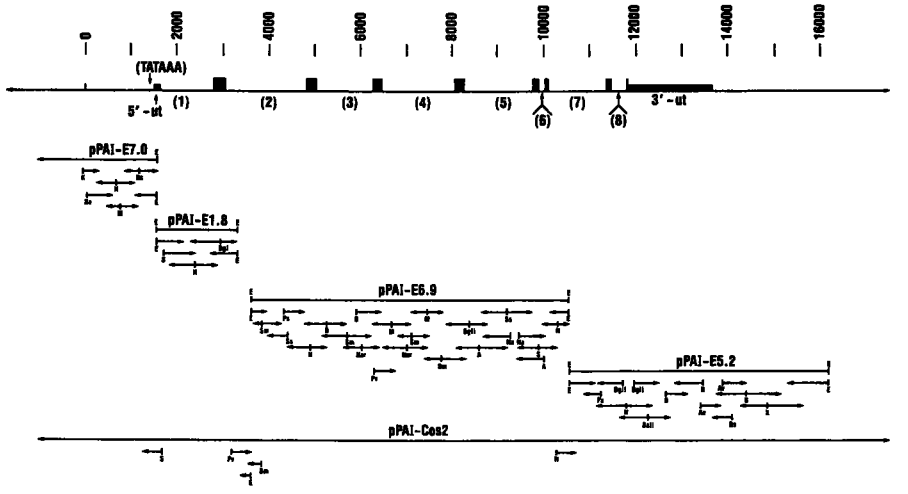


Figure 2. Structure of the PAI-1 gene, subclones, and sequencing strategy.
The top line presents the structure of the PAI-1 gene by showing the location of exons (raised black bars) and introns (regions between raised bars) as determined from the PAI-1 gene sequence presented in Fig. 3. The thicker raised black bars represent coding regions, while the thinner raised bars represent noncoding regions of the transcript. The introns are numbered from 1 to 8. 5'-ut, 5'-untranslated. The complete nucleotide sequence of the PAI-1 gene was determined from the four subclones shown below the gene structure. Nucleotide sequences crossing enzyme sites used for cloning were determined from cosmid clone pPAI-Cos2. The sequencing strategy used is shown below the clones; the enzyme site used and direction and distance sequenced are shown by the horizontal arrows. The restriction enzymes used for sequencing are indicated as follows: A, ApII; Aa, AatII; Av, AvaI; B, BamHI; BgI, BglI; BgII, BglII; Bs, BstEII; D, DraI; E, EcoRI; H, HindIII; K, KpnI; M, MstII; N, NcoI; Na, NaeI; Nar, NarI; Ps, PstI; Pv, PvuII; S, SalI; Sa, SacI; SaII, SacII; Sm, SmaI; Ss, SspI; X, XbaI.

bp, and if longer reads were desired (reads beyond 650 bp) the sequenced samples were run on a 4% polyacrylamide gel fitted with a uniform 0.2-mm spacer and electrophoresed for a total of 45,000 volt-hours. The capacity of these gels could be increased by using a comb with 3-mm slots (available from International Biotechnologies, Inc.) which allowed 32 loads to be made across a 20-cm wide gel; thus, from a single 4% bell-bottom gel loaded once with eight different sequenced fragments, as much as 4,000 bp of nucleotide sequence information could be obtained.

RNA isolation

Total cellular RNA was isolated from cultured endothelial cells as described by Lizardi and Engelberg (1979) with two minor modifications. In the first modification, cells were washed in warm, 37°C, phosphate-buffered saline (6.7 mM KH_2PO_4, 6.7 mM K_2HPO_4, and 150 mM NaCl) and incubated at 37°C for 30 min in lysis buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 7.5 mM EDTA, 0.5% sodium dodecyl sulfate, 150 μg/ml proteinase K) which was applied directly to the culture dish. The second modification was introduced after the ethanol precipitation step as the RNA was purified from contaminating DNA by precipitation in 2 M LiCl for 3 h at 0°C followed by one additional ethanol precipitation. RNA samples were then dissolved in sterile H_2O, and the RNA concentration was determined spectrophotometrically.

Nuclease analysis of 5'-transcript start site

The PAI-1 transcription initiation site was determined by nuclease mapping with either S1 or mung bean nucleases, as described by Weaver and Weissmann (1979). This analysis was done by adding approximately 100,000 cpm of a NcoI-EcoRI restriction enzyme fragment (from positions -795 to +70 (see Fig. 3)), which was 5'-end-labeled at the EcoRI site, to 50 μl of hybridization buffer (0.08 M Pipes (pH 6.4), 2 mM EDTA, 80% formamide). This ^{32}P-labeled DNA probe solution was heated to 95°C for 1.5 min (to denature the DNA duplex) and then chilled on ice, followed by the addition of 50 μl of a high salt solution (0.8 M NaCl, 80% formamide). Total RNA, approximately 15 μg, was dissolved in 20 μl of this DNA probe solution and then sealed in a capillary, or put into a small polypropylene tube under mineral oil, and allowed to anneal with the single-stranded DNA overnight at 50°C. After hybridization, samples were added to 200 μl of nuclease digestion buffer (0.25 M NaCl, 0.03 M sodium acetate, 1 mM $ZnSO_4$) and 5 μg of sonicated calf thymus or salmon sperm DNA. Single-stranded DNA and RNAs were removed by the addition of 400 units of nuclease (either S1 or mung bean) and incubated at room temperature for 20 min. These nuclease-digested samples were then precipitated with ethanol. Reaction samples, control samples which contained either tRNA or no RNA and the sequenced NcoI-EcoI fragment, were analyzed by

electrophoresis through 16 and 6% polyacrylamide sequencing gels. This procedure allows for the identification of protected fragments up to 400 bp 5' of the EcoRI site.

Computer analysis of nucleotide sequences

Computer-aided analyses of the PAI-1 nucleotide sequence were performed using programs supplied by the University of Wisconsin Genetics Computer Group. These programs were designed for use on the VAX computer, and some of the programs have been described by Devereux et al. (1984).

RESULTS AND DISCUSSION

Human PAI-1 gene isolation, characterization, and subcloning

We isolated five cosmid clones by screening a human recombinant cosmid library (1.6 x 10^6 independent clones) with a ^{32}P-labeled DNA probe isolated from a PAI-1 CDNA clone (van den Berg et al., 1987). These cosmid clones were subjected to restriction enzyme site mapping followed by blot hybridization analysis of the resulting gels. These analyses showed that the five cosmid clones represented only two unique 43-kb insert types, designed PAI-Cos1 and PAI-Cos2 (Fig. 1), each of which contains the complete PAI-1 gene. This limited analysis demonstrated that these PAI-1 genes are probably identical, which is consistent with the presence of one PAI-1 gene copy per haploid genome (Ginsburg et al., 1986; and confirmed by our analysis, data not shown). These two cosmid clones could contain non-allelic isolates of the PAI-1 gene, but our present

Figure 3. The complete nucleotide sequence of the human PAI-1 gene and flanking DNA regions. The nucleotide sequence of the human PAI-1 gene and flanking DNAs was determined using the strategy shown in Fig. 2. The PAI-1 transcription initiation site was determined by S1 and mung bean nuclease mapping experiments (see Fig. 4), and the most probable initiation site (A) is marked as position 1. The organization of the PAI-1 exons (indicated by the dashed line) and introns was determined by comparison with the sequence of the many sequenced PAI-1 cDNA clones (see text). Translation initiation codon is indicated by asterisks (***) above the first M and the termination codon is designed TER; the complete PAI-1 amino acid sequence (single-letter code) is shown below the second nucleotide of each codon. Nucleotide sequences which may function as proximal promoter elements, because they share identity with the TATAA and CCAAT elements, are indicated above the sequence line. The location of two large DNA regions capable of forming Z-DNA are indicated by the letter (z) above the sequence line. Vertical arrows indicate exon-intron boundaries, and they all conform to the GT/AT rule (Breathnach et al., 1978) and closely fit the consensus donor and acceptor sequences purposed by Mount (1982). The arrows marked IR-1 and IR-2 indicate the position of the two largest inverted repeats found in the 5'-flanking DNA. In the 3'-untranslated DNA region (shown as a continuous line) sequences which share identity with the consensus polyadenylation signals (AATAAA) are indicated along with a nonconsensus polyadenylation signal sequence (AATAAT) which may be responsible for the shorter PAI-1 transcript (Pannekoek et al., 1986; Ny et al., 1986). The double dashed line above the sequence indicates the location of Alu elements, numbered 1-12; poly purine (Pur) elements, numbered 1-5, are indicated by ////// above the sequence.

Figure 3.

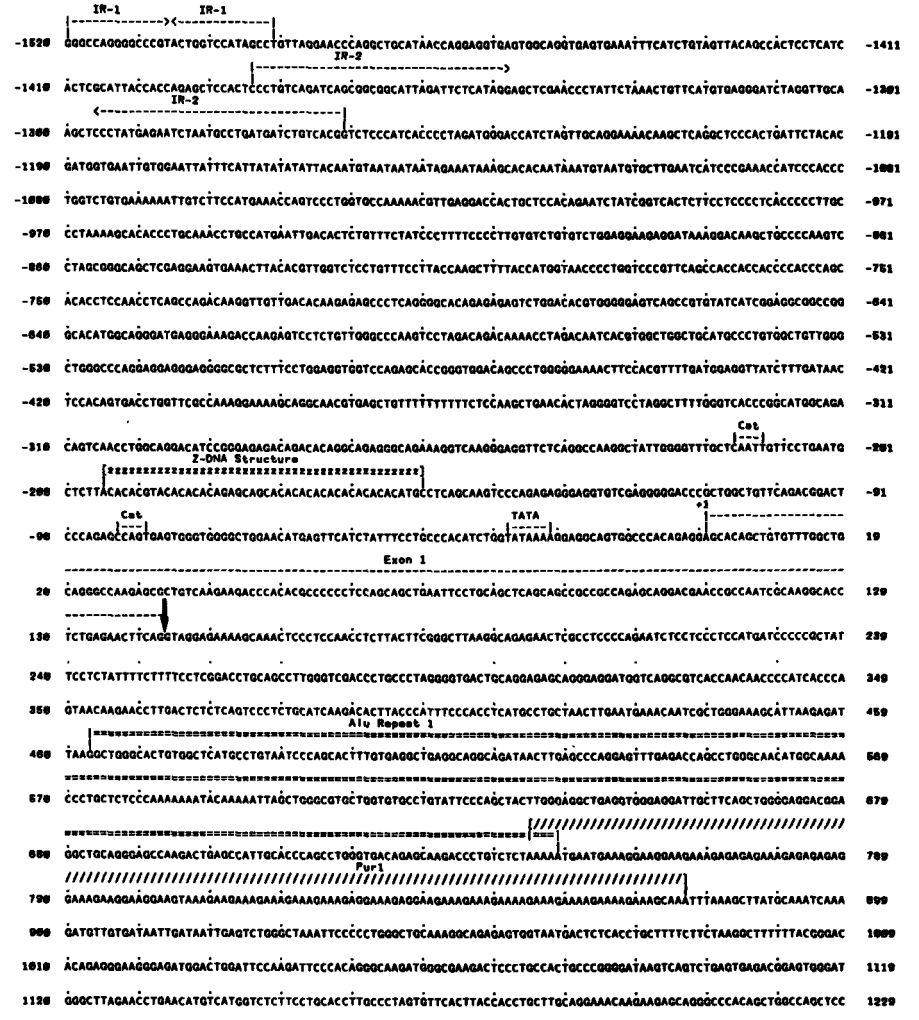

IR-1
IR-1
IR-2
IR-2
Z-DNA Structure
Cat
Cat
TATA
+1
Exon 1
Alu Repeat 1
Pur1

Figure 3 - continued

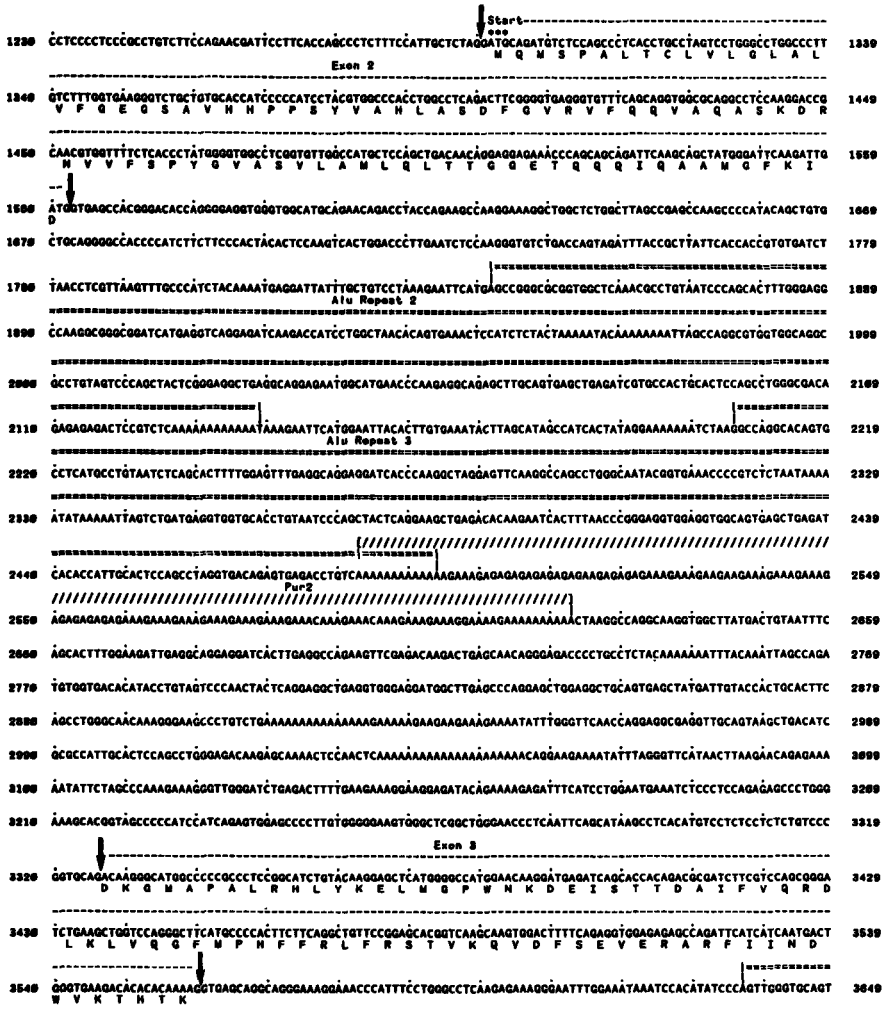


Figure 3 - continued

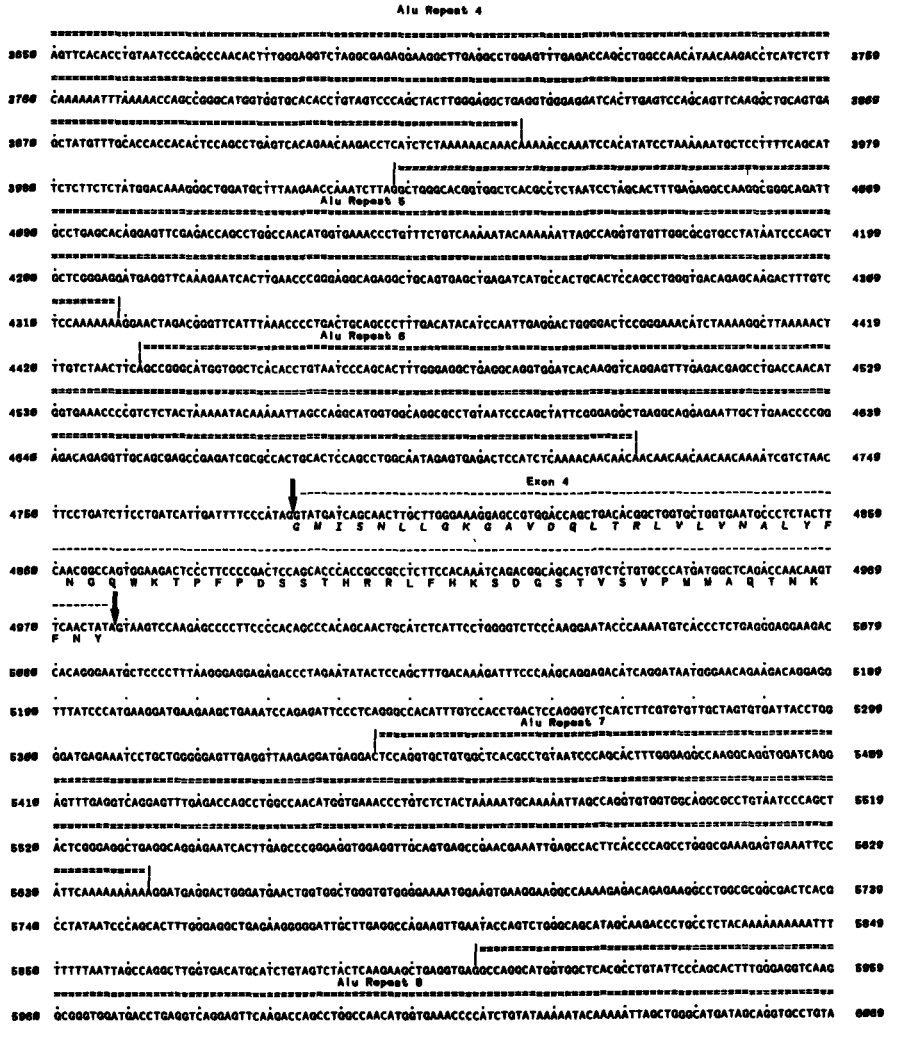


Figure 3 - continued

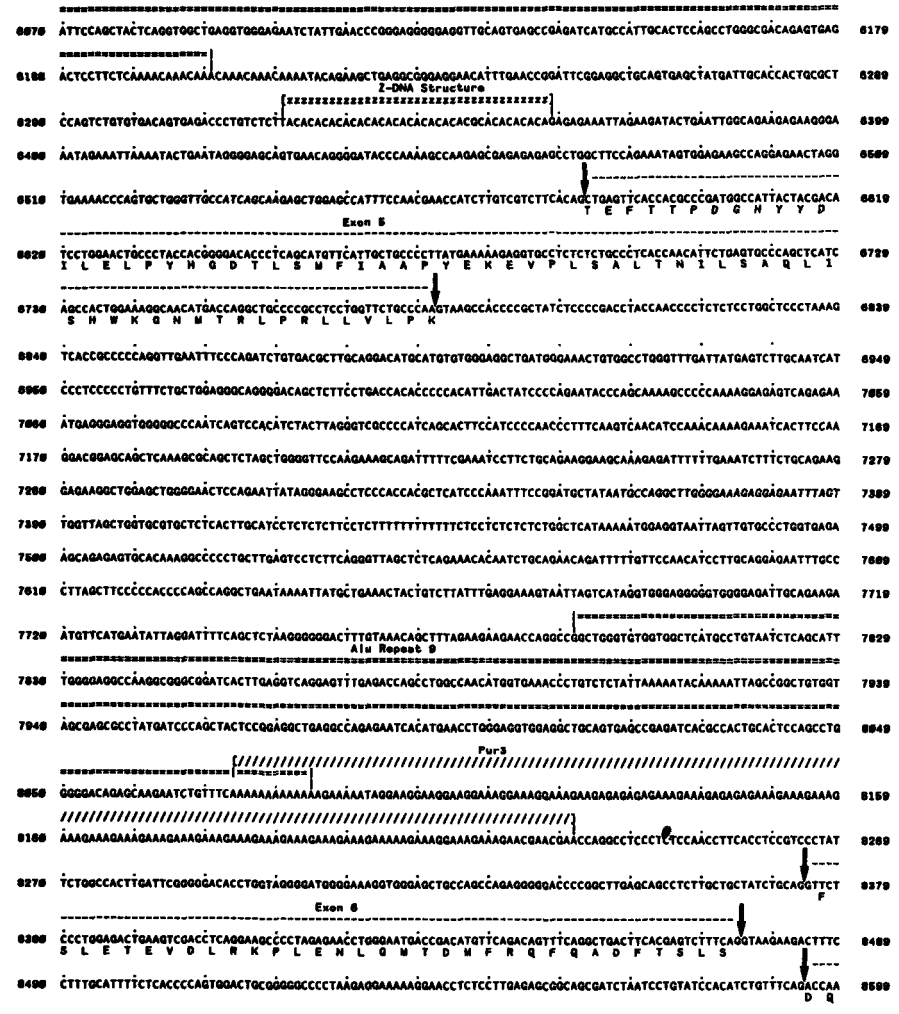


Figure 3 - continued

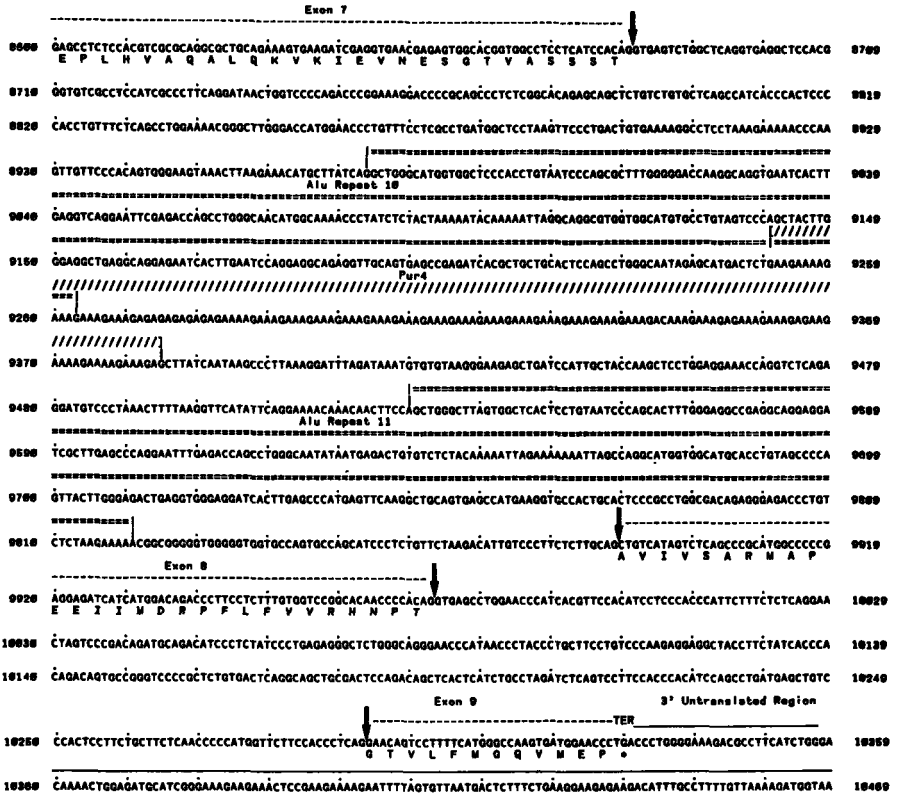
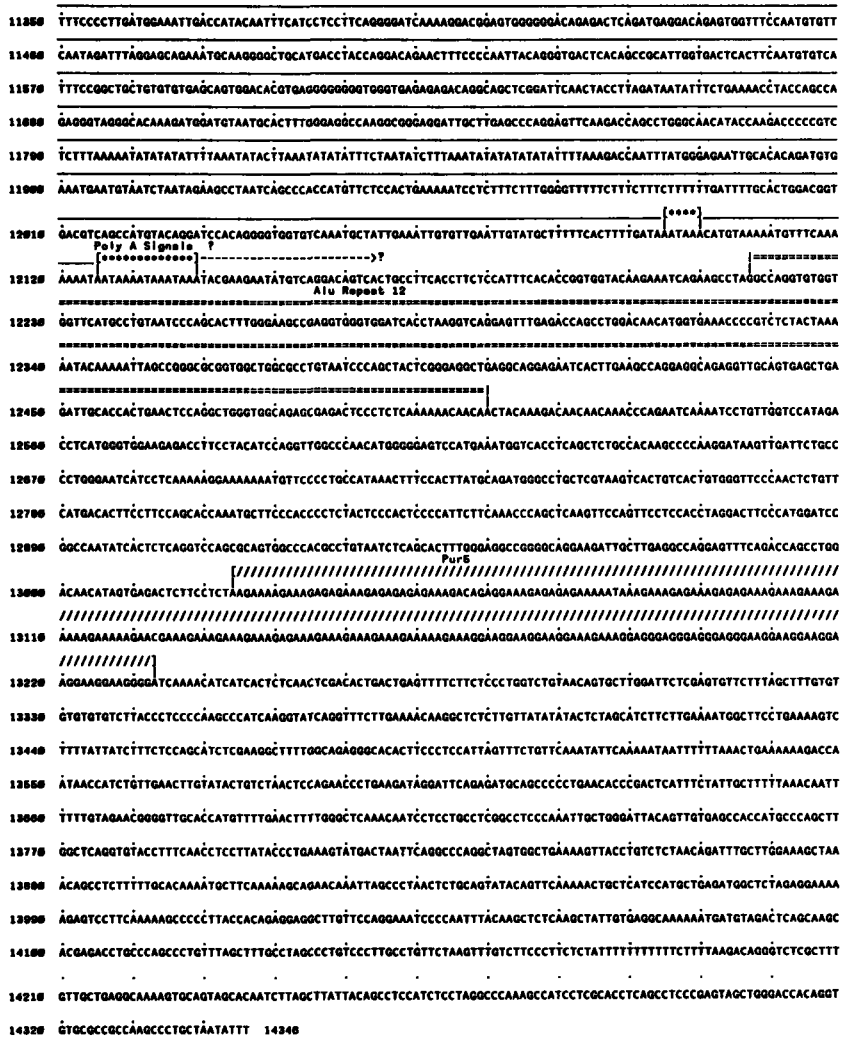


Figure 3 - continued



analysis has not revealed any polymorphic restriction enzyme sites to support this possibility.

Nucleotide sequence analysis of the human PAI-1 gene was facilitated by subcloning restriction enzyme fragments of PAI-Cos2 into either the pUC-derived part of the original cosmid vector (pTCF) or pUC9 using EcoRI restriction enzyme sites. The resulting subclones are shown in Fig. 2, below a detailed map of PAI-Cos2, and they are designated as pPAI-E7.0 (an EcoRI subclone which contains the PAI-1 promoter region), and EcoRI subclones pPAI-E1.8, pPAI-E6.9, and pPAI-E5.2.

Nucleotide sequence of the human PAI-1 gene

The complete nucleotide sequence of the human PAI-1 gene and considerable stretches of its 5'- and 3'-flanking DNA regions were determined using the chemical DNA sequencing procedure described by Maxam and Gilbert (1980). The strategy used to determine this nucleotide sequence is shown in Fig. 2. Most of the sequence was determined from the plasmid subclones except for that needed to cross the non-overlapping EcoRI cloning sites; these sequences were determined directly from the parent cosmid PAI-Cos2 (see Fig. 2). A total of 15,867 bp was determined, and a comparison with PAI-1 cDNA clone sequences (Pannekoek et al., 1986; Ny et al., 1986) shows that the entire PAI-1 structural gene is contained within about 12,165 bp, depending on the assignment of 5'- and 3'-untranslated DNA lengths (see below). In addition, we have sequenced 1,520 bp of 5'-flanking DNA and about 2,200 bp of 3'-flanking DNA; all of these sequences are presented in Fig. 3.

Structural organization of the PAI-1 gene

Because the sequences of several PAI-1 cDNA clones have previously been reported (Pannekoek et al., 1986; Ny et al., 1986; Ginsburg et al., 1986; Wun et al., 1987) determination of the organization of the PAI-1 gene was straightforward. The PAI-1 gene contains nine exons which are separated by eight introns. The location and size of these exons/introns are shown in Figs. 2 and 3, and are listed in Table I. The sites of both exons and intron regions vary considerably: exons range between 84 and 1823 bp, while introns range between 119 and 1764 bp in length. These intron sequences account for 74% of the PAI-1 structural gene length. The first exon contains 144 bp of 5'-untranslated DNA (see below). Exon 2 starts with 1 bp of 5'-untranslated DNA which is followed by the translation initiation site. The PAI-1 5'-untranslated region does not contain any false translation initiation start sites. The sequence flanking this translation initiation site, AGGATGC, only partially conforms to the consensus eukaryotic ribosome binding site (Kozak, 1987) because of the nonconsensus C-nucleotide in position +4.

Exon 9 is the largest exon, but encodes for only 12 amino acids (Fig. 3) as it consists mostly of the long 3'-untranslated DNA region. The remaining exons have an average length of 160 bp, which is consistent with the average length of 150 bp reported for the exons of genes of higher eukaryotes (Naora and Deacon, 1982). The exact length of this 3'-untranslated DNA region cannot be assessed at this time, as all the reported sequences of PAI-1 cDNA clones contain different 3'-untranslated DNA lengths. RNA blot analysis shows the presence of at least two distinct PAI-1 mRNA species, of about 3,500 and 2,350 nucleotides (Pannekoek et al., 1986; Ny et al., 1986; Ginsburg et al., 1986). Restriction enzyme mapping and blot hybridization analyses of PAI-1 exon 9 region by Loskutoff et al. (1987) suggest that these different transcripts are not the result

Table I. Location and size of exons and introns in the human PAI-1 gene

Exon	Nucleotide positions	Length	Amino acids[a]
		bp	
1	1 - 144	144	
2	1,292 - 1,563	272	-23-68
3	3,328 - 3,561	234	69-146
4	4,785 - 4,979	195	147-211
5	6,585 - 6,783	199	212-278
6	8,376 - 8,476	101	279-311
7	8,596 - 8,682	87	312-340
8	9,891 - 9,974	84	341-368
9	10,295 -12,165	1,871	369-379 and 3'-untranslated region

Intron	Type[b]	Nucleotide positions	Length	No. of Alu repeats
			bp	
1		145 - 1,291	1,147	1
2	I	1,564 - 3,327	1,764	2
3	I	3,562 - 4,784	1,223	3
4	I	4,980 - 6,584	1,605	2
5	II	6,784 - 8,375	1,592	1
6	I	8,477 - 8,595	119	0
7	I	8,683 - 9,890	1,208	2
8	I	9,975 -10,294	319	0

[a] Minus amino acids represent the PAI-1 signal peptide.
[b] Intron type is according to Sharp (1981); 0 indicates a splice between codons, I indicates a splice after the first nucleotide of a codon, and II indicates that the splice occurs after the second nucleotide.

of alternative splicing but are due to the presence of multiple polyadenylation splice sites. Our analysis of the PAI-1 3'-untranslated and flanking DNAs confirms this observation because these 3'-untranslated regions of the cDNA and gene sequences are collinear. A check of the PAI-1 sequence (Fig. 3) reveals the presence of several potential polyadenylation signals which fit the consensus sequence AATAAA (Proudfoot and Brownlee, 1976). Four putative polyadenylation signals are located between nucleotides 12,092 and 12,141, but no consensus signal is present at an upstream position which could be responsible for the polyadenylation splice that produces the shorter mRNA species. However, the hexanucleotide sequence AATAAT, which has a 1-bp mismatch, is located at position 11,174 and is surrounded by an A-T-rich region. If this sequence functions as a polyadenylation signal, the resulting mRNA species would be about 900 bp shorter, close to that observed (Pannekoek et al., 1986; Ny et al., 1986; Ginsburg et al., 1986). Additional analyses (nuclease mapping or primer extension experiments) are needed to determine the locations of the functional polyadenylation signals.

The eight introns found in the PAI-1 gene conform to the GT-AG rule (Breathnach et al., 1978), and the sequences surrounding these splice junctions (Table II) conform, but are not identical to, the consensus sequence proposed by Mount (1982). However, the splice junction sequences presented here do differ from those reported by Loskutoff et al. (1987); these differences are listed in Table III. Interestingly, all of these differences are located within the intron part of the acceptor splice site. Whether these differences are due to nucleotide sequence polymorphisms or sequencing errors remains to be determined. A total of nine differences were found in the comparison of less than 100 bp, a level much higher than that expected for sequence polymorphisms (even in neutral DNA); the expected level is about 0.14% (Miyamoto et al., 1987; J.L. Slightom, unpublished data). A recheck of our sequence gels shows no artifacts that could have been responsible for reading errors.

Several nucleotide sequence differences were found between our PAI-1 gene sequence and the cDNA sequences reported previously (Pannekoek et al., 1986; Ny et al., 1986; Ginsburg et al., 1986; Wun et al., 1987), and these differences are listed in Table IV. The 5'-untranslated region of the cDNA sequence presented by Pannekoek et al. (1986) differs at two positions from our sequence data and the 3'-untranslated regions the PAI-1 gene and PAI-1 cDNA clones differ at three positions (see Table IV). In the coding DNA region, these PAI-1 cDNA sequences differ only at gene nucleotide position 3,392 (Fig. 3 and Table IV), where either a T- or C-nucleotide substitution is observed. This nucleotide substitution is silent and thus does not influence the PAI-1 protein structure. The number of nucleotide sequence differences among these representatives of the PAI-1 transcribed regions is only five out of a total of about 12,000 nucleotides compared (assuming that each cDNA and the genomic clone represents a PAI-1 gene

from five unrelated individuals), or a difference of 0.04%. Thus, these differences may represent PAI-1 gene polymorphisms in the human population.

Table II. Intron-exon splice junction sequences in the human PAI-1 gene. Consensus sequence (from Mount (1982)).

```
C     A          TTTTTTTTTTT T
 AG GT AGT... ...           N AG
A     G          CCCCCCCCCCC C
```

Intron	Splice junction sequences		
	Exon	Intron	Exon
1	TCAG	GTAGGA... ...TTTCCATTGCTCTAG	GATG
2	GATG	GTGAGC... ...TCTGTCCCGGTGCAG	ACAA
3	AAAG	GTGAGC... ...TTGATTTTCCCATAG	GTAT
4	TATA	GTAAGT... ...TTGTCGTCTTCACAG	CTGA
5	CCAA	GTAAGC... ...TGCTGCTATCTGCAG	GTTC
6	TCAG	GTAAGA... ...CCACATCTGTTTCAG	ACCA
7	ACAG	GTGAGT... ...TCCCTTCTCTTGCAG	CTGT
8	ACAG	GTGAGC... ...TTCTTCCACCCTCAG	GAAC

Table III. Differences in PAI-1 acceptor intron/exon boundary sequences reported by Loskutoff et al. (1987) (A) and those presented in Fig. 3 (B).

Gene	Intron	Sequence	
A	1	CTTGCTCTAG	GATG
B	1	ATTGCTCTAG	GATG
A	4	CTCTGTACAG	CTGA
B	4	GTCTTCACAG	CTGA
A	5	CCCGTTGCAG	GTTC
B	5	CTATCTGCAG	GTTC
A	6	TCTGTTTTAG	ACCA
B	6	TCTGTTTCAG	ACCA

Table IV. Differences in nucleotide sequence between the previously published PAI-1 cDNA (see cDNA numbers) and that presented in Fig. 3.

The nucleotide at position 3,392 is in the coding region, the other nucleotides are in untranslated regions. Blanks indicate sequence not available.

Nucleotide position (from Fig. 3)	Gene	PAI-1 sequence from			
		cDNA 1[a]	cDNA 2[b]	cDNA 3[c]	cDNA 4[d]
19	T	G			
26	GCCA	GCA			
3,392	T	C	T	T	T
10,693	C		C	T	C
11,163	C		T	T	
11,166	C		T	T	

[a] Pannekoek et al. (1986).
[b] Ny et al. (1986).
[c] Ginsburg et al. (1986).
[d] Wun et al. (1987).

Identification of the transcription initiation site

The longest cloned PAI-1 5'-untranslated region, a length of 126 bp, was reported by Pannekoek et al. (1986). However, no additional evidence, such as nuclease mapping, was presented showing this to be the full length of the 5'-untranslated region. The precise location of the transcriptional start site is needed to support the identification of cis-acting DNA sequence elements. To clarify this, we mapped the PAI-1 transcription initiation site using both S1 and mung bean nuclease protection assays (Weaver and Weissmann, 1979) using a ^{32}P-end-labeled NcoI to EcoRI fragment (extending from position -795 to 70, see Fig. 3). The result of one of these nuclease protection assays is shown in Fig. 4; after a 1.5-bp correction for the difference in mobility found between identical fragments generated by DNA sequencing versus nuclease digesting (Sollner-Webb and Reeder, 1979) the major protected band corresponds to a A-nucleotide, as shown in Fig. 3. Some degree of ambiguity in mapping transcript initiation sites can be expected (Slightom et al., 1985). This result indicates that the full length PAI-1 5'-untranslated region is approximately 145 bp.

Identification of potential cis-acting regulatory elements

A search of the DNA flanking the 5'-side of the human PAI-1 gene revealed the two common DNA sequence elements which are known to be important for proper transcription of many eukaryotic genes, the TATAA and CCAAT elements (Efstratiadis et al., 1980; Maniatis et al., 1987). A consensus TATAA element is located between positions -28 and -24 before the transcriptional start site (Fig. 3), a distance which is consistent with that found for most eukaryotic genes. No consensus CCAAT element was found, but two closely related sequences are located farther upstream; the sequence CCAGT at the expected position, -85 to -81, and the sequence CAATT (which is also present on the opposite strand) located farther upstream, at position -218 to -214 or -217 to -213 (see Fig. 3 and Table V). Which, if any, of these CCAAT-type elements is functional remains to be determined.

One of the important aspects of obtaining the nucleotide sequence of the 5'-flanking DNA region is to examine it for the presence of cis-acting regulatory elements, also referred to as enhancer elements. Such enhancer elements have been associated with modulating the activity of proximal promoter elements (Dynan and Tjian, 1985). However, the identification of enhancer-type elements by comparative analyses (even if computer aided) is difficult because there are many different types of enhancer sequences and their affect is not dependent on orientation or location. They can be located in either 5' or 3' directions and at distances far from the transcription start site. Although consensus sequences for many enhancers exist, these sequences are generally not large, involving less than 15 nucleotides. Thus, the probability of finding sequences which closely match any consensus enhancer sequence is high, and authenticity of any potential enhancer element requires in vitro or in vivo expression analysis.

With the aid of computer search programs we have examined the entire human PAI-1 gene for sequences similar to known enhancers. Many potential enhancer sequence elements were identified and most are listed, by sequence and position, in Table V. This list includes sequences which share identities with the following enhancers: SV40 enhancer (GTGGWWWG) (Gruss, 1984), SV40 Sp1 protein binding site (KGGGCGGRRY) (Dynan and Tjian, 1985), a sequence element common to acute-phase reactant genes (CTGGGA) (Fowlkes et al., 1984; Adrian et al., 1986), and the possible human antithrombin III or immunoglobulin light chain gene enhancer (CTRRWTTG) (Prochownik, 1985). In addition to these potential enhancer elements, a stretch (almost a pure 48 bp) of alternating purine and pyrimidines, Z-DNA, is located between positions -195 to -153 (Fig. 3). (Another Z-DNA stretch, 38 bp, is located in intron 4, see Fig. 3.) Such Z-DNA stretches have been found in the sequences of acute-phase reactant genes, such as human haptoglobin and human C-reactive protein (Cooper et al., 1987). The presence of this Z-DNA region together with the two inverted copies of the acute-phase reactant regulatory sequences (CTGGGA, see Table V) in the PAI-1

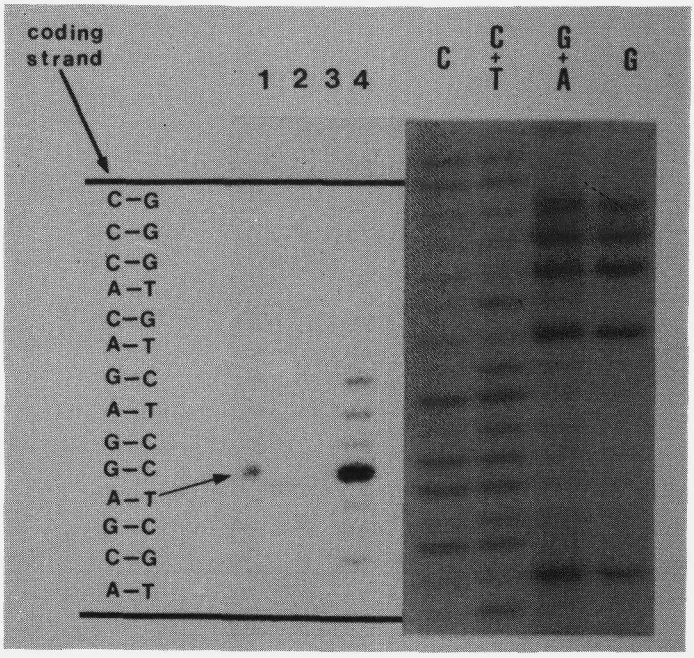


Figure 4. Localization of PAI-1 transcription initiation sites by nuclease mapping.
A 865-bp NcoI-EcoRI fragment spanning the expected PAI-1 promoter region was 5'-end-labeled at the EcoRI site, denatured, and then annealed with total cellular RNA from nontreated (lane 1) and tumor necrosis factor-treated (lane 4) endothelial cells. Lanes 2 and 3 contain control samples annealed with tRNA or no RNA, respectively. After nuclease digestion, these samples were electrophoresed through 16 and 6% polyacrylamide sequencing gels along with the sequenced NcoI-EcoRI fragment. Nuclease-protected DNA fragments were only found at a position about 72 bp 5' of the EcoRI site. The major transcription initiation site is indicated by the arrow, after correction (Sollner-Webb and Reeder, 1979).

5'-flanking DNA are interesting elements to be tested for their involvement in the acute-phase reactant-like behavior of PAI-1 expression. Three more copies of this probable acute-phase reactant regulatory hexanucleotide sequence, one in normal and two in inverted orientation, are found in the first intron (listed in Table V), and several other copies are located in its 3'-flanking DNA region (locations not listed in Table V). Whether any of these potential enhancer-type sequences are involved in regulating the level of human PAI-1 gene expression, or if there are other (yet to be discovered) unique PAI-1 gene enhancer elements, will have to await data from expression experiments.

Table V. Putative regulatory elements found in the human PAI-1 gene.

R stands for A or G. W stands for A or T. K stands for G or T. Y stands for C or T.

Enhancer element	Consensus sequence	Position	PAI-1 sequence
CAT	CCAAT	- 218 to - 214	CAATT
		- 84 to - 81	CCAGT
Inverted Cat	ATTGG	- 217 to - 213	AGTGG
TATAA	TATAA	- 28 to - 24	TATAA
SV40 enhancer core	GTGGWWWG	- 479 to - 469	GTGGACAG
		- 227 to - 220	GGGGTTTG
Acute phase signal	CTGGGA	439 to 444 (intron 1)	CTGGGA
Inverted acute phase	TCCCAG	- 141 to - 136	AGGGTC
		- 92 to - 86	ACGGGTC
		491 to 496 (intron 1)	AGGGTC
		625 to 630 (intron 1)	AGGGTC
Antithrombin III enhance	GTRRWTTG	-1186 to -1179	GTGAATTG
SP-1 binding site	KGGGCGGRRY	- 534 to - 525	TGGGCTGGGC
		- 76 to - 66	TGGGTGGGGC
Z-DNA	$(GT)_n$	- 196 to - 153	$(YR)_{23}$
		6321 to 6358	$(CA)_{18}$

Indirect repeats and shared nucleotide sequences between human t-PA and PAI-1

With the aid of computer search programs, the 1520 bp of PAI-1 5'-flanking DNA was searched for the presence of direct and inverted repeats. Numerous short, imperfect direct and indirect repeats are located in this PAI-1 5'-flanking DNA region (data not shown), but only two imperfect indirect repeats, of at least 15 bp, were found, and their locations are shown in Fig. 3. One inverted repeat, IR-1, is located at the very end of the sequenced 5'-flanking DNA, positions -1520 to -1506 and -1505 to -1491, and contains matches of 11 out of 15 nucleotides. The IR-2 repeat is located nearby, positions -1383 to -1348 and -1295 to -1261, and contains matches of 31 out of 35 nucleotides (but requires two gaps).

Our search for PAI-1 5'-flanking DNA sequence identity in the GenBank database revealed a significant degree of shared identity with only one eukaryotic gene: the 5'-flanking region of the t-PA gene (Friezner Degen et al., 1986). The presence of these regions of shared nucleotides is surprisingly quite extensive, involving an alignment of

521 positions, including only six gaps which were used to maximize identity. These sequence identities are located on opposite DNA strands, PAI-1 noncoding strand nucleotide positions -1520 to -1008 and t-PA coding strand nucleotide positions -3491 to -2977. The degree of shared identity is quite high (81%) and includes both IR-1 and IR-2 imperfect indirect sequence elements, see Fig. 5. It should be noted that the size of the shared PAI-1 and t-PA sequence region could be more extensive than we show because the region of identity, shown in Fig. 5, extends to the end of the PAI-1 5'-flanking DNA sequence presented in Fig. 3. Additional 5' sequencing of this PAI-1 flanking DNA could reveal more shared sequences identity which would extend inward toward the t-PA promoter region. A check of the last 5' sequences (38 bp) of the t-PA sequence (Friezner Degen et al., 1986) shows a much-reduced level of shared identity, suggesting that this PAI-1 and t-PA identity region may not extend farther upstream in the t-PA 5'-flanking DNA, or in the reciprocal direction downstream toward the PAI-1 transcription initiation site. Clearly, delineation of the extent of this shared PAI-1 and t-PA nucleotide region will require additional sequencing of both genes. The t-PA indirect repeat IR-1 shares about the same degree of identity as that found for the IR-1 elements of PAI-1. However, the t-PA IR-2 repeats are shorter than those found in PAI-1; the t-PA repeat involves 29 nucleotides with a match of 23 out of 29, including one gap.

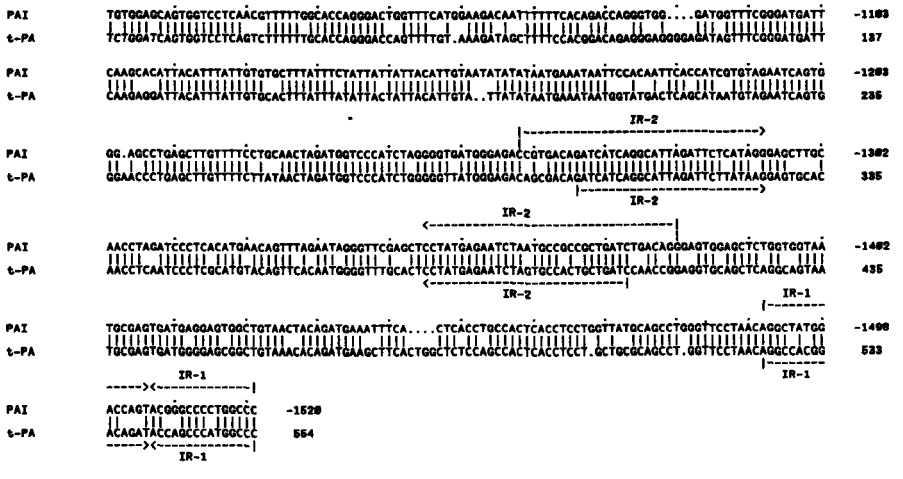


Figure 5. Alignment of shared nucleotide sequences between the human PAI-1 and t-PA genes.
A search of GenBank sequences showed that an extensive region sequence identity is shared between the 5'-flanking regions of the human PAI-1 and t-PA genes. The shared sequences are from opposite strands with the PAI-1 sequences (top line) coming from the noncoding DNA strand, positions -1520 to -1008; while the t-PA sequences (bottom line) are from the DNA strand which encodes the t-PA structural gene, positions -3491 to 2977 (Friezner Degen et al., 1986). The alignment shows a match of 81%, which required the use of only six gaps (shown as dots in the sequence lines). This shared identity also includes the two inverted repeats which are found in both 5'-flanking DNAs.

The existence of shared nucleotide sequence identity, even if on opposite strands, is interesting and suggests that these sequences, or shorter sequence elements within this region, may be important in regulating the coordinate expression of the t-PA and PAI-1 genes. In this respect it might be significant that changes in t-PA levels in plasma in several cases have been accompanied by corresponding changes in the activity of PAI-1. For example, an increase in plasma levels for both t-PA antigen and PAI-1 activity is observed after surgery, myocardial infarction, and severe trauma (Kluft et al., 1985a); a decrease in both t-PA antigen and PAI-1 activity is observed after treatment of healthy volunteers with stanozolol (Verheijen et al., 1984); a very similar diurnal fluctuation pattern was found for t-PA antigen and PAI-1 activity in plasma (Kluft et al., 1985b). Recent in vitro studies also provide support for the coordinate expression of these genes. In cultured endothelial cells, thrombin and histamine stimulate the production of both t-PA and PAI-1 (Hanss and Collen, 1987; Van Hinsbergh et al., 1987b), and in the human fibrosarcoma cell line HT1080, the increased production of t-PA in the presence of dexamethasone is also associated with increased production of PAI-1 (antigen) (Medcalf et al., 1987). The latter authors demonstrated by Northern blot hybridization and nuclear "run-off" transcript assays that the dexamethasone-induced increase of t-PA and PAI-1 synthesis parallels equivalent changes of gene template activity. Thus, the functional significance of the shared t-PA and PAI-1 nucleotide sequence in their regulatory regions clearly deserves further investigation.

Repetitive elements in and around the human PAI-1 gene

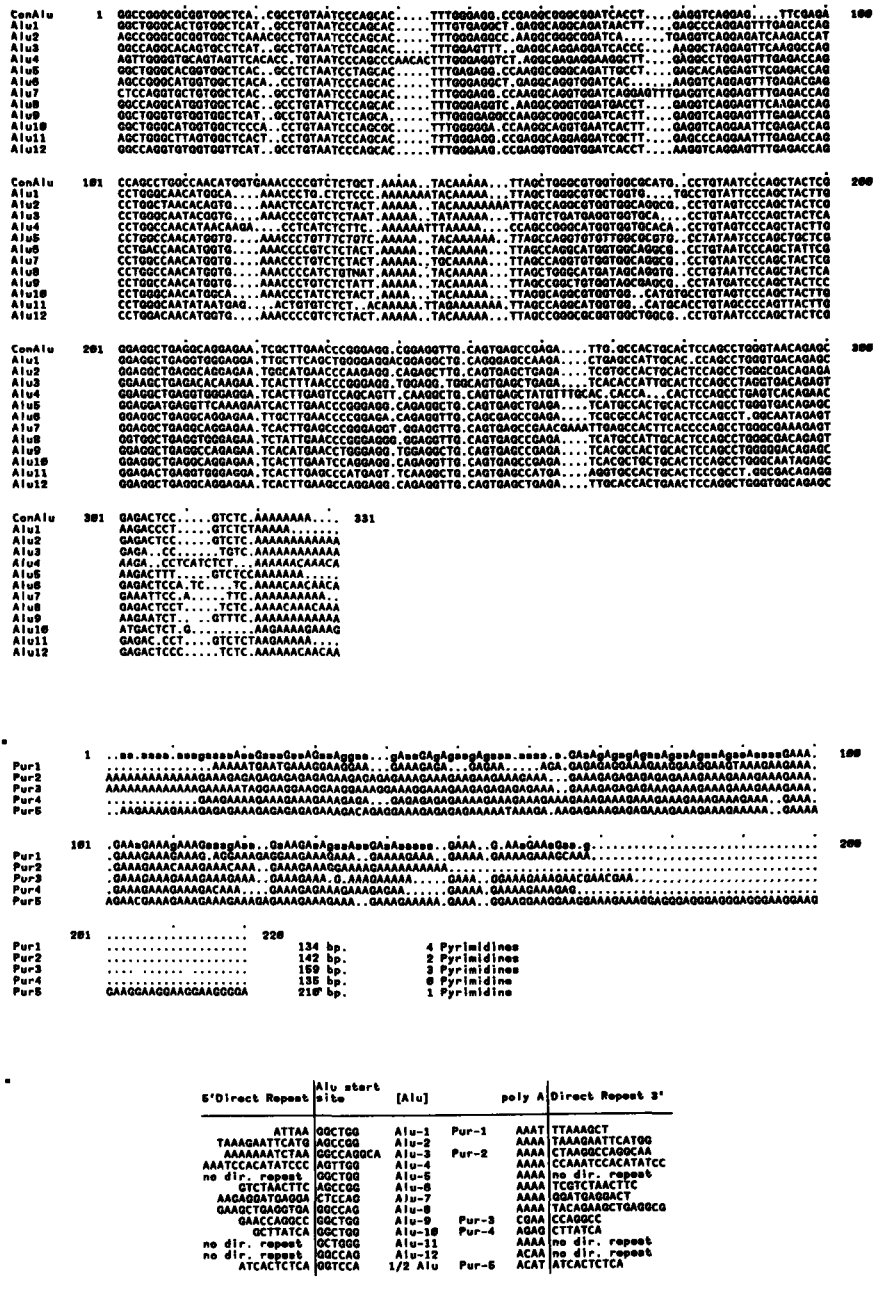
A search of the PAI-1 gene sequence for the presence and location of common repeat elements, such as Alu (Houck et al., 1979), Sau3A (Kiyama et al., 1986), and Kpn (Adams et al., 1980), revealed only sequences which share identity with the Alu element. Higher primate Alu elements are short dimeric sequences (about 300 bp in length) which probably were derived by retroposition of a sequence originally derived from a 7SL RNA gene (Ullu and Tschudi, 1984; Kariya et al., 1987). It has been estimated that the human genome may contain nearly 900,000 copies of the Alu repeat element (Hwu et al., 1986). Fig. 6 presents the results of this Alu element search in the form of an alignment of the 12 PAI-1 gene Alu elements which share between 90 and 77% identity with the

Figure 6. Alignment of repetitive DNA elements found in the PAI-1 gene region and their association with each other.

The nucleotide sequence and possible alignment of Alu elements are presented in A, Pur elements in B, and C presents the direct repeats found flanking the Alu and closely associated Alu-Pur elements. The location of the Alu and Pur elements are shown in Fig. 3, and the numbering of these elements is consistent with that used in Fig. 3. The top line in A and B represents consensus sequences for the respective elements, the consensus Alu sequence is from Koop et al. (1986), while the consensus Pur element was derived from the alignment shown in B. C lists which of the Alu and Pur elements are found in close association; Pur element 5 is associated with a one-half Alu element (see text).

Figure 6.

A.



B.

C.

5'Direct Repeat	Alu start site	[Alu]		poly A	Direct Repeat 3'
ATTAA	GGCTGG	Alu-1	Pur-1	AAAT	TTAAAGCT
TAAAGAATTCATG	AGCCGG	Alu-2		AAAA	TAAAGAATTCATGG
AAAAAAATCTAA	GGCCAGGCA	Alu-3	Pur-2	AAAA	CTAAGGCCAGGCAA
AAATCCACATATCCC	AGTTGG	Alu-4		AAAA	CCAAATCCACATATCC
no dir. repeat	GGCTGG	Alu-5		AAAA	no dir. repeat
GTCTAACTTC	AGCCGG	Alu-6		AAAA	TCGTCTAACTTC
AAGAGGATGAGGA	CTCCAG	Alu-7		AAAA	GGATGAGGACT
GAAGCTGAGGTGA	GGCCAG	Alu-8		AAAA	TACAGAAGCTGAGGCG
GAACCAGGCC	GGCTGG	Alu-9	Pur-3	CGAA	CCAGGCC
GCTTATCA	GGCTGG	Alu-10	Pur-4	AGAG	CTTATCA
no dir. repeat	GCTGGG	Alu-11		AAAA	no dir. repeat
no dir. repeat	GGCCAG	Alu-12		ACAA	no dir. repeat
ATCACTCTCA	GGTCCA	1/2 Alu	Pur-5	ACAT	ATCACTCTCA

consensus Alu element (Koop et al., 1986). None of these Alu elements are located in the 5'-flanking DNA or exon regions, and only one is located in the 3'-flanking DNA. Most are located in intron sequence regions (see Table I), all are located on the coding strand (see Fig. 3 for exact locations) and in many cases several Alu elements are located within close proximity to each other. Whether these Alu elements have any present regulatory function for PAI-1 expression is doubtful because of their presence throughout the human genome (Houck et al., 1979). It has been suggested that these Alu elements may be important because they provide homologous DNA regions which could participate in unequal cross-over events (Jeffreys and Harris, 1982; Rogers, 1985). However, some examples of cross-overs involving Alu repeats have had deleterious results, such as that found in a defective low density lipoprotein receptor gene (Lehrman et al., 1985). The determination of any evolutionary significance which can be assigned to any particular PAI-1 Alu elements will have to await the analysis of the orthologous genes of other primate and mammalian species.

Short direct terminal repeats have been found flanking many Alu elements (Van Arsdell et al., 1981; Ruffner et al., 1987), and these repeats, along with the A + T-rich composition of the surrounding DNA (Daniels and Deininger, 1985), may be important elements in controlling the targeting and orientation of integration of Alu elements. Several of the PAI-1 gene Alu elements are flanked by short perfect direct terminal repeats of at least 5 bp in length, while others are flanked by imperfect direct repeats as large as 14 bp (see Fig. 6). These direct terminal repeats do not appear to be related as they share little or no sequence identity. Direct repeats were not found for three Alu elements (Fig. 6), while the 3'-direct repeats for five other Alu elements appeared to be displaced by the presence of another sequence element, a poly purine sequence (see Figs. 3 and 6).

During the sequencing of the human PAI-1 gene, another type of DNA sequence repeat element became apparent because of its unusual sequence composition. These elements consist of long stretches of essentially pure G and A nucleotides, and they are found only on the coding strand, the same strand preference found for the Alu elements. We refer to these sequence elements as poly purine elements (Pur elements), and the PAI-1 gene contains 5 Pur elements which range in length from 134 to 210 bp (see Figs. 3 and 6). All of the Pur elements are located in intron sequences except Pur element 5 which is located in the 3'-flanking DNA. Alignment of these elements reveals a common core of about 120 bp (Fig. 6), which suggests that they may have a common evolutionary origin. This possibility is supported by the finding that each Pur element, except Pur element 5, overlaps with the 3' end of an Alu element, see Fig. 3. A more detailed analysis of Pur element 5 shows that it overlaps with a one-half Alu element, positions 12,907-13,023 (Fig. 3). The close association of these Alu and Pur elements suggests a common link in their integration mechanism. Such a common link could be: (i) the A-

rich composition of the 5'-end of the Pur element may be a target for Alu element integration, (ii) the A + T-rich composition of the 3'-end of the Alu element may be a target for Pur element integration, or (iii) these Pur elements may represent a different type of Alu repeat element which may have been deposited, as a single event, in the human genome by the Alu retroposition mechanism. If so, this would indicate that in some cases retroposition of Alu elements may involve more than the Alu element itself, the possible retroposition of sequence adjacent to an ancestral Alu element, in this case an adjacent Pur element. This information may be useful for the assignment of Alu element subclasses which may provide additional insight into their evolution. The degree of divergence among the individual Alu elements found in close association with Pur elements ranges between 89 and 77%, essentially the same range as found for the Alu elements (Kariya et al., 1987). This finding suggests that these Alu-Pur elements are not the result of a single burst of retroposition of an ancestral Alu-Pur element. Additional insight in the evolution of these Alu-Pur elements and into the mechanism responsible for their close association can possibly be obtained with the determination of PAI-1 gene sequences from related species.

A search of GenBank sequences for the Pur element showed that this element is present much less frequently than the highly repetitive Alu sequence elements. This is consistent with our hypothesis that Alu-Pur elements represent a small subset of Alu elements. Pur elements in the range of at least 100 bp were found in the sequence of only a few genes, the human apolipoprotein CIII (Protter et al., 1984), in a human actin pseudogene (Moos and Gallwitz, 1983), and even in DNA isolated from an ancient Egyptian mummy (Pääbo, 1985), but none are found in the sequenced t-PA gene region (Friezner Degen et al., 1986). It is extremely interesting to note that the Pur elements found in the apolipoprotein CIII gene and the mummy DNA are also associated with an overlapping Alu element. All of these Alu-Pur element overlaps occur in the same orientation as those presented in Fig. 3, with respect to the Alu poly(A) region. This finding supports these Alu-Pur elements being a small subclass of Alu elements and supports the hypothesis that the Alu-Pur elements may have been incorporated in the genome as a result of a single retroposition event. Additional experiments, such as genomic blot hybridizations and copy number determination, are needed to accurately determine the repetitive nature of the Pur or Alu-Pur element. As in the case with Alu elements, it is doubtful that these Alu-Pur elements are important for the regulation of the PAI-1 gene, but this possibility certainly deserves to be tested.

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CHAPTER 3

FURTHER CHARACTERIZATION OF THE 5'-FLANKING DNA OF THE GENE ENCODING HUMAN PLASMINOGEN ACTIVATOR INHIBITOR-1

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Gene (in press)

SUMMARY

Previous nucleotide (nt) sequence analysis of the 5'-flanking DNA of the gene *(PAI-1)* encoding plasminogen activator inhibitor-1 revealed an extensive region of shared nt sequence identity with the 5'-flanking region of the gene *(t-PA)* encoding tissue-type plasminogen activator [Bosma et al., J. Biol. Chem. 263 (1988) 9129-9141]. Additional sequence (1642 bp) from the *PAI-1* gene 5'-flanking DNA reveals that these *"PAI-1/t-PA"* sequence elements share an alignment that contains a total of 575 positions. This additional *PAI-1* 5'-flanking sequence also contains two *Alu* elements that form inverted repeats. Southern blot analysis using the *PAI-1/t-PA* element as a probe indicates that this element is repeated in the human genome, which supports the classification of this element as a medium reiteration frequency *(MER)* sequence [Jurka, Nucleic Acids Res. 18 (1990) 137-141].

INTRODUCTION

Plasminogen activators (PAs) are serine proteases that catalyze the conversion of plasminogen to plasmin, a broad-spectrum protease. PAs have been implicated in a variety of physiological processes that involve controlled proteolysis, including phenomena as diverse as fibrinolysis, mammary gland involution, angiogenesis, hormone processing, and tumor cell metastasis (see Danø et al., 1985; and references therein). There are two forms of PA, t-PA and u-PA, and a specific inhibitor of both PAs, PAI-1, appears to play an important role in precise regulation of PA activity (Sprengers and Kluft, 1987).

We previously sequenced the *PAI-1* gene, including some 5'- and 3'-flanking DNAs and found that the *PAI-1* and *t-PA* gene 5'-flanking DNAs share an extensive sequence region referred to as the *PAI-1/t-PA* element (Bosma et al., 1988). The exact size of the *PAI-1/t-PA* element was not previously determined, which is why we report here an additional nt sequence of 1642 bp from the 5'-flanking region of the *PAI-1* gene. We have also extended our computer-aided analyses of *PA* and *PAI* 5'-flanking regions to include sequences from the *u-PA* (Verde et al., 1988) and *PAI-2* (Kruithof and Cousin, 1988) genes.

Using computer-aided searches of GenBank, we and Jurka (1990) found examples of sequences that are related to the *PAI/t-PA* element in other human genes. Alignment of these elements reveals two subfamilies, which Jurka (1990) refers to as *MER1a* and *MER1b* elements. The *PAI-1/t-PA* elements are members of the *MER1b* subfamily. The *MER1a* elements are located in genes that are not related to the *PA* or *PAI* genes; these include genes encoding angiogenin, apolipoprotein A4, and immunoglobulin heavy chain

constant region sigma(γ4). We have extended the alignment presented by Jurka (1990) using the additional 5'-flanking DNA sequence made available in this report.

RESULTS AND DISCUSSION

(a) Nt sequence and comparative analyses of the *PAI-1* 5'-flanking region

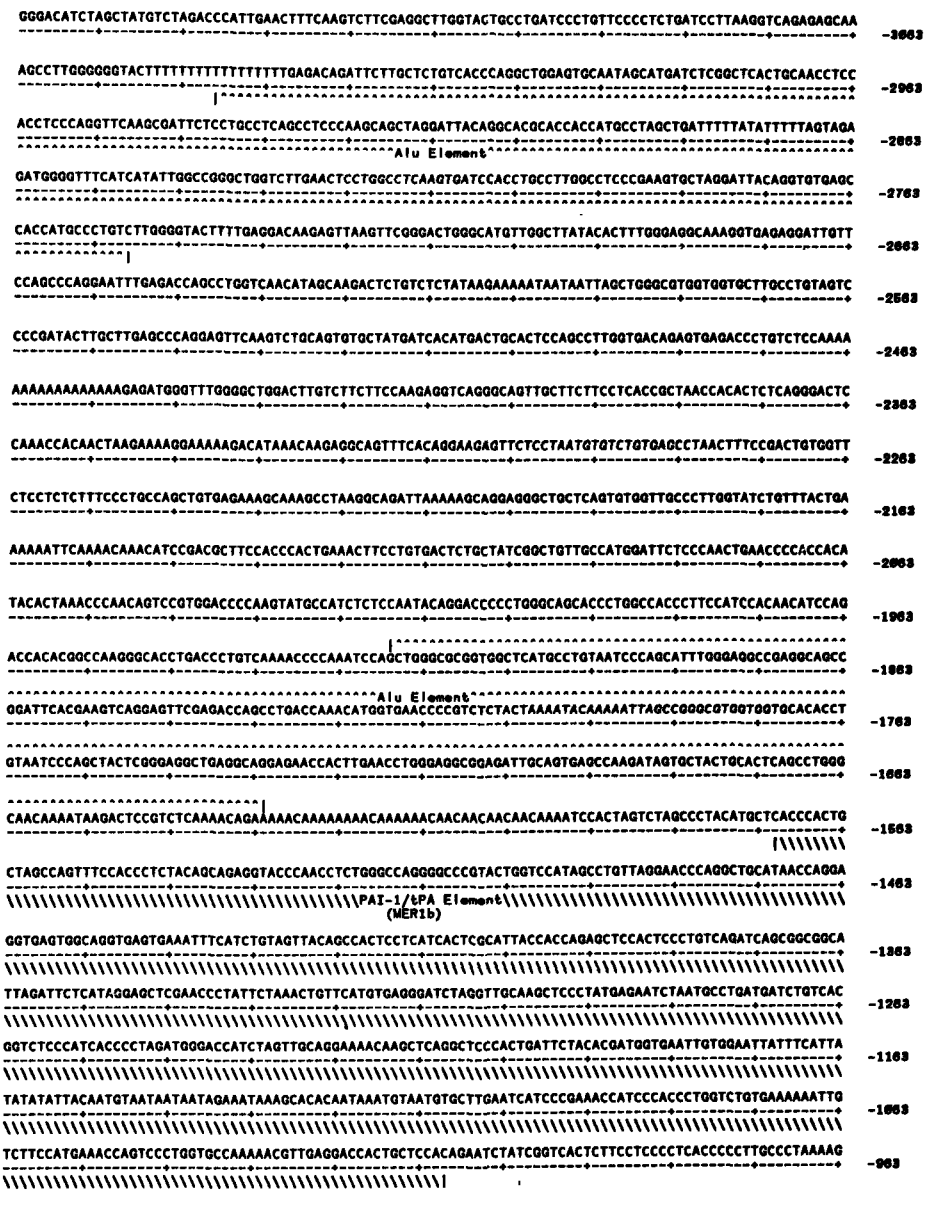
An additional 1642 bp of the *PAI-1* 5'-flanking DNA was determined from subclone pPAI-E7.0 and combined with that previously obtained by Bosma et al. (1988). This nt sequence is presented in Fig. 1, which extends our analysis of the 5'-flanking DNA region to a total of 3162 bp 5' of the *tsp*.

A computer-aided analysis of the additional *PAI-1* 5'-flanking DNA sequence finds that the *PAI-1/t-PA* element extends only an additional 51 bp beyond that described by Bosma et al. (1988). The location of this best alignment of *PAI-1* and *t-PA* shared nt sequences is indicated on the *PAI-1* nt sequence in Fig. 1. The overall degree of sequence identity for the 575 position alignment is about 80%. Outside of the shared nt region indicated in Fig. 1, i.e., 5' of nt -1571 and 3' of nt -1010, the degree of sequence identity between the *PAI-1* and *t-PA* 5'-flanking DNAs drops to about 25% to 30% (data not shown), levels expected for randomly aligned nt sequences. Comparative analyses of the *PAI-1* 5'-flanking DNA sequence with that from the *u-PA* gene (2798 bp) (Verde et al., 1988) or from the *PAI-2* gene (2170 bp) (Kruithof and Cousin, 1988) reveal the absence of any extensive region of shared nt similar to the *PAI-1/t-PA* element. Comparisons of the structure of the rat and human *PAI-1* genes (Zeheb and Gelehrter, 1988; Bruzdzinski et al., 1990) shows a strict conservation of the exon-intron structure and two regions of shared sequence identity in the 5'-flanking DNAs. However, the 5'-flanking DNA of the rat *PAI-1* gene does not contain sequences that share identity with the *PAI-1/t-PA* element (Bruzdzinksi et al., 1990).

(b) Organization and comparison of *Alu* elements in *PA* and *PAI* genes

The *PAI-1* promoter 5'-flanking regions show the presence of two *Alu* elements, which are located 5' of the *PAI-1/t-PA* element, as indicated in Fig. 1. One *Alu* element is located in the 5'-flanking region of *t-PA* gene (Friezner Degen et al., 1986) and two *Alu* elements are located in the 5'-flanking region of the *PAI-2* gene (data not shown). A partial *Alu* element of 83 bp, which shares 96% identity with the consensus *Alu* element (Koop et al., 1986), is present at the extreme 5'-end of the *u-PA* 5'-flanking DNA (Verde et al., 1988). Presumably, if additional sequences were obtained from the *u-PA* 5'-flanking DNA a complete *Alu* element would be revealed. The percentage match between each of the *Alu* elements identified in the *PA* and *PAI* genes and the consensus

Figure 1.


GGGACATCTAGCTATGTCTAGACCCATTGAACTTTCAAGTCTTCGAGGCTTGGTACTGCCTGATCCCTGTTCCCCTCTGATCCTTAAGGTCAGAGAGCAA
-3663
AGCCTTGGGGGGTACTTTTTTTTTTTTTTTTTTGAGACAGATTCTTGCTCTGTCACCCAGGCTGGAGTGCAATAGCATGATCTCGGCTCACTGCAACCTCC
-2963
ACCTCCCAGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCAAGCAGCTAGGATTACAGGCACGCACCACCATGCCTAGCTGATTTTTATATTTTTAGTAGA
Alu Element
-2863
GATGGGGTTTCATCATATTGGCCGGGCTGGTCTTGAACTCCTGGCCTCAAGTGATCCACCTGCCTTGGCCTCCCGAAGTGCTAGGATTACAGGTGTGAGC
-2763
CACCATGCCCTGTCTTGGGGTACTTTGAGGACAAGAGTTAAGTTCGGGACTGGGCATGTTGGCTTATACACTTTGGGAGGCAAAGGTGAGAGGATTGTT
-2663
CCAGCCCAGGAATTTGAGACCAGCCTGGTCAACATAGCAAGACTCTGTCTCTATAAGAAAAATAATAATTAGCTGGGCGTGGTGGTGCTTGCCTGTAGTC
-2563
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-2463
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-2363
CAAACCACAACTAAGAAAAGGAAAAAGACATAAACAAGAGGCAGTTTCACAGGAAGAGTTCTCCTAATGTGTCTGTGAGCCTAACTTTCCGACTGTGGTT
-2263
CTCCTCTCTTTCCCTGCCAGCTGTGAGAAAGCAAAGCCTAAGGCAGATTAAAAAGCAGGAGGGCTGCTCAGTGTGGTTGCCCTTGGTATCTGTTTACTGA
-2163
AAAAATTCAAAACAAACATCCGACGCTTCCACCCACTGAAACTTCCTGTGACTCTGCTATCGGCTGTTGCCATGGATTCTCCCAACTGAACCCCACCACA
-2063
TACACTAAACCCAACAGTCCGTGGACCCCAAGTATGCCATCTCTCCAATACAGGACCCCCTGGGCAGCACCCTGGCCACCCTTCCATCCACAACATCCAG
-1963
ACCACACGGCCAAGGGCACCTGACCCTGTCAAAACCCCAAATCCAGCTGGGCGCGGTGGCTCATGCCTGTAATCCCAGCATTTGGGAGGCCGAGGCAGCC
Alu Element
-1863
GGATTCACGAAGTCAGGAGTTCGAGACCAGCCTGACCAAACATGGTGAACCCCGTCTCTACTAAAATACAAAAATTAGCCGGGCGTGGTGGTGCACACCT
-1763
GTAATCCCAGCTACTCGGGAGGCTGAGGCAGGAGAACCACTTGAACCTGGGAGGCGGAGATTGCAGTGAGCCAAGATAGTGCTACTGCACTCAGCCTGGG
-1663
CAACAAAATAAGACTCCGTCTCAAAACAGAAAAACAAAAAAAACAAAAAACAACAACAACAACAAAATCCACTAGTCTAGCCCTACATGCTCACCCACTG
-1563
CTAGCCAGTTTCCACCCTCTACAGCAGAGGTACCCAACCTCTGGGCCAGGGGCCCGTACTGGTCCATAGCCTGTTAGGAACCCAGGCTGCATAACCAGGA
PAI-1/tPA Element
(MER1b)
-1463
GGTGAGTGGCAGGTGAGTGAAATTTCATCTGTAGTTACAGCCACTCCTCATCACTCGCATTACCACCAGAGCTCCACTCCCTGTCAGATCAGCGGCGGCA
-1363
TTAGATTCTCATAGGAGCTCGAACCCTATTCTAAACTGTTCATGTGAGGGATCTAGGTTGCAAGCTCCCTATGAGAATCTAATGCCTGATGATCTGTCAC
-1263
GGTCTCCCATCACCCCTAGATGGGACCATCTAGTTGCAGGAAAACAAGCTCAGGCTCCCACTGATTCTACACGATGGTGAATTGTGGAATTATTTCATTA
-1163
TATATATTACAATGTAATAATAATAGAAATAAAGCACACAATAAATGTAATGTGCTTGAATCATCCCGAAACCATCCCACCCTGGTCTGTGAAAAAATTG
-1063
TCTTCCATGAAACCAGTCCCTGGTGCCAAAAACGTTGAGGACCACTGCTCCACAGAATCTATCGGTCACTCTTCCTCCCCTCACCCCCTTGCCCTAAAAG
-963

Alu element derived by Koop et al. (1986) ranges between 87.2 to 91.2%, which is consistent with the range found for *Alu* elements of higher primates (Koop et al., 1986).

c) The *PAI-1/t-PA* element is a repetitive element in the human genome

A computer-aided search of human gene sequences in GenBank identified several genes that contain partial copies of the *PAI-1/t-PA* element. Partial *PAI-1/t-PA* elements were found in the 5'-flanking DNA of the gene encoding the immunoglobulin heavy chain sigma(γ4) (Akahori et al., 1988), the gene encoding angiogenin (Kurschi et al., 1985), and the gene encoding apolipoprotein CIII (Protter et al., 1984). The alignment of the *PAI-1/t-PA* elements from the genes encoding PAI and t-PA, along with the related sequences from these other genes is shown in Fig. 2. A similar computer-aided search of GenBank by Jurka (1990) also revealed the same *PAI-1/t-PA* related elements, although he did not show the alignment of the element from the gene encoding the immunoglobulin heavy chain constant region sigma(γ4). Jurka (1990) has suggested that the *PAI-1/t-PA* elements be referred to as the *MER1* elements. These *MER1* elements appear to belong to two different subfamilies, the *MER1a* elements, which include elements from HUMAGG, HUMAPOAI1, and HUMSIGMG4 in Fig. 2 (sharing about 77% identity), and the *MER1b* elements, which thus far have only been found in the genes encoding t-PA and PAI-1, HUMTPA and HUMPAIA respectively, in Fig. 2. The major difference between these two subfamilies is in their length, which appears to be due to the presence of two extra sequences in the *MER1b* relative to the *MER1a* elements (Jurka, 1990). The nt sequence alignment shown in Fig. 2 conforms to the alignment presented by Jurka (1990), but our alignment includes the additional *PAI-1* 5'-flanking DNA sequences (51 bp) and an additional 6 bp not included in the previously reported alignment (nt 570 to 575, in Fig. 2).

The finding of five *MER1* elements represented in the data base (GenBank version 55) has led Jurka (1990) to postulate that the human genome contains about 4500 copies of the *MER1* element. This estimate may be somewhat high as our search of GenBank version 62 did not reveal any additional *MER1* elements. This suggestion was tested experimentally by Southern blot hybridization using the *PAI-1 MER1b* element (nt -1562 to -1171, in Fig. 1) as a ^{32}P-labeled probe and hybridizing it against total human DNA

Figure 1. Additional nt sequence of the 5'-flanking region of the human *PAI-1* gene.
The nt sequence extends the *PAI-1* 5'-flanking DNA sequence described by Bosma et al. (1988) by 1642 bp. DNA sequences were obtained as described by Bosma et al. (1988) using the subclone pPAI-E7.0. Positions (nt) are numbered negatively with the -1 position being the nt 5'-adjacent to the *tsp* (position +1) reported by Bosma et al. (1988) and extends to nt -3162. The position of the *PAI-1/t-PA* (or *MER1b*) element is indicated by the hashed (\\\\\\\\\\) line below the nt sequence, which indicates that the element is located on the strand not shown. The locations of *Alu* elements are indicated above or below the nt sequence, depending on whether the element is located on the *PAI-1* gene coding or non-coding strand (Bosma et al., 1988). The nt sequence reported here is available from GenBank/EMBO data banks, accession No. M 55991.

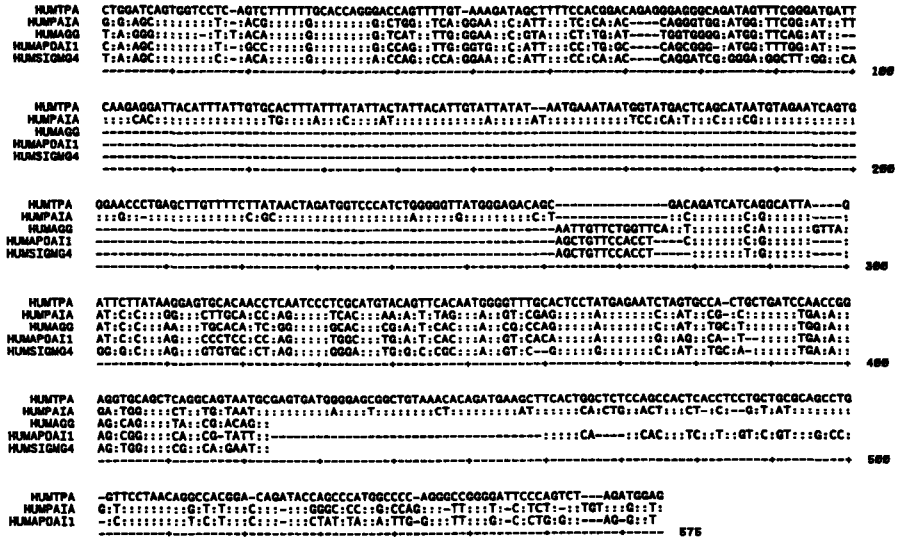

Figure 2. Alignment of *PAI-1/t-PA* or *MER1* elements from the genes encoding PAI-1 and t-PA and from related elements found in GenBank.

The alignment used is similar to that reported by Jurka (1990). Sequence identity in positions is indicated by a colon (:) and gaps, used to maximize identity, are indicated by a dash (-). The nt from each element is shown for positions that contain a nt difference. The gene sequences from which the *MER1* elements were obtained are listed by their GenBank nomenclature: HUMTPA, gene encoding human t-PA (nt 40 to 585, Friezner Degen et al., 1986); HUMPAIA, gene encoding human PAI-1 (nt -1010 to -1571, this report); HUMAGG, gene encoding human angiogenin (nt -716 to -970, Kurachi et al., 1985); HUMAPOAI1, gene encoding human apolipoprotein A4 (nt 6395 to 6051, Protter et al., 1984); and HUMSIGMG4, gene encoding immunoglobulin heavy chain constant region sigma(γ4) (nt 176 to 420, Akahori et al., 1988).

digested with either *Hind*III or *Eco*RI. The hybridization results (Fig. 3) show a large number of hybridizing bands (even after several washes). Fig. 3 also show the hybridization results expected for a single copy gene when a similar blot was probed with the cloned *PAI-1* cDNA insert (van den Berg et al., 1988). Thus, the results shown in Fig. 3 support the suggestion that the *MER1* element, or some part of it, is represented many time in the human genome.

(d) The *PAI-1 MER1b* element appears not to be important for expression

Previously we speculated that the *PAI-1/t-PA* or *MER1b* element in the *PAI-1* 5'-flanking DNA may play a role in regulating the expression of this gene. Various *PAI-1* promoter constructions, using nt upto -800 (Fig. 1, Bosma et al., 1988), have been tested in several different types of cell lines, including HT-1080 cells (Riccio et al., 1988),

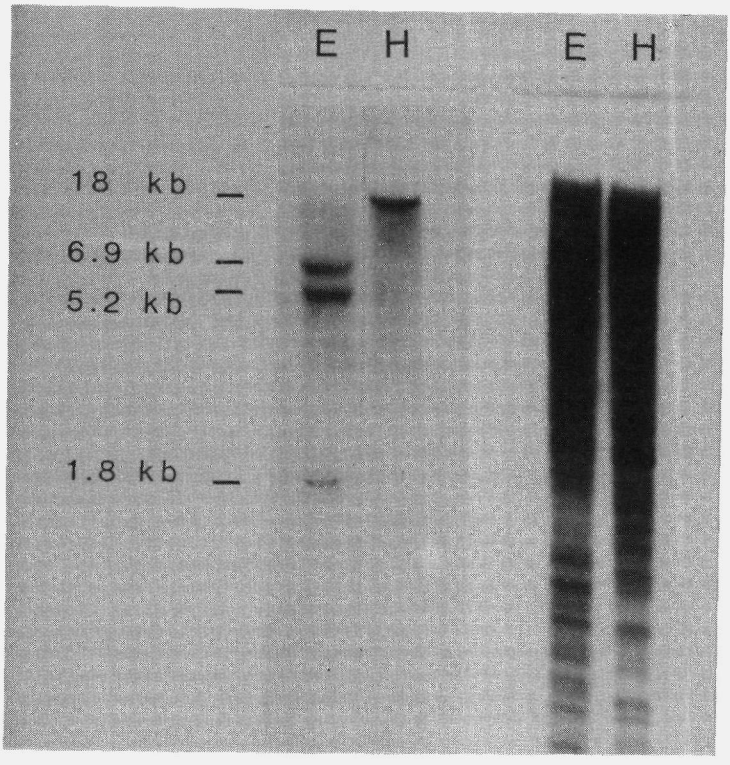

Figure 3. *Genomic Southern blot hybridization using the PAI-1/t-PA or MER1b element as probe.* Ten μg total human DNA isolated from leukocytes (Maniatis et al., 1982), was digested with either *Eco*RI (E) or *Hind*III (H). After electrophoresis the DNA was blotted onto Hybond N and hybridized overnight against either ^{32}P-labeled*PAI-1* cDNA (van den Berg et al., 1988) (shown on the left) or the *PAI-1 MER1b* element contained within the DNA fragment starting at the *Nhe*I site at nt -1562 to the *Xmn*I site at nt -1171 (shown on the right). These DNA fragments were ^{32}P-labeled using the random primer kit obtained from Amersham. Hybridizations were performed using 1 ng/ml of ^{32}P-labeled probe (2 x 10^8 cpm/μg) at 65°C in 1 mM EDTA; 7% SDS; 0.5 M $NaHPO_4$-NaH_2PO_4(pH 7.2). After hybridization for 18 hours, the blot was washed twice (15 min each) at 65°C with each of the following solutions: 2 x SSC, 1% SDS; 1x SSC, 1% SDS; 0.4 x SSC, 1% SDS, and 0.1 x SSC, 1% SDS. The filter was then exposed to Kodak XAR film using a Du Pont Quanta III intensifying screen for 24 h.

(HeLa and rat FTO2B cell lines (van Zonneveld et al., 1988)), and COS cells (Follo and Ginsburg, 1989). We have also tested *PAI-1* promoter constructions using nt between -4.3 to -114 bp to drive the expression of the gene encoding chloramphenicol acetyltransferase in hepatoma, endothelial, and fibrosarcoma cell lines (Bosma, 1991). Expression levels obtained from these various *PAI-1* promoter constructions showed that the presence or absence of the *PAI-1 MER1b* element (nt -1571 to -1010, in Fig. 1) does not substantially affect basal *PAI-1* expression levels in these cell lines. A similar conclusion can be deduced from the results described for *PAI-1* expression in HeLa and

rat FT 028 cells (Van Zonneveld et al., 1988). Thus the *PAI-1 MER1b* element appears not to play a role in regulating the expression of the *PAI* gene; however, we can not rule out the possibility that these results are due to the cell systems used, because these cell systems may not represent the correct cellular environment. *In vivo*, analyses have revealed a correlation between PAI-1 and t-PA levels in plasma (Juhan-Vague et al., 1987) and a diurnal fluctuation in their activities (Kluft et al., 1985). Thus, additional experiments, possibly using transgenic animal models may be needed to definitively determine if the *PAI-1 MER1b* element plays a role in *PAI-1* expression or if its positioning in the 5'-flanking region of this gene (and presumably others) is merely coincidental.

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Appendix to Chapter 3.

FUNCTIONAL ANALYSIS OF PAI-1 5'-FLANKING DNA

INTRODUCTION

To identify the DNA regions involved in the regulation of (basal) PAI-1 gene expression, we dissected the 5'-flanking region of this gene by progressively deleting the upstream sequences in the 5' to 3' direction. The ability of these segments to drive the expression of the bacterial gene coding for chloramphenicol acetyl transferase (CAT) was tested in short-term (transient) expression experiments. Plasmid constructs containing the progressively deleted PAI-1 regulatory regions fused to the cat gene were transfected into the human hepatocellular cell line Hep G2 (Knowles et al., 1980), the human endothelial hybrid cell line EA.hy 926 (Emeis and Edgell, 1988), and the human fibrosarcoma cell line HT-1080. Two of these cell lines, Hep G2 and EA.hy 926, were chosen because they might represent the cell types involved in the production of plasma PAI-1 (Van Hinsbergh et al., 1988; Kooistra 1990; Emeis and Edgell, 1988). The third cell line, HT-1080, served as a reference because of its frequent use in PAI-1 regulatory studies (Andreasen et al., 1987; Riccio et al., 1988).
The relative promoter activities of the various deletion mutants were determined by enzymatic CAT assay.

MATERIALS AND METHODS

Construction of PAI-1/CAT plasmids

The various PAI-1/CAT fusion constructs were made by ligation of HindIII linkers to the EcoRI site, made blunt by endfilling, of a 4.4 kb BglII-EcoRI fragment of the PAI-1 promoter. The resulting HindIII-BglII fragment was cloned into the HindIII/BamHI sites of pKTCAT, a plasmid containing the BamHI/HindIII CAT fragment of pSVO CAT (Gorman et al., 1982) cloned into the AATII-HindIII sites of pUC18. From the resulting plasmid, pKTPAI smaller constructions were generated, using the AvaII sites at -114, -343, -489 and -1023, the ApaI sites at -524 and -594, and the NheI site at -1561. After restriction enzyme digestion the sites were made blunt by endfilling with Klenow. All of the constructs have identical 3'-ends (the EcoRI site at position +75; Bosma et al., 1988).

Presentation of CAT assay data

Between the different transfection experiments, a considerable variation in CAT activity per mg of cell protein was found. Similar observations, among others for Hep G2, were reported by Ginot et al. (1989). To overcome variations due to differences in transfection efficiency in independent experiments and to normalize the expression of the different gene constructs, two approaches were chosen. Firstly, in all experiments each plasmid was co-transfected with a plasmid containing the E.coli β-galactosidase gene. β-Galactosidase activity was assayed spectrophotometrically, using O-nitrophenyl-D-galactosidase as a substrate. Secondly, promoter activity of the various constructs was expressed as a percentage value of maximal cat gene expression within an experiment. With both approaches, inter-experimental variation was minimized, and a very consistent cat gene expression profile was obtained for each of the three cell lines. In general, β-galactosidase expression was too low in EA.hy 926 cells for accurate correction of differences in transfection efficiency. For that reason, promoter activity of each construct is given as a percentage value of the most efficient construct within each cell line.

RESULTS AND DISCUSSION

The results of the expression studies are summarized in Fig. 1. This experiment showed that in Hep G2 cells, progressive deletions in the 5' to 3' direction from -4.3 kb down to position -343 do not substantially alter promoter activity. Deletion of the sequences from position -343 to -114 causes a 10-fold decrease of expression. In EA.hy 926 and HT-1080 cell lines, the results are for the most part similar to that found in the Hep G2 cell line, except that no sharp drop in promoter activity was observed following deletion of the sequences from position -343 to -114 (see Fig. 1). The discrepancy with respect to the promoter activity of the PAI-1 region between positions -343 to -114 in Hep G2 cells and EA.hy 926 and HT-1080 cells might be due to the absence of detectable activator protein-2 (AP-2) activity in Hep G2 cell line (Imagawa et al., 1987). The transcription factor AP-2 can bind to AP-2 binding elements of a gene and be responsible for basal activity of a promoter (Imagawa et al., 1987). Two putative AP-2 binding elements are located between positions -114 to +75 of the PAI-1 promoter and thus could act as basal enhancers in EA.hy 926 and HT-1080 cells. The lack of AP-2 activity in Hep G2 would prevent such action and thereby keep expression at a low level. If this truly explains the results found in the Hep G2 cell line, it would suggest that sequence elements important for PAI-1 gene expression are located between positions -343 to -114.

The analysis presented here clearly show that the presence or absence of the PAI-1/t-PA element, located between position -1593 and -1019, does not substantially affect basal PAI-1 expression.

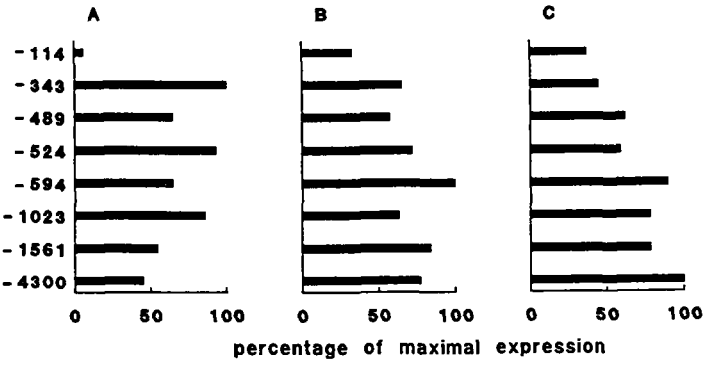

Figure 1. The relative CAT activity of the PAI-1-cat promoter constructs transfected into Hep G2 (Frame A), HT-1080 (Frame B), and EA.hy 926 (Frame C) cell lines.
Two days prior to transfection, cells were seeded out at a density of 2 to 4 x 10^5 cells per well (10 cm^2), and the medium [Dulbecco's modification of Eagle's medium, supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2 mM)] was changed 24 hr before transfection. All three cell lines were transfected with the phosphate precipitation method as described by Graham and Van der Eb (1973). The DNA-calcium phosphate precipitate was prepared with 10 µg of supercoiled plasmid DNA. Per well, 1 µg of DNA was added, and 48 hr after transfection the cells were harvested and protein extracted, the CAT activity was determined according to the procedure described by Gorman et al. (1982). Per assay 1 to 5 µg of protein, as determined by a standard Bradford assay (Bio Rad), was used and the reaction was allowed to proceed for 0.5 to 2 hr. Before measuring incorporation of ^{14}C-chloramphenicol, samples were heated to 65°C for 5 min to destroy deacetylating activity (Crabb and Dixon, 1987). The numbers on the left of the figure refer to the position (from Fig. 1) of the 5'-end for each PAI-1-cat construct tested. The CAT activities of each construct are given as a percentage of the most efficient construct within each cell line. Each bar represents the mean, out of four independent transfection experiments carried out in triplicate, the standard deviation in all cases was less than 30%.

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CHAPTER 4

PLASMINOGEN ACTIVATOR INHIBITOR 1: BIOSYNTHESIS AND mRNA LEVEL ARE INCREASED BY INSULIN IN CULTURED HUMAN HEPATOCYTES

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SUMMARY

Clinical studies have shown that plasma insulin levels are closely related to plasma plasminogen activator inhibitor 1 (PAI-1) levels. To investigate a possible involvement of hepatocytes we have studied the effect of insulin on PAI-1 production by primary cultures of human hepatocytes. We have isolated human hepatocytes from seven left liver lobes. PAI-1 activity measured in 24 hours conditioned medium varied considerably between the various hepatocyte preparations (from 2.9 to 8.5 units per 5 cm^2 of cells) possibly as a result of interindividual variability in basal PAI-1 production by hepatocytes from different donors. In all cases, however, the relative extent, time profile and dose-dependency of the insulin-induced increase in PAI-1 synthesis were consistent. Up to about 7 nM, insulin dose-dependently increased both PAI-1 activity and PAI-1 antigen production. The increase in PAI-1 synthesis became measurable between 4 and 8 hours after addition of the hormone, and maximally reached two-fold control values. The increase in PAI-1 synthesis could be fully explained by a concomitant increase in PAI-1 mRNA levels. The effect of insulin seems fairly specific for the synthesis of PAI-1: overall protein synthesis and mRNA levels of some control proteins (albumin and fibrinogen) did not markedly change after insulin addition. These results, obtained with primary cultures of human hepatocytes, are fully comparable with those obtained with the hepatocellular carcinoma cell line Hep G2. They strengthen the suggestion that the elevated level of PAI-1 in high insulin plasma might be the result of increased hepatic synthesis of PAI-1.

INTRODUCTION

Plasminogen activator inhibitors may serve an important regulatory role in controlling the fibrinolytic activity in blood. Plasminogen activator inhibitor 1 (PAI-1) appears to be the main physiological inhibitor of PA activity in plasma (1). Elevated levels of PAI-1 are frequently associated with thrombotic state including deep venous thrombosis (2-5), coronary artery disease (6) and the post-operative phase (7). A report by Hamsten et al. (8) suggests that increased plasma levels of PAI-1 have a predictive value for the recurrence of myocardial infarction. For future design of rational therapeutic approaches for the prevention of thrombotic diseases, therefore, insight into the regulation of PAI-1 levels in plasma is required.

Clinical studies have indicated that plasma insulin levels are closely correlated to PAI-1 levels (9,10). In recent studies it has been demonstrated that insulin time- and dose-dependently stimulates PAI-1 synthesis in the human hepatocellular carcinoma cell line Hep G2, but not in cultured human umbilical vein endothelial cells (11). Although the

cell line Hep G2 has retained the capacity to synthesize a variety of plasma proteins, including those of fibrinolysis and coagulation (12-15), it remains to be confirmed that the same regulatory mechanisms are operative in normal human hepatocytes. In this report we demonstrate that insulin enhances PAI-1 production in primary cultures of normal human hepatocytes in a very similar way to that found with Hep G2 cells. We also show that the insulin-induced increase in PAI-1 synthesis in both the normal and the transformed hepatocyte cultures is accompanied by elevated PAI-1 mRNA levels.

MATERIALS AND METHODS

Materials

Fetal bovine serum, kanamycin and cell culture media were purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, UK. Penicillin and streptomycin were obtained from Boehringer Mannheim, Mannheim, FRG. 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was from Merck, Darmstadt, FRG. Plastics were obtained from Costar, Cambridge, MA. Collagenase (type 1), bovine serum albumin, insulin (from bovine pancreas), and dexamethasone were purchased from Sigma Chemical Company, St. Louis, MO. [^{35}S]Methionine (1084 Ci/mol) was supplied by New England Nuclear Corp., Boston, MA, and deoxycytidine 5[α-^{32}P] triphosphate from Amersham, International plc, Buckinghamshire, England, UK.

t-PA (> 99% two-chain) was purified from Bowes-melanoma-cell culture medium as described by Kluft et al. (16). The activity is expressed as international units (i.u.), using the first international standard of the World Health Organisation, code 83/517, as a standard (17). Our t-PA preparation has a specific activity of 500,000 i.u./mg.

An enzyme immunoassay research kit for determination of human PAI-1 antigen was kindly provided by Biopool, Umeå, Sweden. All other chemicals were of the highest quality commercially available.

Cells

Hep G2 cells, an established cell line derived from a human liver tumour, were kindly provided by Dr. B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA). Cells were grown in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU of penicillin/ml and 100 μg of streptomycin/ml under 5% CO_2/95% air atmosphere (18).

Human hepatocytes were isolated from pieces of the left lobes of livers, essentially as described before (19-21). The conditions described below for the isolation of hepatocytes from human liver reflect the accumulated experience with isolation and culture of these cells. The pieces of liver were obtained through the Auxiliary Partial Liver

Transplantation Program carried out at the University Hospital Dijkzigt at Rotterdam, The Netherlands. Permission for use of the non transplanted part of the donor liver for scientific research was given by the Medical Ethical Committee. The livers were taken from seven physically healthy organ donors, who died after brain hemorrhages or severe traumatic brain injury. Informed consent of relatives of the donors was obtained. Relevant information available on the liver donors is summarized in Table 1. A 120-450 g segment of liver tissue was excised and placed in ice-cold buffer containing 10 mM HEPES, pH 7.4, 132 mM NaCl, 6.7 mM KCl, and 20 mM glucose. Care was taken that the removed pieces were surrounded by a hepatic capsule on all sides except for one cut surface. Perfusion of the liver with 3 litres of the HEPES buffer at a rate of 150 ml per min (30-40 ml/min per catheter) was started after insertion of four polyethylene catheters (14-18 gauge) into vascular orifices at the cut surface. After the preperfusion, the liver was perfused successively with 500 ml of a HEPES buffer, pH 7.6, containing 100 mM HEPES, 67 mM NaCl, 6.7 mM KCl, and 5 mM $CaCl_2$ without recirculation, and with 200 ml of this buffer containing 0.05% and 0.1% collagenase respectively with recirculation for 20 min and 20-40 min, respectively. After the perfusion was completed, the tissue was dissociated with sterile scalpels in Hanks buffer containing 2% bovine

Table 1. Human hepatocytes isolated from physically healthy organ donors. Characteristics of the donors, cell yield and viability, and PAI-1 activity in 24 h conditioned medium.

Donor	Age (years)	Male/ female	Cause of death	Cell yield	Viability (%)	Cell protein ($\mu g/5\ cm^2$)	PAI-1 act. (u/ml)	PAI-1 act. (u/mg cell protein)
A	18	F	trauma^{+}	13.0×10^8	70	236	8.3	35.2
B	35	F	hemorrhage^{++}	13.0×10^8	81	286	3.9	13.6
C	15	M	trauma	17.0×10^8	74	265	2.9	10.9
C*						N.D.	3.9	
C**						N.D.	3.3	
D*	42	M	hemorrhage	48.0×10^8	75	530	4.6	8.7
E*	19	M	trauma	8.4×10^8	77	167	5.9	35.3
F*	29	M	trauma	6.4×10^8	53	247	8.5	34.4
G*	36	F	trauma	32.0×10^8	74	328	3.2	9.8

$^{+}$ = severe traumatic brain injury

$^{++}$ = brain hemorrhage

* = fibronectin coating (30 min at 37°C with a 50 µg/ml fibronectin solution)

** = gelatine coating (30 min at 20°C with a 1% gelatine solution)

N.D. not determined

serum albumin. Cells were filtered through a 200 μm filter, and hepatocytes were purified by differential centrifugation (75 × g for 1 min), followed by three washes in cold (4 °C) Williams E medium to remove damaged and residual non-parenchymal cells. Total cell yields, as estimated by counting the cells in a hemocytometer chamber, varied from 6.4-48 × 10^8 cells for the various isolations. Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%), was 53-81% for the seven hepatocyte isolations (Table 1). The cells were seeded on tissue culture dishes at a density of 1.25 × 10^5 cells per cm^2 and were maintained in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum (30 min at 56 °C), 2 mM L-glutamine, 20 mU insulin per ml (135 nM), 50 nM dexamethasone, 100 IU penicillin per ml, 100 μg kanamycin per ml and 100 μg streptomycin per ml at 37 °C in a 5% CO_2/95% air atmosphere. After 10-16 hours, the non-adherent cells were washed from the plates; the same culture medium as above, but without insulin and dexamethasone, was added, and renewed every 24 h thereafter. The presence of both insulin and dexamethasone during the attachment period was found to be beneficial for cell attachment and viability of the isolated cells. The initially spherical hepatocytes aggregated and took their characteristic polygonal shape within 24 to 40 hours after isolation.

The cells were characterized as hepatocytes on the basis of their morphology and the synthesis of albumin, fibrinogen and apolipoprotein A-1. Hepatocyte cultures were at least 95% pure, as judged by phase contrast microscopy after 72 h of culture.

Cell culture experiments

With Hep G2, confluent cell cultures were used, and cells were always refed the day before the experiment with the appropriate incubation medium, i.e. DMEM, supplemented with 10% heat-inactivated fetal bovine serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Conditioned media (CM) were obtained by incubating cells in 5 cm^2 dishes with 1 ml of incubation medium in the absence or presence of test compound for various times up till 48 h at 37 °C. CM were centrifuged for 2 min in a Beckman Microfuge centrifuge to remove cells and cellular debris, and samples were frozen at -20 °C until use. Cell lysates were obtained by washing the cells twice with ice-cold phosphate-buffered saline (PBS), and scraping the cells off the dish with a rubber policeman in the presence of ice-cold buffer, containing 0.5% (w/v) Triton X-100. Cell lysates were also frozen at -20 °C until use. In some cases, hepatocytes were dissolved in 0.2 M NaOH.

Human hepatocytes were used for experiments after maintaining the cells for 24 h in culture medium without dexamethasone and insulin. Incubation medium consisted of Williams E medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU penicillin per ml, 100 μg kanamycin per ml and 100 μg streptomycin per ml, with or without test compound. Insulin concentrations in various commercial lots

of fetal bovine serum have been reported to be 10.1 ± 4.5 μU/ml (mean ± S.D.; n = 7) (22) which was considered negligible as compared to insulin concentrations used in our experiments. CM and cell lysates were obtained as described above for Hep G2.

During the incubation period used in our experiments, we and other investigators have shown that human hepatocytes retain specialized biochemical functions, like linear or increasing albumin synthesis (23,24), constant cytochrome P-450 content (24,25), modulation of LDL (21,26,27) and insulin (28) receptors, and the induction of the synthesis of acute phase proteins by interleukin-6 (29,30). Furthermore, cells were viable during this culture period, as judged by leakage of the cytoplasmic enzyme lactate dehydrogenase (21).

Protein synthesis

Overall protein synthesis was determined by measuring the incorporation of [^{35}S]methionine into the 10% (w/v) trichloroacetic acid precipitable fraction of radiolabelled CM and cell lysates.

Assays

PAI-1 activity was determined by the method of Verheijen et al. (31): samples were titrated with increasing amounts of t-PA, followed by spectrophotometric measurement of the residual t-PA activity. One unit of PAI-1 is defined as the amount of inhibitor that neutralizes one i.u. of t-PA activity.

PAI-1 antigen determination was performed with an EIA (research kit provided by Biopool). This assay detects both active and "latent" (inactive) forms of PAI-1. The detection level is below 1 ng/ml in the undiluted sample.

Protein in the cell lysates was determined according to Lowry et al. (32) or, if Triton X-100 was present in the sample, by a modification of this method as described by Wang and Smith (33). In both cases, bovine serum albumin was used as a standard.

RNA hybridization

Total RNA was extracted from hepatocytes as described by Lizardi and Engelberg (34), with some minor modifications. After washing the cells with warm (37°C) PBS, lysis buffer (50 mM Tris•HCl, pH 7.4; 100 mM NaCl; 7.5 mM EDTA; 0.5% SDS; 150 μg/ml proteinase K) was added directly to the cells in the culture dish, followed by an incubation for 30 min at 37°C. RNA was collected by ethanol precipitation and purified from contaminating DNA by precipitation in 2 M LiCl for 3 hours at 0°C. After a second ethanol precipitation step, RNA samples were dissolved in H_2O. The RNA concentration in each sample was determined either spectrophotometrically or with the aid of a specific rRNA cDNA probe (gift of Dr. B. Bakker, Department of Human Genetics, State University Leiden, The Netherlands).

Equal amounts of total RNA from the different dishes were analyzed for their PAI-1, albumin and fibrinogen mRNA content by RNA dotblot and Northern blot hybridization.

With Northern blotting, RNA samples were subjected to gel electrophoresis in formaldehyde agarose gels, as outlined in Maniatis et al. (35). After electrophoresis, RNA was transferred to Genescreen-plus or Hybond N according to the instructions of the manufacturer. Pre-hybridization and hybridization were at 60°C (1 mM EDTA; 5% SDS, 0.5 M NaH_2PO_4-Na_2HPO_4; pH 7.2) (modified from 36). DNA fragments to be used as a probe were isolated from low melting agarose (35). Hybridization was usually performed with 1 ng/ml of probe labelled by random prime labelling to approximately 2×10^8 cpm/μg DNA (35).

The probes used were a 2500 bp PAI-1 cDNA fragment described by Van den Berg et al. (37), a cDNA probe for albumin (38; gift of Dr. H. Pannekoek), and a cDNA probe for fibrinogen [gift of Dr. S. Lord (39)].

The filters were washed at a stringency of 0.2 × SSC and 1% SDS for 2 × 15 min at 60°C (35). The membranes were subsequently exposed to a Kodak XAR5 or XRP film with an intensifying screen at -80°C. For the quantification of the relative amounts of mRNA, densitometry was used. In short, a scan of the bands was made on a CS 910 Shimadzu scanner and the areas under the peaks were integrated and plotted with the aid of a United Technology Packard data processor. The concentration of the mRNA for PAI-1, albumin and fibrinogen in hepatocytes after incubation with insulin was expressed relative to corresponding levels of mRNA in control cells.

With dot blot hybridization, serial dilutions of total RNA preparations of cultured hepatocytes were applied directly to Hybond N using a manifold filtration apparatus (Schleicher & Schuell). Hybridization and quantification were performed exactly as described above for Northern blotting hybridization.

Statistical methods

Statistical analyses were carried out using Student's t-test. All data are shown as mean ± S.D. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Isolation and culture of human hepatocytes

The information available on the history of the liver donors and the characteristics of seven human hepatocyte preparations are shown in Table 1. Cell yield varied from 6.4 to 48 × 10^8 hepatocytes with a viability of 53 to 81%. Examination of the cultured hepatocyte monolayers by phase-contrast microscopy at 6 hours after seeding showed that the hepatocytes had attached to the plastic, but that they still had a rounded contour. At

40 hours after seeding, the hepatocytes had aggregated and spread out with a characteristic polygonal shape. Attachment of the cells to the dishes was found to be strikingly enhanced if the culture dishes had been coated with gelatine of fibronectin. In addition, the subsequent spreading of the cells was promoted by the presence of gelatine or fibronectin. As the coating of the dishes had no differential effect on the insulin-stimulated PAI-1 synthesis, fibronectin coating of the culture dishes was routinely introduced into the culture protocol.

Effect of insulin on PAI-1 production by human hepatocytes

Figure 1 shows a typical experiment, in which the accumulation of PAI-1 activity in human hepatocyte CM is followed in time. After an initial rise, PAI-1 activity reaches a plateau level as a result of instability of the inhibitor molecule (19). The amount of PAI-1 activity measured in 24 hours CM of the various hepatocyte preparations differed from 2.9 to 8.5 units per 5 cm^2 of cells (Table 1). The difference in PAI-1 production cannot be explained on the basis of variation in cell density, as measured by protein content, nor can it be explained by the sex or age of the donors. Apparently, interindividual variability exists in basal PAI-1 production by hepatocytes from different donors.

When the hepatocytes were incubated with insulin, an enhanced PAI-1 secretion started about 4 to 8 hours after addition of the hormone (Fig. 1). The effect of insulin was dose-dependent up to about 7 nM, reaching a maximal stimulation of PAI-1 activity in 24 hours CM of about two-fold (Fig. 2). The insulin-induced increase in PAI-1 accumulation in the medium was accompanied by similar increases in PAI-1 activity in the corresponding cell extracts: in six independent experiments with insulin-treated hepatocytes, PAI-1 activity levels in 24 hours CM and cell extracts were 177 ± 14% and 212 ± 13% of control values (mean ± S.D.), respectively.

The identity of the PA-inhibitory activity in CM from control and insulin-treated hepatocytes was verified in an experiment in which specific monoclonal antibodies raised against PAI-1 were added to the CM prior to the activity assay: in this case complete quenching of the PA-inhibitory activity occurred, indicating that we are dealing with PAI-1 activity only.

PAI-1 in hepatocyte CM can exist in two forms: an active and an inactive form (19). To exclude the possibility that the effect of insulin is the result of a change in the ratio active vs inactive PAI-1, rather than a change in net synthesis, we also analysed the CM of a number of representative experiments for the presence of PAI-1 antigen (Table 2). 135 nM insulin-treated cells produced 1.9 ± 1.0 (mean ± S.D.; n = 5) times more PAI-1 antigen and 2.2 ± 0.9 (mean ± S.D.; n = 5) times more PAI-1 activity than control cells. These values are not significantly different, and indicate an increase in net PAI-1

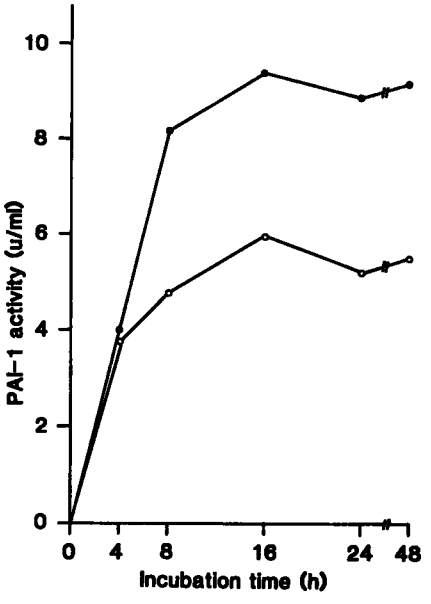

Figure 1. Time course of accumulation of PAI-1 activity in conditioned medium of primary cultures of human hepatocytes incubated in the presence (●) or absence (○) of 135 nM insulin. The values represent the mean value of a duplicate determination for a single, representative experiment.

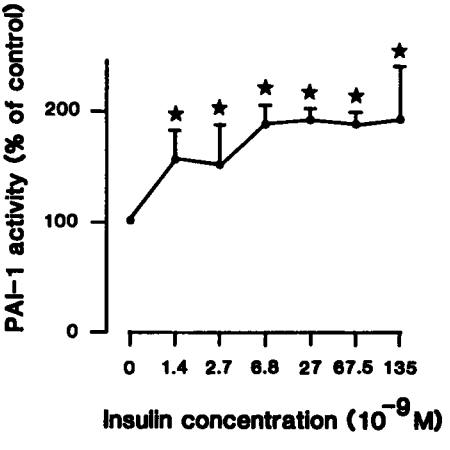

Figure 2. Effect of insulin (1.4 to 135 nM) on PAI-1 activity in 24 hr conditioned medium of primary cultures of human hepatocytes. Data are expressed as % values relative to control values. Each value represents the mean and the vertical bar S.D. of 5-17 incubations with cells from 3-7 isolations. *Statistical significance of increase in production of PAI-1 activity for each insulin concentration tested as compared to control: $p < 0.001$.

synthesis. Concordantly, the ratio between the amount of active PAI-1 and PAI-1 antigen (expressed as units per ng of PAI-1 protein) is not significantly different between 24 hours CM from control cells and insulin-treated cells (means ± S.D. were 6.3 ± 1.6 × 10^{-2} (n = 5) and 7.5 ± 2.0 × 10^{-2} (n = 5) u/ng, respectively).

To eliminate the possibility that the changes in PAI-1 production were the result solely of a general change in protein synthesis, the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable protein in cells and medium was measured. There was little effect of insulin on total protein synthesis (values in the range of 83%-107% of the control after 24 hours of incubation in the presence of 135 nM insulin) that could explain the observed results, suggesting that the action of insulin is a restricted and a fairly specific one.

PAI-1 mRNA in insulin-treated Hep G2 and hepatocytes

To investigate whether the insulin-increased PAI-1 synthesis is the result of a specific increase in PAI-1 mRNA, Hep G2 and hepatocytes were incubated with or without 135 nM insulin, and RNA was extracted for mRNA analysis. The RNA on the Northern blots and dot blots were hybridized with PAI-1 cDNA and, as a control, with cDNAs for albumin and fibrinogen (Figs. 3 and 4). In a similar manner to that of human endothelial cells (37), the PAI-1 gene in Hep G2 and human hepatocytes is transcribed into two major mRNA's of 2.3 and 3.2 kb, respectively; in a number of cases, another, smaller

Table 2. Effect of insulin on production of PAI-1 activity and PAI-1 antigen by cultured human hepatocytes. Data represent analysis of 24 h conditioned media of five independent experiments.

Sample	PAI-1 activity (u/ml)		PAI-1 antigen (ng/ml)	
	control	+ 135 nM insulin	control	+ 135 nM insulin
I	7.9	11.0	150	184
II	9.5	17.4	184	259
III	6.4	10.4	108	189
IV	3.8	10.0	63	99
V	3.5	12.9	38	142

In the presence of 135 nM insulin, the production of PAI-1 activity and PAI-1 antigen increases 2.2 ± 0.9 and 1.9 ± 1.0 times, respectively (mean ± S.D.; n = 5). These values are not significantly different.
The ratio between active PAI-1 and PAI-1 antigen (expressed as u per ng of protein) in conditioned media from control and insulin-treated cells is 6.3 ± 1.6 x 10^{-2} and 7.5 ± 2.0 x 10^{-2}, respectively (mean ± S.D.; n = 5). These values are not significantly different.

mRNA species was detected (Fig. 3). Both the upper and lower PAI-1 mRNA band increased on incubation of Hep G2 and hepatocytes with insulin, and as shown for human hepatocytes, this profile is maintained during various incubation times (Fig. 3).

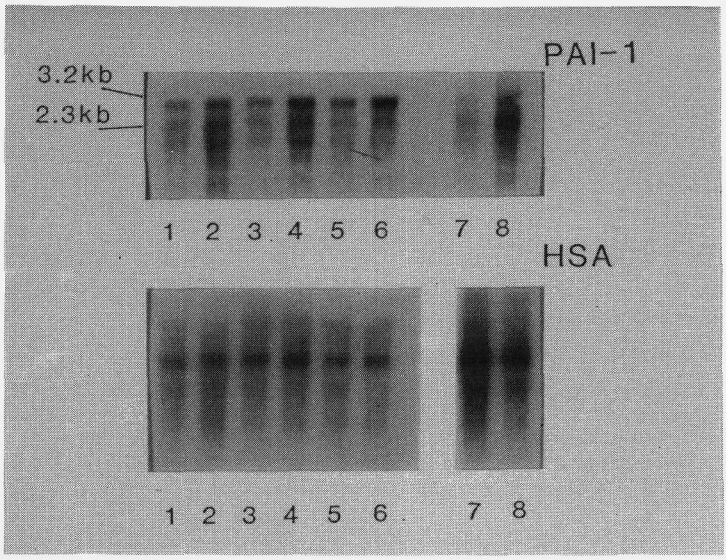

Figure 3. Effect of insulin on PAI-1 mRNA and human serum albumin mRNA levels in primary cultures of human hepatocytes (lanes 1-6) and in Hep G2 (lanes 7 and 8) as visualized by Northern blot analysis. Total cellular RNA was isolated from cells incubated for various times in the presence or absence of 135 nM insulin and hybridized to PAI-1 or albumin cDNA. Approximately 2 μg of RNA were loaded in each lane. Lane 1: control (4 h); lane 2: insulin (4 h); lane 3: control (8 h); lane 4: insulin (8 h); lane 5: control (16 h); lane 6: insulin (16 h); lane 7: control (16 h); lane 8: insulin (16 h).

For a reference, the (constant) albumin mRNA levels in the same samples are also shown. Figure 4 shows the time profile of the effect of 135 nM insulin on PAI-1 mRNA content in Hep G2 and human hepatocytes for a representative experiment in which mRNA levels were quantified by densitometry. With both cell cultures, an enhanced PAI-1 mRNA level was already detectable 4 hours after addition of insulin. This is in agreement with the observed increase in PAI-1 activity in the medium between 4 and 8 hours after addition of insulin as shown in Fig. 1. Similarly, the maximally 2- to 3-fold increase in the level of PAI-1 mRNA is comparable with the observed increases in PAI-1 activity and PAI-1 antigen as shown in Figs. 1-2 and Table 2. Insulin had no marked effect on steady-state levels of albumin mRNA and fibrinogen mRNA during a 24-hours

incubation period. For example, in three experiments with insulin-treated hepatocytes, albumin mRNA and fibrinogen mRNA levels were 119 ± 17% and 106 ± 29% of control values (means ± S.D.), respectively.

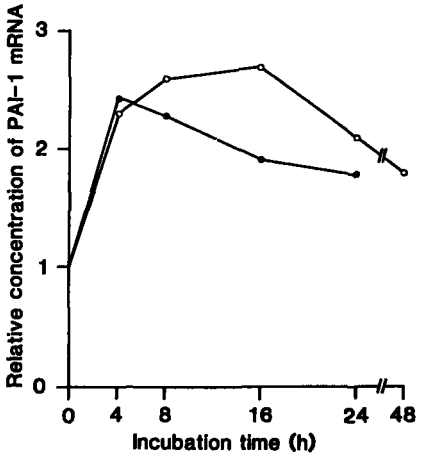

Figure 4. Time course of PAI-1 mRNA levels in primary cultures of human hepatocytes (●) and in the hepatoma cell line Hep G2 (○) when incubated in the presence of 135 nM insulin. Quantification of mRNA levels was performed as described in the section Materials and Methods. The data shown are from a representative experiment in which PAI-1 mRNA levels in insulin-treated cells are expressed relative to PAI-1 mRNA levels in cells incubated for the same time but without insulin.

DISCUSSION

In a previous paper (11), insulin was found to stimulate PAI-1 synthesis in the hepatocellular cell line Hep G2. This finding led to two major questions. First, since Hep G2 cells represent transformed liver cells and might, therefore, possess an altered response pattern, would normal adult human hepatocytes react similarly to insulin? Secondly, at what level is PAI-1 synthesis regulated? The existence of the auxiliary liver transplantation program (40) enabled us to investigate these questions by providing us with pieces from seven livers for hepatocyte isolation. Although the basal production of PAI-1 varied considerably among the various hepatocyte preparations (Table 1), possibly as a result of interindividual variety between the donors, the relative extent and the time- and dose-profile of the insulin-induced increase in PAI-1 production were fairly consistent and very comparable to previous findings obtained with Hep G2 (11). In both cell culture systems, the insulin-induced increase in PAI-1 synthesis was about two-fold and dose-dependent in the nanomolar range (Figs. 1 and 2), a concentration range compatible with insulin levels in rat postprandial portal vein plasma (41). The increase

in PAI-1 production became measurable in the CM after a lag period of about 4 hours (Fig. 1). In both hepatocyte cultures, the observed increase in PAI-1 synthesis can be fully explained at the mRNA level: the onset of enhanced PAI-1 synthesis is preceded by an increase in PAI-1 mRNA level and insulin enhances PAI-1 synthesis and PAI-1 mRNA levels to the same extent. No significant influence of insulin on overall protein synthesis could be demonstrated. Moreover, the increase in PAI-1 mRNA levels was without a concomitant change in two arbitrary chosen controls, albumin and fibrinogen mRNA. The effect of insulin thus seems fairly specific for PAI-1.

PAI is known to exist in an active and an inactive form (1,11,19,42). The insulin-induced increase in PAI-1 activity in hepatocyte CM could, however, be confirmed at the level of antigen (Table 2), indicating that insulin enhanced net synthesis of PAI-1 rather than the ratio active over inactive PAI-1. Assuming that 1 unit of PAI-1 activity corresponds to 1.4 ng of antigen (11), it can be calculated from Table 2 that on average about 10% of PAI-1 in 24 hour conditioned human hepatocyte medium represents active PAI-1. The remaining part of the PAI-1 antigen probably represents predominantly free but inactive PAI-1. This finding is in agreement with the idea that PAI-1 is rapidly inactivated upon secretion from cultured cells (42).

It has been found that two mediators of the acute phase response, viz. interleukin-1 (α and β) and tumor necrosis factor, can also induce an increase in plasma PAI-1 levels in vivo (43,44). In contrast to insulin, however, interleukin-1 and tumor necrosis factor increased the synthesis of PAI-1 by cultured human endothelial cells, but did not change PAI-1 production in cultured hepatocytes (11,43,44). These data suggest that endothelial cells and hepatocytes can each be responsible for an increase in plasma PAI-1 levels, with the type of inducer as a discriminating factor.

In conclusion, our results indicate that the elevated level of PAI-1 activity in high insulin plasma may be the result of a specific increase in hepatic PAI-1 mRNA, resulting in increased hepatic PAI-1 synthesis. The strong similarity between human hepatocytes and Hep G2 in expressing the effect of insulin on PAI-1 production suggests that the Hep G2 cell line may be a suitable model system to study regulation of synthesis of PAI-1 and perhaps other fibrinolytic parameters.

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CHAPTER 5

SERUM AND PHORBOL ESTER INCREASE TRANSCRIPTION OF PLASMINOGEN ACTIVATOR INHIBITOR 1 GENE IN HUMAN HEPATOMA CELLS

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SUMMARY

In this study, we have compared the effect of serum and a specific activator of protein kinase C (PKC), phorbol-12-myristate-13-acetate (PMA), on PAI-1 gene expression in the human hepatoma cell line, Hep G2. The following observations support the hypothesis that serum and PMA might act via the same regulatory pathway.

After a lag period of 1 h, both fetal bovine serum (10%) and PMA (100 nM) transiently enhanced PAI-1 mRNA levels in serum-starved Hep G2 cells, albeit with a different time profile and to a different extent. After addition of serum, total PAI-1 mRNA levels increased upto 9-fold at 4 h, remained enhanced upto 8 h, and then decreased to basal levels. With PMA, PAI-1 mRNA levels reached a maximally 32-fold increase after 4 h, followed by an immediate decrease to pre-stimulatory levels. Run-on analysis showed that the increases in PAI-1 mRNA levels by serum and PMA can be fully accounted for by comparable increases in PAI-1 gene transcription rates. With serum, a 10-fold increase in transcription rate is reached after 2.5 h, followed by a gradual decrease to a still 5-fold elevated transcription rate at 7.5 h. With PMA, a 36-fold increased transcription is reached at 2 h, immediately followed by a rapid decline to basal levels at 7.5 h.

Inhibition of protein synthesis by cycloheximide (10 μg/ml) strongly quenched the subsequent induction of PAI-1 gene expression by serum and PMA, suggesting the involvement of newly-synthesized transcription factor(s) in this induction. Since serum and PMA effects on gene transcription are often mediated by transcription factor AP-1, for which an almost perfect binding site is present in the PAI-1 promoter region, we investigated their effect on expression of c-jun and c-fos, the genes coding for the factors contributing to AP-1. It is shown that c-jun and c-fos mRNA levels are transiently increased prior to PAI-1 induction, with the response to serum lower and more persistent than that to PMA.

The results indicate that serum might induce increased PAI-1 gene transcription through an (PKC-dependent) increase in AP-1 synthesis.

INTRODUCTION

Plasminogen activator inhibitor 1 (PAI-1) is one of the major inhibitors of the fibrinolytic system (for reviews see Sprengers and Kluft, 1986; Schleef and Loskutoff, 1988). The association of high plasma PAI-1 levels with deep venous thrombosis (Nguyen et al., 1988), with a high mortality in patients with septic shock (Pralong et al., 1989), and with an increased risk or recurrent myocardial infarction (Hamsten et al., 1987), as well as reports on decreased functional PAI-1 activity in plasma of patients with a bleeding

tendency (Francis et al., 1986; Schleef et al., 1989), suggest an important role for regulation of PAI-1 expression.

Among the cell types that may contribute to plasma PAI-1 levels, the human hepatocyte is a likely candidate (Kooistra, 1990). Primary cultures of human hepatocytes and the human hepatoma cell line Hep G2 show a basal expression of PAI-1, which can be stimulated by various serum growth factors, like insulin (Alessi et al., 1988), EGF (Lucore et al., 1988) and TGF-β (Fujii et al., 1989).

The mechanism by which the increase in PAI-1 production is induced by these serum factors is not well defined. Serum effects can often be mimicked by activators of protein kinase C (Angel et al., 1988; Imbra et al., 1987; Lamph et al., 1988). To gain more insight into the regulation of PAI-1 synthesis in human hepatocytes, we have examined the effect of serum on PAI-1 gene expression in Hep G2 cells, and have compared it with the effect of a specific protein kinase C activator, 12-phorbol-13-myristate acetate (PMA). The increases in PAI-1 mRNA levels by both serum and PMA were paralelled by comparable increases in transcription rates and could be blunted by prior addition of the protein synthesis inhibitor cycloheximide, suggesting that synthesis of nuclear factors may be required for the activation of the PAI-1 gene. In several cell types, serum and PMA have been found to induce the genes of c-jun, c-fos and c-myc, which code for transcriptional activators. We therefore conducted experiments to investigate whether these activators could also be involved in the induction of PAI-1 gene expression by serum and PMA in Hep G2.

MATERIALS AND METHODS

Materials

Hybond N, random primer kit (multi-prime), [^{32}P]-labeled dCTP and UTP, and hyperfilm were from Amersham Corporation. Cycloheximide (CHX) was purchased from Sigma. Enzyme immunoassay kits for determination of human PAI-1 antigen (Imulyse) were obtained from Biopool. The gene clean kit was from Bio 101, Inc, and all tissue culture materials were from Costar.

Cell culture experiments

The human hepatoma cell line Hep G2 (Knowles et al., 1980) was cultured in Dulbecco's modification of Eagles medium (DMEM), supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and glutamine (2 mM) under 5% CO_2/95% air atmosphere at 37 °C (Havekes et al., 1983). For the experiments, confluent cells were used that were treated according to the following protocol. Fourty h before the start of an experiment, fresh complete medium was added to the cells.

Sixteen h before the start, the cells were washed twice with serum-free medium (DMEM, containing 0.1% human serum albumin (HSA), glutamine, penicillin and streptomycin) was added. At the start of an experiment the cells were rinsed once with fresh serum-free medium and fresh medium with test compounds was added.

mRNA analysis

For mRNA analysis the cells were rinsed with phosphate-buffered saline (PBS) and total cellular RNA was isolated according to the method of Chomczynski and Sacchi (1987). 5-10 μg of total cellular RNA, as determined spectrophotometrically, was subjected to gel electrophoresis in formaldehyde agarose cells (Maniatis et al., 1982). After electrophoresis, the RNA was transferred to Hybond N according to the instructions of the manufacturer. Hybridization was with 2 ng/ml of cDNA probes, labeled with ^{32}P by the random primer method to approximately 5 x 10^8 cpm/μg DNA, at 65°C in 0.5 M NaH_2PO_4-Na_2HPO_4 (pH 7.2), 7% SDS, 1 mM EDTA (modified from Church and Gilbert, 1984). After hybridization the filters were washed in three steps. In each step the filters were incubated twice for 30 mins at 65°C in respectively: 2 x SSC, 1% SDS; 1 x SSC, 1% SDS; 0.1 x SSC, 1% SDS (1 x SSC is 0.15 M NaCl, 0.015 M Na-citrate). Quantification was performed by exposing the membranes to hyperfilm with an intensifying screen. The relative intensities of the bands were determined by densitometric scanning.

cDNA probes

The probes used were a 2.5 kb EcoRI PAI-1 cDNA fragment (Van den Berg et al., 1988), a 1.2 kb PstI cDNA fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.3 kb c-myc genomic DNA fragment spanning most of exon 3, a 1.9 kb c-jun cDNA fragment and a 2.6 kb c-fos cDNA fragment, kindly provided by Drs. H. Van Dam, R. Offringa and I. Laird Offringa, University of Leiden (Fort et al., 1985; Van Beveren et al., 1984; Colby et al., 1983). All probes were isolated from low melting agarose and purified using a gene clean kit.

Run-on analysis

Preparation of nuclei for run-on assays was performed as described by Greenberg and Ziff (1984). After isolation, the nuclei were resuspended in glycerol buffer (50 mM Tris, pH 8.3, 40% glycerol, 5 mM $MgGl_2$, 0.1 mM EDTA), frozen in N_2 and stored at -80°C until use. Per run-on assay approximately 5 x 10^6 nuclei were used. The elongation reaction and mRNA isolation were performed according to Nevins (1987), with some minor modifications. In short, the nuclei were incubated for 20 min at 30°C in 200 μl reaction buffer, containing: 20 mM Tris, pH 7.9; 20% glycerol; 140 mM KCl; 10 mM MgCl; 1 mM DTT; 1 mM each of ATP, CTP, GTP; 2 μM [α-^{32}P]-UTP (800 Ci/mM (1

Ci = 3.7 x 10^{10} Bq)); 10 mM creatine phosphate; 20 U/ml creatine kinase and 1000 U/ml RNAsin. The incubation was ended by adding 800 µl HSB buffer: 10 mM Tris, pH 7.4; 0.5 M NaCl; 50 mM $MgCl_2$; 2 mM $CaCl_2$ and 10 U/ml DNAse 1. As soon as the viscosity had disappeared 1 ml of extraction buffer was added: 10 mM Tris, pH 7.4; 1% SDS; 20 mM EDTA. The RNA was isolated by hot fenol extraction. Contaminating DNA was further degraded by DNAse treatment for 20 min at 37°C in 20 mM Tris, pH 7.7; 1 mM $MgCl_2$ and DNAse 1, 50 µg/ml. After fenol extraction the RNA was partially degraded by incubation for 20 min with ice-cold 0.2 M NaOH, and neutralized with HEPES. To remove the unincorporated label, repeated ethanol precipitations with high salt were performed.

Hybridization of labeled RNA to DNA

Linearized DNA (2 µg) was blotted onto Hybond N Filter in alkali blotting solution (1.5 M NaCl, 0.25 NaOH) using a minifold filtration apparatus. Prehybridization and hybridization were performed at 65°C in 0.5 M NaH_2PO_4-Na_2HPO_4, pH 7.2, 7% SDS; 1 mM EDTA. The RNA was hybridized for 65 h, whereafter the filters were washed several times with 2 x SSC and treated with RNAse and proteinase K according to Nevins (1987). Quantification was performed by exposing the filter to Hyperfilm followed by densitometric scanning of the dots.

RESULTS

Time course of serum- and PMA-induced PAI-1 gene expression

Incubation of serum-starved Hep G2 cells for 8 h with increasing concentrations of serum (0 to 40%) or PMA (0 to 200 nM) showed a concentration-dependent increase in PAI-1 mRNA levels, reaching maximal induction at 10-20% serum and 100-200 nM PMA respectively (results not shown). Further experiments were performed with optimally inducing concentrations only, i.e. 10% serum and 100 nM PMA. As shown in Fig. 1, with both serum (A) and PMA (B) PAI-1 mRNA levels are transiently enhanced after a lag period of 1 h, but with a different time profile and to a different extent. After serum addition total PAI-1 mRNA (i.e. 3.2 kb and 2.4 kb) reaches maximally 9-fold increased levels at 4 h, remains at this enhanced level upto about 8 h, and then returns to prestimulatory levels with an apparent halflife time of about 4 h. The induction of PAI-1 mRNA by PMA is much stronger and shorter, reaching maximally a 32-fold increase after 4 h, followed by an immediate decline to prestimulatory levels with an apparent halflife time of about 3 h. Autoradiographs of the Northern blots revealed that the relative abundance of the two PAI-1 mRNA transcripts changed with time after PMA addition (see inset Fig. 1B): at 4 h the 3.2 kb form is predominant, while at later times

the smaller species is more abundant. Such a shift in ratio between the two PAI-1 mRNAs is not seen after serum induction (see inset Fig. 1A).

The effects of serum and PMA were exerted at the level of PAI-1 gene transcription as indicated by the results of nuclear run-on experiments (Fig. 1). With serum, the transcription of the PAI-1 gene is maximally about 10-fold increased at 2.5 h, then decreases slowly, and is still 5-fold enhanced at 7.5 h. The transcription of the PAI-1 gene is maximally 36-fold enhanced by PMA after 2 h, and is immediately followed by a rapid decline to prestimulatory levels at 7.5 h.

Thus the increased transcription rates can account for the majority of the serum/PMA effects on PAI-1 mRNA levels.

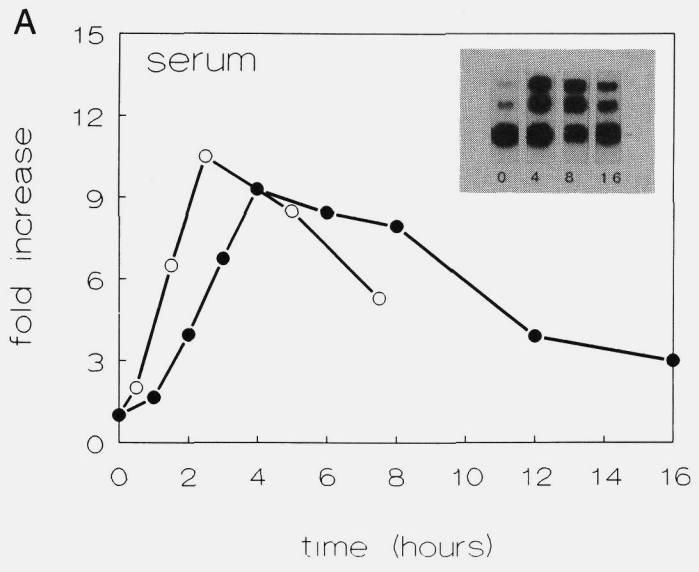
Effect of protein synthesis inhibition on PAI-1 mRNA induction

To establish whether the induction of PAI-1 mRNA by serum and PMA required protein synthesis, Hep G2 cells were incubated with 10 μg/ml cycloheximide (CHX), a concentration sufficient to inhibit protein synthesis for over 95% (not shown). When added 1 hour prior to the inducers, the induction of PAI-1 mRNA seen at 3 h after addition of serum or PMA is completely suppressed by CHX (Fig. 2). Thus the rapid induction of PAI-1 gene by serum and PMA is dependent on protein synthesis. In control cells incubated with CHX alone no effect on PAI-1 mRNA level nor on its transcription was seen (not shown).

Induction of c-jun, c-fos and c-myc mRNA levels

As the serum- and PMA-induced increases in PAI-1 gene transcription required protein synthesis, we sought for possible mediators of this response. Recent evidence indicates that induction of gene transcription by serum and PMA is often mediated by the transcription factor AP-1 (Angel et al., 1988; Lamph et al., 1987; Curran and Franza, 1988). AP-1 is a homo- or heterodimer of two proto-oncogene products, Jun and Fos, for which specific binding sites have been identified (Angel et al., 1987, 1988; Rauscher et

Figure 1. Time course of the increase of PAI-1 mRNA (●) and gene transcription rate (○) by serum (A) and PMA (B) in Hep G2 cells.
Hep G2 cells were preincubated with serum-free medium for 16 h, and then treated with serum (10%) or PMA (100 nM). At the times indicated RNAs were isolated and 5 μg of total cellular RNA was analyzed by Northern blot hybridization. The northern blots were exposed to Amersham hyperfilm (see inset), and the bands were quantified by densitometric scanning. The amount of PAI-1 mRNA present at the different times is given relative to that found at t = 0. The insets show the two PAI-1 mRNA bands (two upperbands) at various times and the band of a constant probe GAPDH (lower band), which shows that the RNA loading in each lane is equal. To determine the transcription rate, nuclei were isolated from cells incubated with serum or PMA for the times indicated. Run-on assays were performed by elongation of the nuclear transcripts in the presence of [α-^{32}P]-UTP. The labeled RNA was isolated and hybridized to linearized plasmid DNA of PAI-1 and GAPDH, a constant probe. The blots were exposed to Amersham hyperfilm and quantified by densitometric scanning.


A
serum
fold increase
time (hours)
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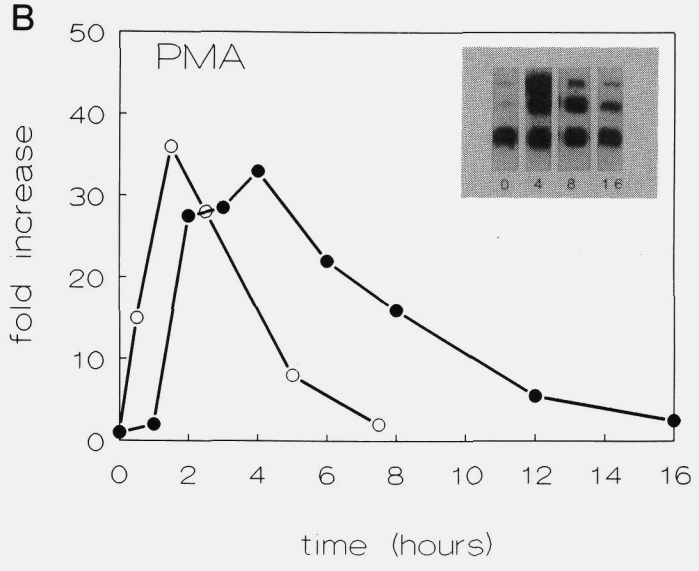
B
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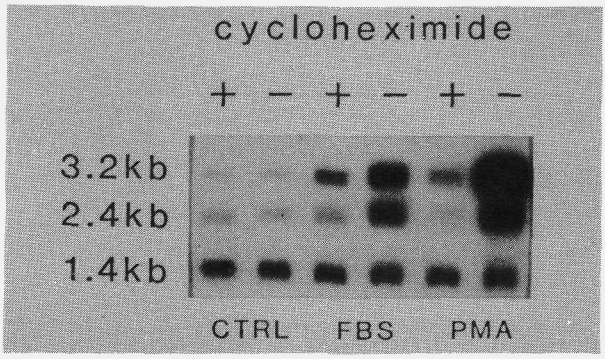

Figure 2. Northern blotting analysis of the effect of cycloheximide (CHX) on the induction of the PAI-1 gene by serum and PMA in Hep G2 cells.
Hep G2 cells were preincubated with serum-free medium for 16 h, and then fresh medium with or without CHX (10 μg/ml) was added. After 1 h, serum (10%) or PMA (100 nM) was added. 3 h after the addition of serum or PMA, cells were rinsed with PBS and RNA was isolated and analyzed by Northern blotting. After hybridization to ^{32}P-labeled DNA fragments of human PAI-1 and rat GAPDH, filters were washed and exposed to hyperfilm of Amersham.

al., 1988). Since the 5'flanking DNA of the human PAI-1 gene contains an almost perfectly conserved AP-1 binding site (Riccio et al., 1988; Bosma et al., 1988), we have investigated the effect of serum and PMA on c-jun and c-fos expression. Figure 3 represents the time course of the increase of these two proto-oncogenes in Hep G2 cells by serum and PMA. Under basal conditions Hep G2 c-jun and c-fos levels are at a low, undetectable level. After addition of serum or PMA a transient increase of c-jun and c-fos mRNA is seen, but with a different time profile and to a different extent for each compound. c-jun mRNA after serum addition starts to increase after 1 hour, reaches its maximal level at 2.5 h, and then returns to prestimulatory levels at 6 h. Upon PMA addition c-jun mRNA increases more rapidly, reaching maximal levels already at 1 hour, followed by a rapid decline to basal level at 3 h. In addition, the maximal level of c-jun mRNA induced by PMA is 2 to 3 times higher than the maximal level reached with serum. Addition of serum results only in a minor increase in c-fos mRNA, whereas with PMA a strong and rapid induction of this messenger is seen, reaching its peak after 1 h and then returning to basal levels already after 2 h. These results indicate that AP-1 is activated differently by serum and PMA, which might explain the different induction of the PAI-1 gene by these compounds in Hep G2.

Recently it has been reported that the proto-oncogene c-myc modulates the expression of the PAI-1 gene by a nuclear post-transcriptional mechanism (Prendergast et al., 1989, 1990). We therefore also determined the effect of serum and PMA on c-myc mRNA levels in Hep G2. In contrast to c-jun and c-fos, c-myc mRNA is already

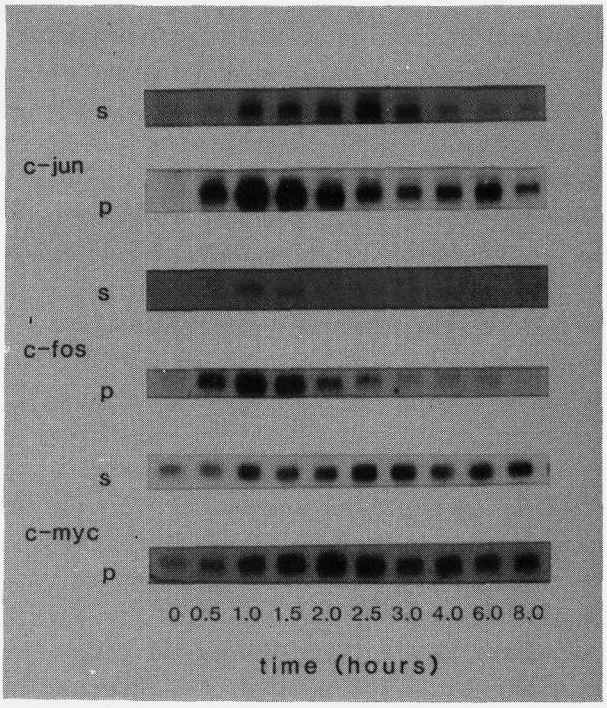

Figure 3. Northern blotting analysis of c-jun, c-fos and c-myc mRNA induction by serum (S) and PMA (P) in Hep G2 cells.
Hep G2 cells were preincubated with serum-free medium for 16 h, and the fresh medium containing serum (10%) or PMA (100 nM) was added. At the times indicated RNA was isolated and 5 μg total RNA was analyzed by Northern blotting. After hybridization of the filters to ^{32}P-labeledDNA fragments of c-jun, c-fos or c-myc, the radioactivity was visualized by autoradiography.

detectable in non-stimulated Hep G2 cells. c-myc mRNA is induced by serum and PMA with a similar time profile but to a different extent. Upon addition of serum or PMA, the level of c-myc starts to increase after 1 hour. The maximally enhanced level, a 4-fold increase with serum and a 10-fold increase with PMA, is reached after about 2.5 h and is followed by a gradual decline. These results suggest that induction of c-myc by serum and PMA is too slow to play a role in the induction of PAI-1 gene transcription.

DISCUSSION

In this paper we show that serum-starved Hep G2 cells transiently increase PAI-1 mRNA levels in response to the addition of serum or PMA. While both serum and PMA activate protein kinase C (Nishizuka, 1984, 1986), the time courses and potencies of their effects are quite different. The induction by PMA is very strong and of short duration. In contrast, the response to serum is longer lasting, and peak levels are lower. These differences in PAI-1 gene expression are also seen at the transcriptional level, and on the induction of c-jun, c-fos and c-myc mRNA, where induction by serum is lower but more persistent than by PMA. The basis for these differences is not clear, but may be related to downregulation of protein kinase C after prolonged exposure to PMA (Young et al., 1987; Isakov et al., 1990). In addition, serum growth factors may also operate via other mechanisms not involving PKC, such as Ca^{2+} or cAMP.

The induction of PAI-1 is dependent on protein synthesis, possibly transcriptional factors. The polypeptides encoded by the c-jun and c-fos mRNAs are "leucine-zipper"-containing DNA binding proteins that function as transcriptional activators. DNA transfection, micro-injection, and anti-sense nucleic acid experiments indicate an important role for these polypeptides in mediating PMA and serum responses (Curran and Franza, 1988; McDonnell et al., 1990). As shown in Fig. 3, serum or PMA addition to Hep G2 cells results in a rapid and transient increase of c-jun and c-fos mRNA levels. Jun and fos are known to form dimers, the AP-1 complex, for which an almost perfect binding site is present in the 5'flanking DNA of the PAI-1 gene. The induction of c-jun and c-fos mRNAs precedes that of PAI-1 mRNA, suggesting that accumulation of jun and fos may be necessary for the subsequent induction of the PAI-1 gene. Also, the prolonged increased expression of c-jun by serum compared to the very transient induction by PMA may explain the more persistent stimulation of the PAI-1 gene by serum. An additional level of control in transcriptional regulation by the jun/AP-1 family of transcription factors may involve homo- and heterodimerization. Since, Jun-Fos heterodimers have a greater affinity for the AP-1 site and are a more potent transcriptional activator than Jun homodimers, both Jun and Fos are required for optimal transcriptional activation by AP-1 (Sassone-Corsi et al., 1988; Kouzarides and Ziff, 1988; Schuermann et al., 1989; Halazonetis et al., 1988; Chiu et al., 1988). The minor induction of c-fos by serum could limit the formation of fos/jun heterodimeric transcription complexes, which could explain the weaker induction of the PAI-1 gene by serum as compared to PMA. Further studies (i.e. expression studies and footprinting experiments) are needed however to definitely establish the role of jun and fos in the regulation of PAI-1 gene expression in Hep G2. In contrast to what is seen for c-jun and c-fos, the increase of another proto-oncogene, c-myc, by serum and PMA does not precede that of PAI-1 mRNA but runs more or less parallel to it, making a role for c-

myc in the induction of PAI-1 gene transcription by these compounds unlikely. Whether c-myc is involved in post-transcriptional regulation processes, as shown for 3T3 cells and c-myc-immortalized primary rodent fibroblasts by Prendergast et al. (1990), is not clear from our work.

It is noteworthy that after induction by PMA, but not after serum, a shift in ratio between the two PAI-1 mRNAs towards the 2.4 kb mRNA species is seen (Fig. 1). Whether the different regulation of the two PAI-1 mRNAs by PMA in Hep G2 cells is the result of the transcriptional effects or if posttranscriptional effects like RNA processing, nucleocytoplasmic transport or mRNA turnover in the nucleus or cytoplasm, are involved, remains to be determined.

In conclusion, induction of PAI-1 gene expression by serum and PMA is qualitatively very similar and might both be mediated by the transcription factors, Jun and Fos. Further experiments are required to establish such a role for AP-1 factors.

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CHAPTER 6

DIFFERENT INDUCTION OF TWO PLASMINOGEN ACTIVATOR INHIBITOR 1 mRNA SPECIES BY PHORBOL ESTER IN HUMAN HEPATOMA CELLS. ROLE OF 3'-UNTRANSLATED REGION IN mRNA STABILITY AND TRANSLATION

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SUMMARY

In man, the plasminogen activator inhibitor 1 (PAI-1) gene codes for two mRNA species, one of 3.2 kb and the other of 2.4 kb. We report that the protein kinase C activating phorbol ester, phorbol 12-myristate-13-acetate (PMA), causes a different induction of the two PAI-1 mRNA species in the human hepatoma cell line Hep G2. Upon addition of 100 nM PMA, the level of the 3.2 kb PAI-1 mRNA species increased to 25-fold after 3 hours, and then declined rapidly. The level of the 2.4 kb species increased more slowly and reached a maximal 18-fold stimulation after 6 hours, followed by a gradual decrease towards control levels. Run-on analysis showed that PMA induces a transient, 40-fold increase in PAI-1 gene transcription rate, without markedly changing the transcription termination site. PMA shifts the relative concentration of the two PAI-1 mRNA species in the nuclei of Hep G2 towards the 2.4 kb form, suggesting that changes in post-transcriptional nuclear processing might contribute to their different accumulation. Also, the two mRNAs differ in turnover rate, with a half-life of about 0.85 h for the 3.2 kb form and a half-life of about 2.5 h for the 2.4 kb form. By itself, cycloheximide (CHX) had no effect on PAI-1 mRNA levels, but when CHX was added 2 h after PMA, it increased the stability of the 3.2 kb form. A similar effect was seen with inhibitors of transcription, indicating that the rapid decay of the 3.2 kb mRNA is mediated by a PMA-induced labile protein factor coded for by a labile mRNA. When added prior to PMA, CHX prevented the induction of PAI-1 mRNA, which suggests that PMA exerts its stimulating transcriptional activity through a regulatory protein. The two PAI-1 mRNAs are equally efficient in the synthesis of PAI-1 protein, both in Hep G2 cells and in an *in vitro* translation system. We conclude that the two PAI-1 mRNAs are functionally similar but may differ in stability.

INTRODUCTION

Plasminogen activation provides an important source of localized proteolytic activity, not only during fibrinolysis (Collen, 1980), but also during ovulation (Beers et al., 1975), cell migration (Strickland et al., 1976), tumor invasion and metastasis (Ossowski and Reich, 1983), hormone processing (Virji et al.,1980), collagenase activation (Danø et al., 1985), angiogenesis (Gross et al., 1983), and a variety of other physiological processes. Precise regulation of plasminogen activator (PA) activity thus constitutes a critical feature of many biological processes. This control may occur at various levels, including the synthesis and secretion of PAs, and the interaction with specific PA inhibitors, PAIs (Sprengers and Kluft, 1987). PAI-1 is the major PAI found in blood, and has been shown to be expressed in many body tissues (Quax et al., 1990; Erickson et al., 1990). It is a 50

kD glycoprotein, belonging to the serpin family (Pannekoek et al.,1986; Ny et al., 1986), and a specific inhibitor of both tissue-type PA (t-PA) and urokinase-type PA (u-PA). PAI-1 has also been detected in platelet releasates and in the conditioned media of a variety of cells including endothelial cells, hepatocytes, smooth muscle cells and several tumor cell lines (see Sprengers and Kluft, 1987). Recently, PAI-1 has been identified as a matrix protein (Rheinwald et al., 1987; Pöllänen et al., 1987; Knudsen et al., 1987).

The biosynthesis of PAI-1 appears to be highly regulated. For example, PAI-1 synthesis can be induced by endotoxin (Colucci et al., 1985; Emeis and Kooistra, 1986), inflammatory mediators (Emeis and Kooistra, 1986; Van Hinsbergh et al., 1988; Van den Berg et al., 1988; Schleef et al., 1988; Sawdey et al., 1989), glucocorticoids (Gelehrter et al., 1983; Andreasen et al., 1987), insulin (Alessi et al., 1988; Kooistra et al., 1989) and phorbol ester (Mayer et al., 1988). Understanding of the complex regulation of PAI-1 biosynthesis at the molecular level is complicated by the existence in human cells of two distinct PAI-1 mRNA species, one of 3.2 and one of 2.4 kb (Ginsburg et al., 1986; Ny et al., 1986). Both PAI-1 mRNAs have an identical coding region, but differ in the length of their 3' untranslated region, probably as a result of alternative polyadenylation (Loskutoff et al., 1987; Bosma et al., 1988). Interestingly, the 3.2 kb species, but not the 2.4 kb species, contains an AU-rich sequence, which has been associated with message lability (Shaw and Kamen, 1986; Caput et al., 1986; Fort et al., 1987). Although several authors have noticed a variation in the relative concentrations of the two mRNAs (Van den Berg et al., 1988; Schleef et al., 1988; Lucore et al., 1988), no study has previously been performed to address such mRNA parameters as stability and translational capacity.

In this paper we have studied these important questions. As part of a continuing study on the regulation of PAI-1 expression, we observed that the protein kinase C activator, phorbol 12-myristate-13-acetate (PMA), strongly enhances the levels of the two PAI-1 mRNA species in the human hepatoma cell line, Hep G2. A striking aspect of this induction is that the levels of the two messengers each increase with a different time profile and to a different extent. Further experiments were directed at the role of transcriptional activation and mRNA stability in determining the levels of the two PAI-1 mRNA species. We report that PMA induces a strong, but transient increase in the transcription of the PAI-1 gene and an accelerated decay of the 3.2 kb species. Both processes require de novo RNA and protein synthesis, as suggested by experiments with inhibitors of transcription and translation. A comparison of the induction profile of the two mRNAs with the production of PAI-1 protein indicated that both mRNA forms are translated with about the same efficiency in Hep G2. Similarly, in an *in vitro* translation system, both mRNA species are equally efficient in directing the synthesis of a 40,000-Dalton protein.

MATERIALS AND METHODS

Materials

Hybond N, random primer kit (multi prime), [^{32}P]labeled dCTP and UTP, and [^{35}S]Methionine were from Amersham Corporation. [^{35}S]Methionine for *in vitro* translation experiments was obtained from New England Nuclear. Cycloheximide (CHX), H-7, actinomycin D and phorbol 12-myristate-13-acetate (PMA) were purchased from Sigma. Stock solutions of PMA (10^{-4} M) were prepared in ethanol. 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) was obtained from Boehringer Mannheim. t-PA (> 99% two-chain) was purified from Bowes melanoma cell culture medium as described by Kluft et al. (1983). Enzyme immunoassay kits for determination of human PAI-1 antigen ("Imulyse") were obtained from Biopool. The riboprobe system pSP64 and the rabbit reticulocyte lysate used for *in vitro* transcription and translation, respectively, were obtained from Promega. Other materials used in the methods described below have been specified in detail in the relating references.

Cell culture experiments

The human hepatoma cell line Hep G2 (Knowles et al., 1980) was cultured in Dulbecco's modification of Eagles medium (DMEM), supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycine (100 μg/ml) and glutamine (2 mM) under 5% CO_2/95% air atmosphere at 37 °C (Havekes et al., 1983). For the experiments, confluent cells were used. Fresh complete medium was added to the cells 40 hours before the start of the experiments. 16 hours prior to the experiments, the medium was changed to DMEM supplemented with 0.1% human serum albumin, glutamine, penicillin and streptomycin. Cells were incubated in the presence of test compounds for various times up to 24 hours. The conditioned media were centrifuged for 2 minutes in a Beckmann microfuge centrifuge to remove cells and cellular debris and were stored at -20 °C until use. Cell lysates (cells plus matrix) were prepared by rinsing the cells with phosphate buffered saline (PBS) and scraping them with a rubber policeman in the presence of ice cold PBS containing 0.5% (w/v) Triton X-100. The cell lysates were also stored at -20 °C until use. Preparation of nuclei for RNA isolation was performed as described by Marzluff and Huang (1984).

Protein synthesis

Overall protein synthesis was determined by measuring the incorporation of [^{35}S]methionine into the 10% (w/v) trichloroacetic acid precipitable fraction of radiolabeled CM and cell lysates.

Assays

PAI-1 activity was determined by the method of Verheijen et al. (1984): samples were titrated with increasing amounts of t-PA, followed by spectrophotometric measurement of the residual t-PA activity. PAI-1 antigen determinations were performed by a commercially available enzyme immunoassay kit. This assay of human PAI-1 antigen detects both active and "latent" (inactive) forms of PAI-1.

mRNA analysis

Total cellular RNA and nuclear RNA was isolated according to the method of Chomczynski and Sacchi (1987). 4-10 μg of RNA, as determined spectrophotometrically, was subjected to gel electrophoresis in formaldehyde agarose gels (Maniatis et al., 1982). After electrophoresis, the RNA was transferred to Hybond N according to the instructions of the manufacturer. Pre-hybridization and hybridization were done at 65 °C in 0.5 M NaH_2PO_4-Na_2HPO_4 (pH 7.2), 7% SDS, 1 mM EDTA (modified from Church and Gilbert, 1984). Hybridization was performed with cDNA probes (1 ng/ml), labeled to approximately 5 x 10^8 cpm/μg DNA by the random primer method. After hybridization the filters were washed twice for 15 mins at 65 °C, with respectively: 2 x SSC, 1% SDS; 1 x SSC; 1% SDS; and 0.1% SSC, 1% SDS. Quantification was performed by cutting the bands, and counting them in scintillation fluid or by exposing the membranes to KODAK XAR5 film with an intensifying screen. The relative intensities of the bands were determined by densitometric scanning. PAI-1 mRNA preparations for *in vitro* translation experiments were quantified by dot blot analysis, using *in vitro* transcribed PAI-1 SP6 RNA as a standard.

The probes used were a 2.5 kb EcoRI PAI-1 cDNA fragment (Van den Berg et al., 1988), a 1.2 kb EcoRI PAI-2 cDNA fragment provided by Dr. E.K.O. Kruithof (Schleuning et al., 1987), and a 1.2 kb Pst1 cDNA fragment of rat glyceraldehyde -3-phosphate dehydrogenase (GAPDH), a kind gift from Dr. R. Offringa (Fort et al., 1988). All probes were isolated from low melting agarose and purified by using the gene clean kit (Bio 101, Inc.).

Run-on analysis

Preparation of nuclei for run-on analysis was performed as described by Greenberg and Ziff (1984). After isolation, the nuclei were resuspended in glycerol buffer (50 mM Tris, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), frozen in N_2, and stored at -80 °C until use. Per run-on assay, approximately 5 x 10^6 nuclei were used. The elongation reaction and RNA isolation were performed according to Nevins (1987). In short, the nuclei were incubated for 20 min at 30 °C in 200 μl reaction buffer, containing: 20 mM Tris, pH 7.9; 20% glycerol; 140 mM KCl; 10 mM MgCl; 1 mM DTT; 1 mM each of ATP, CTP, GTP; 2 μM[α-^{32}P] UTP (800 Ci/mM (1 Ci = 3.7 x 10^{10} Bq); 10 mM creatine

phosphate; 20 U/ml creatine kinase and 1000 U/ml RNAsin. At the end of the incubation, 800 µl HSB buffer: 10 mM Tris, pH 7.4; 0.5 M NaCl; 50 mM MgCl; 2 mM CaCl and 10 U/ml DNAse 1, was added, followed by the addition of 1 ml of extraction buffer: 10 mM Tris, pH 7.4; 1 % SDS; 20 mM EDTA, as soon as the viscosity had disappeared. The RNA was isolated by hot fenol extraction. Contaminating DNA was further degraded by a DNAse treatment for 20 min at 37°C in 20 mM Tris, pH 7.7; 1 mM MgCl and DNAse 1, 50 µg/ml. After fenol extraction, the RNA was incubated for 20 min in ice-cold 0.2 M NaOH. The solution was then neutralized with HEPES buffer pH 7.4. To remove unincorporated label, repeated ethanol precipitations with high salt were performed.

Hybridization of labeled RNA to DNA

DNA (2 µg) was blotted onto Hybond N filter using a minifold filtration apparatus in alkali blotting solution (1.5 M NaCl, 0.25 M NaOH). Pre-hybridization and hybridization were performed at 65°C in 0.5 M NaH_2PO_4 - Na_2HPO_4, pH 7.2; 7% SDS, 1 mM EDTA. The RNA was hybridized for 65 hours. After the hybridization, the filters were washed several times with 2 x SSC and treated with RNAse and proteinase K as described (Nevins, 1987). Quantification was carried out by exposing the filters to KODAK XAR5 film followed by densitometric scanning.

In vitro transcription and translation

The two PAI-1 mRNA species were synthesized by *in vitro* transcription using the riboprobe system pSP64 (Promega). A PAI-1 cDNA fragment, starting at the PstI site at position +78 up to the Bam HI site at position +3051, was cloned into the Pst-I/BamH-I sites of the polylinker of pSP64(poly A). The resulting plasmid pSP6-large represents the 3.2 kb PAI-1 mRNA species, containing the entire mRNA coding region and 1704 bp of 3' non-coding region. From this plasmid, pSP6-small was generated by deletion of 845 bp from its 3'-end, i.e. from the DraI at position +2207 up to the BamH-I site at position +3051. The resulting plasmid pSP6-small corresponds to the 2.4 kb PAI-1 mRNA form and has a 3' non-coding region of only 860 bp. After linearization of these plasmids, using the EcoRI site in the SP64 vector behind the 30 bp poly(A) stretch, both mRNAs were transcribed from the SP6 polymerase promoter. The size of the two mRNA species, both containing a poly(A) track of 30 bp, was verified by Northern blot analysis.

Preparations of the two PAI-1 mRNA forms synthesized *in vitro* or poly(A) mRNA with different ratios of 3.2 kb and 2.4 kb species, isolated from Hep G2 cells, were used for *in vitro* translation experiments in a rabbit reticulocyte lysate system (Promega). Both SP6 constructs gave a linear dose- and time-dependent incorporation of [^{35}S]-methionine, as determined by TCA precipitation, up to 50 ng of RNA and 30 min of incubation. For the poly(A) RNA preparations, a linear response up to 2 µg of RNA was seen during a

15 min incubation. To determine the translational capacity of the two mRNA species we chose for a 10 min incubation of 20 ng SP6 or 2 μg poly(A) RNA. The amount of PAI-1 produced by *in vitro* translation was quantified by precipitation with a specific, surface-bound monoclonal antibody directed against PAI-1, followed by liquid scintillation counting of the complex. *In vitro* translation products from both PAI-1 SP6 constructs were also analyzed on 10% SDS-polyacrylamide gels (Laemmli, 1970). Molecular weight markers (Bio-Rad) were used for calibration of the gels. For autoradiofluorography, the gel was treated with an autoradiofluorography enhancer (En^{3}hance, New England Nuclear) according to the manufacturer's instructions, dried, and placed on X-ray film (Kodak XAR5).

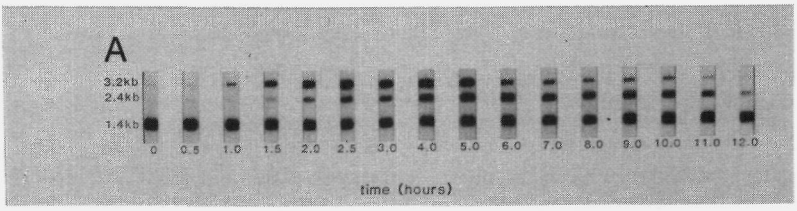
RESULTS

Time course of PMA-induced increases in PAI-1 mRNA in HepG2

Treatment of Hep G2 cells for 8 h with 0.01 - 200 nM PMA showed a dose-dependent increase in PAI-1 mRNA levels, reaching a maximal stimulation at 100 - 200 nM (results not shown). Fig. 1 represents the time course of the transient increase of the two PAI-1 mRNAs, with lengths of 3.2 and 2.4 kb, by 100 nM PMA. After a lag-period of 45 min, the level of both mRNAs starts to increase, but with a different time profile and to a different extent. The 3.2 kb species is maximally 25- to 30-fold stimulated after about 3 h, and then rapidly declines again to the prestimulatory level. The 2.4 kb messenger reaches maximally 18 times control values after 6 h, and then declines, more slowly than the 3.2 kb species, to basal levels. In the same experiment no significant effect of PMA on GAPDH mRNA content in the cells was seen (Fig. 1A).

Run-on analysis

To investigate whether the induction of the PAI-1 mRNAs by PMA is at the level of transcription, nuclear run on experiments were performed. For that purpose nuclei from Hep G2 cells that had been incubated with PMA for various times, were isolated. Nascent nuclear transcripts were elongated in the presence of [α-^{32}P] UTP and hybridized to various cDNA probes immobilized on Hybond N membranes. As shown in Fig. 2A and B, PMA causes a strong and transient increase in the transcription of the PAI-1 gene. As early as 30 min after the addition of PMA to Hep G2, the transcription of the PAI-1 gene is enhanced. After 2 h the transcription reaches its maximal 40-fold induction, followed by a rapid return back to pre-stimulatory levels at 8 h after PMA addition. In control cells, and in cells incubated with CHX for 4 h, PAI-1 transcripts stayed at the low prestimulatory level (not shown). PMA incubation had no effect on the transcription of the GAPDH gene. No detectable signal was given by the PAI-2 cDNA



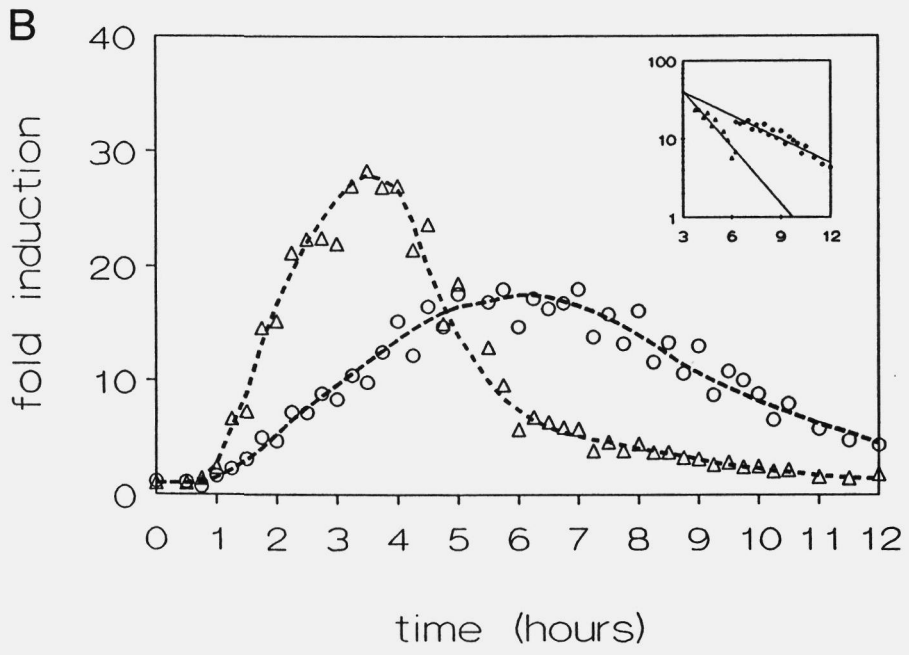
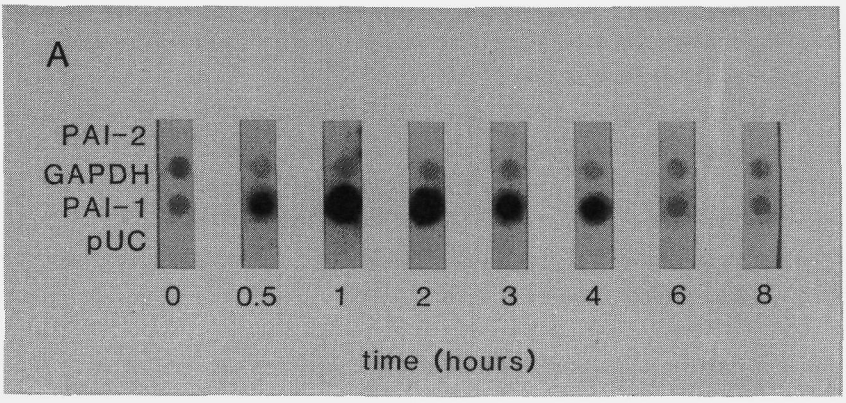
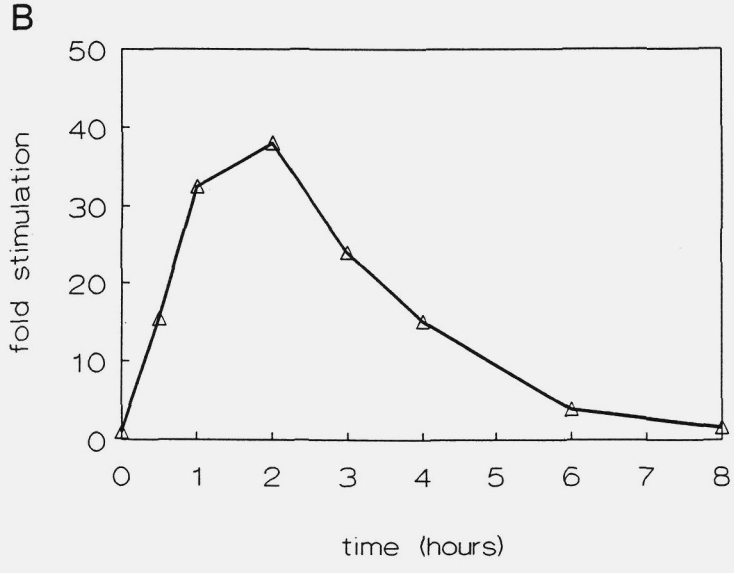


Figure 1. Time course of the induction of PAI-1 mRNA by PMA in Hep G2 cells.
Hep G2 cells were preincubated with serum free medium for 16 h, and then treated with PMA (100 nM) in serum free medium for the times indicated. RNAs were isolated, and 5 μg of total cellular RNA was analyzed by Northern blotting followed by hybridization to ^{32}P-labeled cDNA fragments of human PAI-1 and/or GAPDH (A). Quantification of the 3.2 (▵) and 2.4 (○) kb PAI-1 mRNA species was performed by cutting the bands and counting them in a liquid scintilation counter. The amount of each mRNA species present at the various time points is presented as -fold induction of the amount present at t = 0 (B). In the inset the decline of both mRNA species on a semi-log scale is shown.

Figure 2. Analysis of the PAI-1 gene transcription rate after PMA stimulation.
Hep G2 cells were incubated with PMA (100 nM) for various times upto 8 h, and then nuclei were isolated. Run-on assays were performed by elongation of the nuclear transcripts in the presence of [α^{32}P]UTP. The [^{32}P]-labeled RNA was isolated and hybridized to plasmid DNA of: PAI-1; PAI-2, as an eukaryotic negative control; GAPDH, as a control for variation in mRNA labeling; and, as a prokaryotic control, pUC. All probes were blotted on Hybond N using a minifold filtration apparatus. The radioactivity is visualized by autoradiography (A). The intensity of the dots is determined by densitometric scanning (B). Values are given relative to corresponding t = 0 values, and are means of two independent experiments. For two time points, 1.5 h and 5 h after PMA addition, the labeled RNA was hybridized to a 1.1 kb 5'-end, or 1.1 kb 3'-end PAI-1 cDNA probe and to GAPDH (C).


A
PAI-2
GAPDH
PAI-1
pUC
0
0.5
1
2
3
4
6
8
time (hours)


B
fold stimulation
50
40
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0
1
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3
4
5
6
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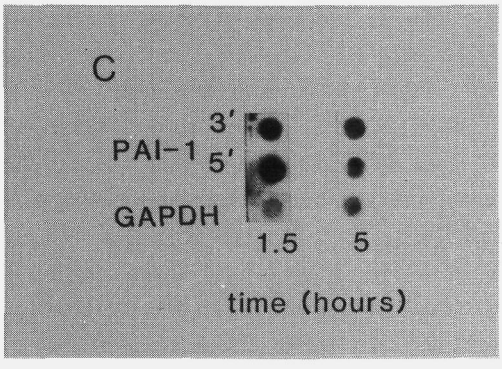
8
time (hours)


C
PAI-1
3'
5'
GAPDH
1.5
5
time (hours)

and the pUC 18 probe, which served as negative controls. In the presence of the RNA polymerase II inhibitor α-amanitine (10 μg/ml) no transcription of the PAI-1 or GAPDH gene was seen (results not shown).

As the different induction of the two PAI-1 mRNAs could also be the result of a change in transcription termination, we compared hybridization of the radiolabeled nuclear transcripts to a 1.1 kb 5' and a 1.1 kb 3' PAI-1 cDNA probe. At the times tested, 1.5 and 5 h after PMA addition, by and large similar amounts of labeled RNA hybridized to each probe, indicating that the transcription termination essentially remains the same throughout the incubation period (Fig. 2C).

Analysis of PAI-1 mRNAs in nuclei from PMA-treated Hep G2 cells

To investigate the possibility that differential accumulation of the 3.2 and 2.4 kb mRNAs is the result of nuclear (RNA processing and/or transport) effects, we analyzed PAI-1 mRNA levels in nuclei from Hep G2 cells incubated for various times with PMA. As shown in Fig. 3, the induction pattern of the two PAI-1 mRNAs in the nuclei is comparable with, but not identical to, that seen with total cellular RNA, with the 3.2 kb species at maximal levels after 2 h and the 2.4 kb species peaking at 4 h. This similarity in induction profile cannot be explained by contamination of the nuclear RNA preparations with cytosolic RNA, since the amount of GAPDH messenger in nuclear RNA is less than 1% of total, i.e. mainly cytosolic, GAPDH mRNA, whereas the amount of nuclear PAI-1 mRNA constitutes at least 5% of total PAI-1 mRNA. In addition, at 4 h and 6 h the ratio between the 3.2 kb form and the 2.4 kb form in the nuclear RNA fraction is 1:1 and 1:2, respectively, and differs from that in total RNA, which is 1:2 and 1:7, respectively.

Stability of the two PAI-1 mRNAs

To assess the role of mRNA stability in the observed changes in the levels of the two PAI-1 mRNAs, we determined their half-lives from the data presented in Figs. 1 and 2. On the assumption that PAI-1 mRNA synthesis is negligible after 6 h (Fig. 2), a half-life of 2.5 h can be calculated for the 2.4 kb mRNA species from its subsequent decline (see inset Fig. 1). Very similar half-life times were calculated from experiments in which the disappearance of the 2.4 kb mRNA form was determined following addition of actinomycin D (5 μg/ml) or DRB (200 μM) at 4 h after induction with PMA (results not shown). For the 3.2 kb messenger a more rapid decline is seen, with an apparent half-life time of 0.85 h between 4 and 6 h after PMA addition (see inset Fig. 1). This half-life time for the 3.2 kb mRNA species is likely to be a slight overestimation of the actual half-life, since PAI-1 mRNA synthesis is still elevated during this period. However, the 3.2 kb mRNA levels in the following period, when PAI-1 gene transcription rates had returned to basal levels, were too low to allow accurate estimates. Blocking of RNA

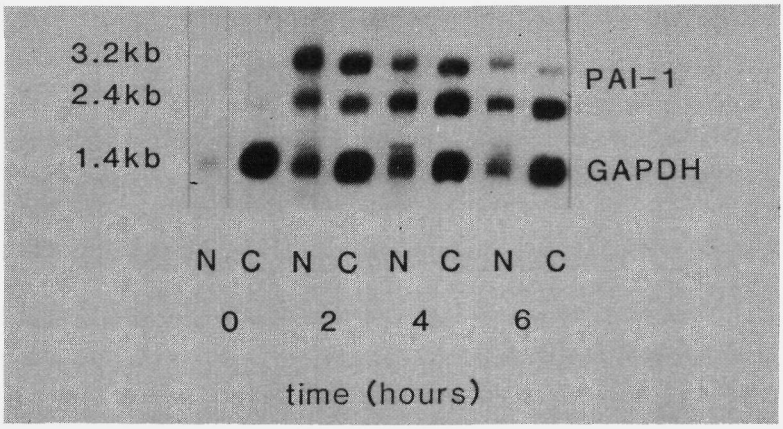


Figure 3. Northern blotting analysis of PAI-1 mRNA induction by PMA in Hep G2 cells and nuclei. Hep G2 cells were preincubated with serum-free medium for 16 h, and then treated with PMA (100 nM) in serum-free medium for the times indicated. 4 μg of RNA isolated from nuclei (N) or cells (C) was analyzed by Northern blotting followed by hybridization to ^{32}P-labeledDNA fragments of human PAI-1 and GAPDH.

synthesis by actinomycin D or DRB could not be used in these experiments, since these compounds induced stabilization of the messenger (see below). Due to the low preinduction levels of both PAI-1 mRNAs, no determination of stability was possible before induction with PMA.

Effect of cycloheximide on the induction profile of PAI-1 mRNA

Although CHX alone had no effect on PAI-1 gene transcription (see above), several papers suggest that inhibition of protein synthesis may lead to PAI-1 mRNA accumulation (Mayer et al., 1988, Van den Berg, 1988). We therefore tested the effect of CHX (10 μg/ml) on PAI-1 mRNA levels in Hep G2, both under basal and PMA-stimulated conditions, when protein synthesis was found to be inhibited for over 95%. In control cells, incubation with CHX up to 12 h neither altered total PAI-1 mRNA levels nor did it change the relative concentration of the two PAI-1 mRNA forms. Thus, in unstimulated Hep G2 cells, addition of CHX has no effect on the stability or synthesis of PAI-1 mRNAs. When CHX was added 1 h prior to PMA the rapid induction of both mRNA species was completely suppressed (results not shown). However, when CHX was added two hours after PMA, when transcription rate is maximal (see Fig. 2), both messengers reach even higher levels than with PMA alone, and maximal mRNA levels are maintained for a longer period (Fig. 4). In fact, the induction profiles of the two species become very similar. Whereas with PMA alone, the 3.2 kb species reaches its peak value after 3 h and then declines with a half-life of about 0.85 h, in the presence of both PMA and CHX, the 3.2 kb mRNA continues to increase, reaching 1.5-fold

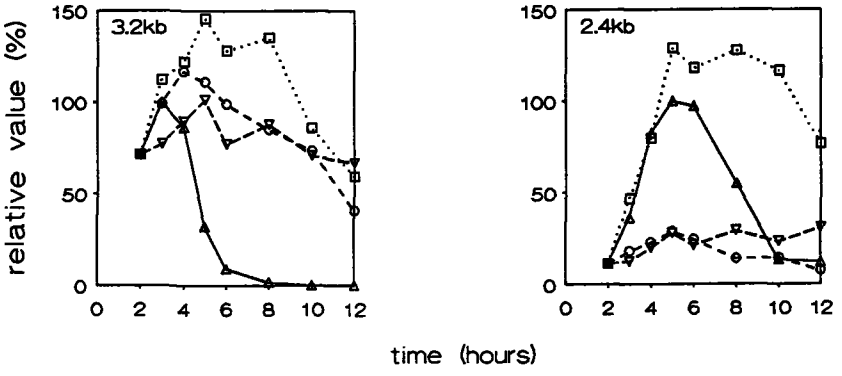


Figure 4. Effect of inhibitors of translation and transcription on the induction of PAI-1 mRNA by PMA. Hep G2 cells were preincubated with serum-free medium for 16 h, and then treated with PMA (100 nM). 2 h after PMA addition, CHX (10 μg/ml), actinomycin D (5 μg/ml) or DRB (250 μM) was added. At various times (2 h to 12 h after PMA addition), RNAs were isolated and analysed by northern blot hybridization. The northern blots were exposed to Kodak XAR film, and the bands were quantified by densitometric scanning. The amounts found of each mRNA are given as percentage values of the maximally enhanced levels in the presence of PMA alone, i.e. the amount of the 3.2 kb species at 3 hours and of the 2.4 kb species at 6 hours. PMA (△); PMA + CHX (□); PMA + Act.D (○) and PMA + DRB (▽).

maximal PMA-induced values after 5 h. This plateau level is maintained unto 8 h, and then the 3.2 kb PAI-1 mRNA level slowly declines with an apparent half-life very similar to that of the 2.4 kb species. The 2.4 kb mRNA species reaches its peak value after 6 h with PMA alone and then declines with a half-life of 2.5 h. In the presence of both PMA and CHX, the 2.4 kb species reaches (a slightly higher) maximal level after 6 h again, but the decline of this species is delayed: the fall in 2.4 kb mRNA concentration starts after 10 h, although its apparent half-life time (2.5 h) seems unchanged. Thus, the addition of CHX to Hep G2, 2 h after adding PMA, not only results in an increased stability of the 3.2 kb mRNA, but maybe also in keeping up the strongly induced transcription rate and/or in changing post-transcriptional processes.

Effect of actinomycin D and DRB on the induction profile of PAI-1 mRNA

In Fig. 4 the effect of 5 μg/ml actinomycin D, an inhibitor of total DNA transcription, and of 200 μM DRB, a specific inhibitor of RNA polymerase II (Mittleman et al., 1983), on the induction of PAI-1 mRNA is also shown. At the concentrations used, these inhibitors showed no effect on protein synthesis, but when added 1 h prior to PMA, these inhibitors completely blocked the induction of PAI-1 mRNA (results not shown). When actinomycin D or DRB were added 2 h after PMA, the rapid decay of the 3.2 kb mRNA is blocked and this messenger stays at the high induced level; the 2.4 kb species remains at a low level. These results suggest that for the rapid decay of the 3.2 kb mRNA ongoing RNA synthesis is required.

Dependence on activated protein kinase C

To investigate the dependence of the induction of PAI-1 on the presence of activated PKC, PMA was washed out from the cells 1 h after its addition by rinsing the cells several times with fresh medium. To ensure complete inhibition of residual PKC activity, 100 μM H7, a specific PKC inhibitor, was added. This procedure completely suppressed the induction of both PAI-1 mRNAs, as measured at 3 h and 6 h after the start of the experiment (Fig. 5). When the same procedure was performed 2 h after the addition of PMA, the 3.2 kb mRNA species is induced normally, but the 2.4 kb species reaches at most 30% of the level induced in the continuous presence of PMA. Thus, to obtain complete induction of the PAI-1 gene, activated PKC appears to be necessary during at least the first 2 h after the addition of PMA. Fig. 5 shows that the removal of PMA together with the addition of H7 after 2 h has no effect on the level of the 3.2 kb mRNA found after 6 h. Thus, the rapid, specific degradation of this mRNA species does not require the presence of activated PKC.

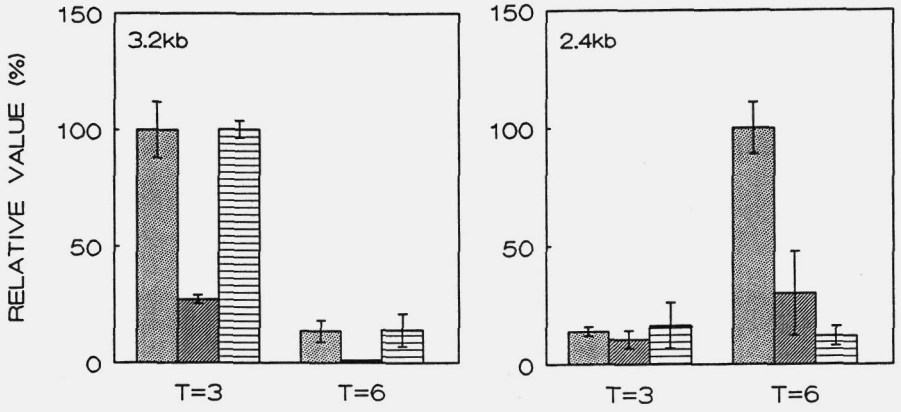


Figure 5. Requirement of active PKC for the induction of PAI-1 mRNA by PMA in Hep G2 cells. Hep G2 cells were preincubated with serum-free medium for 16 h and then treated with PMA (100 nM) for 3 h or 6 h (▨), or treated in the following way: at 1 h (▨) or 2 h (☰) after the addition of PMA, the cells were washed three times and fresh incubation medium containing 100 μM of H7, a specific inhibitor of PKC, was added; 3 h or 6 h after the start of the experiment (i.e., the addition of PMA) the cells were collected, and RNAs were isolated and analysed by Northern blot hybridization. The Northern blots were exposed to Kodak XAR film, and the bands were quantified by densitometric scanning. The concentrations of the 3.2 and 2.4 kb mRNA species in the cells treated with H7 are given as percentage values of the corresponding mRNA values at 3 h (3.2 kb species) or 6 h (2.4 kb species) in cells incubated continuously in the presence of PMA. The results given are the mean out of two experiments carried out in duplicate.

Translation efficiency of the two PAI-1 mRNA species

The different induction profile of the two PAI-1 mRNAs results in large variations in the ratio between the two species. 2 h after the addition of PMA, the ratio between the 3.2 and the 2.4 kb mRNA is 3:1 while at 8 h this is 1:3 (see Fig. 1). To learn whether

both mRNAs are translated into PAI-1 protein with the same efficiency, we measured PAI-1 antigen levels at different times after the addition of PMA, and compared total PAI-1 mRNA levels (3.2 kb plus 2.4 kb species) with PAI-1 antigen increments. As shown in Fig. 6, the increase in PAI-1 antigen over time reflects total PAI-1 mRNA levels, irrespective of the relative concentration of each mRNA form. The same results were found when PAI-1 activity was measured instead of PAI-1 antigen (results not shown). These results suggest, but do not unequivocally prove, an equally efficient translation of both PAI-1 mRNA species.

Direct determination of the translational capacity of both PAI-1 mRNA forms was performed with two different RNA preparations in a rabbit reticulocyte lysate system. Firstly, two synthetic SP6 mRNAs representing the 3.2 and 2.4 kb PAI-1 mRNA forms were used. Translation products of both mRNA species were analyzed on a 10% SDS-polyacrylamide gel. Increasing amounts (0-100 ng) of the two PAI-1 mRNA constructs code, with the same efficiency, for a 40 kD-polypeptide (Fig. 7). An identical translational efficiency of the two PAI-1 mRNA species was also shown by determining TCA-precipitable [^{35}S]-methionine labeled product or immunoprecipitable product in the lysate (Table I).

Table I. *In vitro* translation of the two PAI-1 mRNAs.

20 ng of pSP6-large or pSP6-small RNA, corresponding to the 3.2 kb and 2.4 kb mRNA species, respectively, and 2 µg of poly(A) RNA isolated from Hep G2 cells incubated with PMA for 2 h or 7 h, were translated in a rabbit reticulocyte lysate for 10 min in the presence of [^{35}S]-methionine. Radiolabeled translation products were quantified by TCA precipitation or immunoprecipitation, as described in the Methods section, followed by liquid scintillation counting. The data represent mean values of duplicate experiments or mean ± SD of four experiments, and are expressed as cpm/nmol PAI-1 mRNA or cpm/µg poly(A) RNA.

RNA sample	TCA precipitation	Immunoprecipitation
	10^6 x cpm/nmol PAI-1 mRNA	10^6 x cpm/nmol PAI-1 mRNA
pSP6-large	7.5 ± 1.2	7.9
pSP6-small	6.3 ± 1.4	6.1
	10^6 x cpm/µg poly(A) RNA	10^6 x cpm/nmol PAI-1 mRNA
poly(A) RNA (t = 2 h) (ratio 3.2 : 2.4 = 2 : 1)	6.0	10.6*/29.0**
poly(A) RNA (t = 7 h) (ratio 3.2 : 2.4 = 1 : 5)	5.4	8.8*/19.4**

*, ** Values of two separate, independent experiments.

Secondly, we used poly(A) RNA from Hep G2 cells that had been incubated with PMA for 2 or 7 h, and differed in the relative concentration of the two PAI-1 mRNA forms but not in total amount of PAI-1 mRNA: at 2 h, the ratio between the 3.2 kb and 2.4 kb form was 2:1, at 7 h it was 1:5. No marked difference in total (i.e. TCA-precipitable) protein synthesis (per μg of total RNA) or PAI-1 (i.e. immuno-precipitable) synthesis (per nmol PAI-1 mRNA) was seen with the two RNA preparations (Table I). These results suggest again, as was seen with the synthetic forms, that the 3.2 kb and 2.4 kb PAI-1 mRNA are translated with a similar efficiency.

DISCUSSION

In man, two distinct PAI-1 mRNA species are found, one of 3.2 kb and one of 2.4 kb (Ginsburg et al., 1986; Ny et al., 1986), which differ in the length of their 3' untranslated region, probably as a result of alternative polyadenylation (Loskutoff et al., 1987; Bosma et al., 1988). In this paper we show that treatment of Hep G2 cells with PMA resulted in a strong and transient increase in both PAI-1 mRNAs, but each species with a different induction profile: at early time points the 3.2 kb species predominates, whereas at later time points the 2.4 kb species is more abundant. It was found that PAI-1 mRNA accumulation was preceded by a strongly increased rate of transcription and that it was dependent on de novo protein synthesis: CHX, when added 1 h prior to PMA, completely inhibited the accumulation of PAI-1 mRNA. Thus the induction of PAI-1 mRNA levels may be dependent on the synthesis of transcription factors, which enhance PAI-1 gene expression.

We have found that PMA also affects the ratio between the two PAI-1 mRNAs in the nuclei: in time a shift towards the 2.4 kb species is seen (Fig. 3). This cannot be explained by a change in the termination site of PAI-1 gene transcription (Fig. 2), but could be the result of regulation at the level of poly(A) site selection or another post-transcriptional nuclear event. It is interesting in this respect, that the induction of PAI-1 expression in Hep G2 is accompanied by a transient increase in c-myc mRNA (Bosma and Kooistra, unpublished). PAI-1 gene expression has been shown to be regulated by c-myc protein, which may act at the level of RNA export, splicing, or nuclear RNA turnover (Prendergast et al., 1989, 1990).

Besides nuclear post-transcriptional processes, the different stability of the two PAI-1 mRNAs may contribute to the different induction profile of the two PAI-1 mRNAs: after PMA addition to Hep G2, the 3.2 kb species shows a half-life time of about 0.85 h, which is at least three times shorter than the 2.5 h calculated for the 2.4 kb messenger. The enhanced lability of the 3.2 kb species in comparison to the 2.4 kb species may be due to the presence of an AU rich sequence in the 3' untranslated region of the 3.2 kb form.

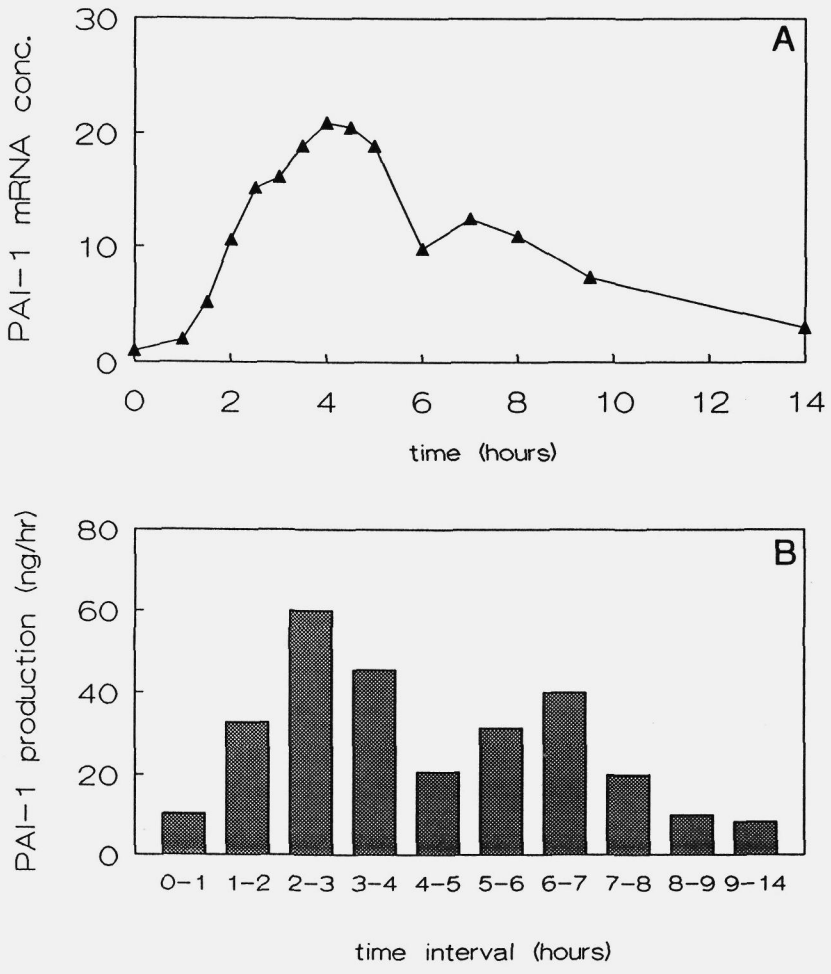
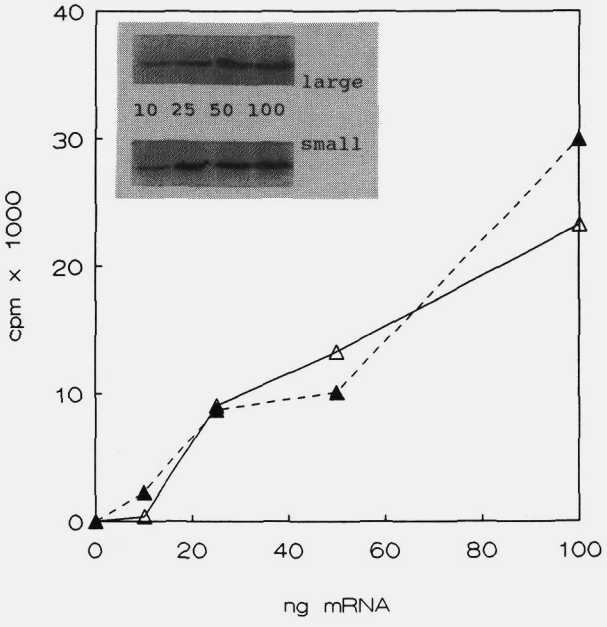
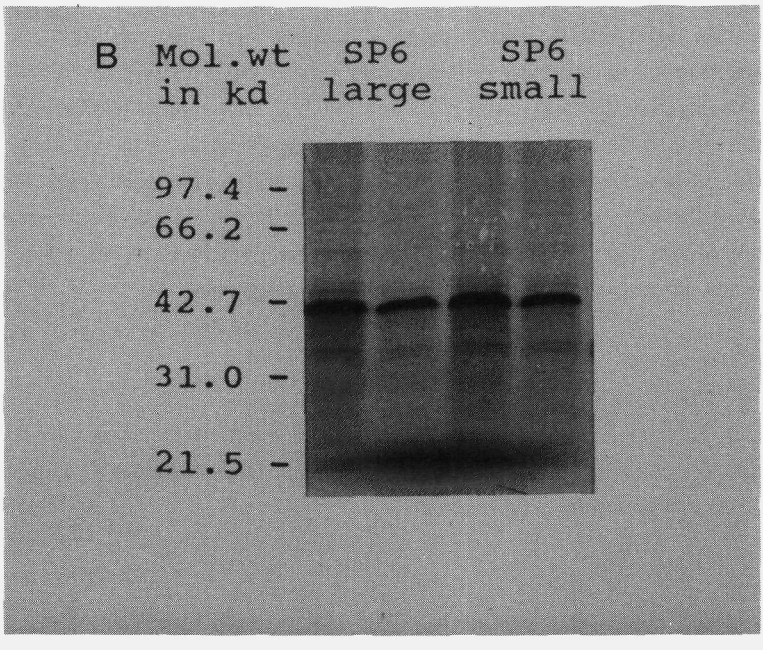


Figure 6. Time course of PAI-1 mRNA levels and PAI-1 antigen production in PMA-treated Hep G2 cells. Hep G2 cells were preincubated with serum-free medium for 16 h and then treated with PMA (100 nM). At the times indicated, total cellular RNA was isolated and analysed by Northern blotting. Total PAI-1 mRNA (3.2 kb and 2.4 kb) is given as relative concentration to the amount present at t = 0 (A). Production of PAI-1 antigen was calculated from the total PAI-1 antigen levels in cell lysate and medium at different time points and is expressed as the increase in ng PAI-1/h per dish (B).

Figure 7. *In vitro* translation of two SP6 PAI-1 mRNAs.
A. Varying amounts of pSP6-large (▵) and pSP6-small (▲) RNA, corresponding to the 3.2 kb and the 2.4 kb PAI-1 mRNA species, respectively, were translated in a rabbit reticulocyte lysate for 30 min in the presence of [^{35}S]methionine. Radiolabeled translation products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, or by TCA precipitation, followed by liquid scintillation counting.
B. 20 ng of each PAI-1 mRNA was translated and radiolabeled. Translation products of two separate experiments were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The migration and sizes of molecular weight standards separated on the same gel are indicated.

A


large
10 25 50 100
small
40
30
20
10
0
cpm x 1000
0
20
40
60
80
100
ng mRNA


B Mol.wt in kd
SP6 large
SP6 small
97.4 -
66.2 -
42.7 -
31.0 -
21.5 -

Such a sequence, absent in the 2.4 kb PAI-1 mRNA, has been reported to be associated with the rapid turnover of the mRNAs coding for lymphokines, cytokines and proto-oncogenes (Shaw and Kamen, 1986; Caput et al., 1986; Fort et al., 1987). The accelerated decay of the PAI-1 3.2 kb species in Hep G2 is dependent on ongoing transcription and translation (Fig. 4), suggesting that it is mediated by a labile protein factor coded for by an instable mRNA. Similar results have been reported for other rapidly degraded, AU sequence containing mRNAs, for example c-fos mRNA (Shyu et al., 1989). In fact, a cytosolic protein has recently been identified that binds specifically to RNA molecules containing an AU rich sequence (Malter, 1989). In control cells no stabilization of the 3.2 kb mRNA by CHX is seen, suggesting that the factor responsible for the rapid decay of this messenger is not always present in Hep G2 cells but is induced by PMA. The induction of this factor seems to be very rapid as already 2 h after PMA addition stabilization of the larger mRNA by CHX, actinomycin D and DRB is seen. Similar results were reported for HT1080 cells (Andreasen et al., 1987) and K562 cells (Alitalo et al., 1989), but are contrast with data reported by other investigators: addition of CHX to human endothelial cells or rhabdosarcoma cells enhances the relative concentration of the 3.2 kb mRNA species (Van den Berg et al. 1988; Schleef et al. 1988; Mayer et al. 1988), indicating that in these cells the factor may also be present under apparently unstimulated conditions. The short half-life of the 3.2 kb species was shown to be insensitive to H7, indicating that after the factor is induced by PMA it is independent of active PKC (Fig. 5).

The 2.4 kb PAI-1 mRNA is only found in humans and higher primates: the extra polyadenylation site in the PAI-1 gene generating this smaller mRNA has been recently acquired during primate evolution (Cicilia et al., 1989). Although the two PAI-1 mRNAs are translated with the same efficiency into a 40 kD-polypeptide (Figs 6,7, Table I), the appearance of a second messenger form in humans, which is insensitive to the factor destabilizing the larger mRNA, can be anticipated to have physiological consequences: stimulation of PAI-1 gene expression may result in prolonged biosynthesis of PAI-1, and, consequently, in prolonged inhibition of plasminogen activation.

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CHAPTER 7

ROLE OF PROTEIN KINASE C AND cAMP IN THE REGULATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR, PLASMINOGEN ACTIVATOR INHIBITOR 1 AND PLATELET-DERIVED GROWTH FACTOR mRNA LEVELS IN HUMAN ENDOTHELIAL CELLS. POSSIBLE INVOLVEMENT OF PROTO-ONCOGENES C-JUN AND C-FOS

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SUMMARY

Activation of protein kinase C (PKC) leads to a strong induction of tissue-type plasminogen activator (t-PA) expression in endothelial cells. With endothelial cells from human umbilical vein (HUVEC) and human aorta (HAEC) we have studied this regulation of t-PA and its inhibitor, PAI-1, at the mRNA level and have compared their induction with the expression of platelet-derived growth factor A and B (PDGF-A and PDGF-B) and the proto-oncogenes, c-jun and c-fos. Treatment of HUVEC with exogenous bacterial phospholipase C or the synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol, led respectively to a three-and two-fold increase in t-PA concentration in 24 hours conditioned medium. Similarly, the more stable PKC activator, phorbol-12-myristate-13-acetate (PMA), caused an increase of about ten-fold in t-PA antigen levels. This effect of PMA is maximal between 8 and 16 hours at a concentration of 10 nM, and is fully accounted for by parallel increases in t-PA mRNA levels. An increase in intracellular cAMP levels by forskolin (10 μM) slightly diminished t-PA expression, but further enhanced the PMA-induced increases in t-PA synthesis and mRNA levels by at least two-fold. PMA also enhanced the mRNA levels of two other important endothelium-expressed genes, PDGF-A and PDGF-B, with a similar time-profile as seen with t-PA, and peak values about 5-fold higher than control values. Forskolin did not further stimulate this PMA-induced PDGF expression in HUVEC, which suggests a regulatory mechanism different from that of t-PA. Qualitatively very similar induction patterns of t-PA, PDGF-A and PDGF-B were seen with HAEC. In contrast to t-PA and PDGF, PAI-1 mRNA and antigen levels only slightly increased upon treatment of HUVEC or HAEC with PMA; forskolin alone or in combination with PMA diminished the expression of PAI-1.

The induction of t-PA mRNA by PMA was dependent on protein synthesis and preceded by a strong, transient increase in c-jun and c-fos mRNA levels; the induction of c-fos, but not c-jun, was potentiated by forskolin. Since the products of these two proto-oncogenes form dimeric complexes, for which specific binding sites are present in the t-PA promoter region, they might mediate the PKC-dependent increase in t-PA gene expression, including the stimulating action of cAMP.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) plays a crucial role in initiating fibrinolysis by mediating conversion of the zymogen plasminogen to the active serine protease plasmin, which can degrade fibrin. Accordingly, the fibrinolytic capacity in plasma is subject to regulation by mechanisms that determine t-PA activity in the circulation. The

vascular endothelium is actively involved in controlling plasma t-PA activity by producing t-PA as well as a specific plasminogen activator inhibitor,
PAI-1. Knowledge about the regulation of synthesis of t-PA and PAI-1 may be helpful in developing drugs that may increase insufficient endogenous production of t-PA or that may diminish overproduction of either t-PA or PAI-1.

A variety of agents has been shown to increase t-PA and/or PAI-1 synthesis in cultured human endothelial cells (1,2), but only recently some initial insight into the intracellular regulatory mechanisms responsible for altered t-PA expression has been obtained (3-6). In these studies activation of protein kinase C (PKC) has been implicated in the induction of t-PA expression in endothelial cells from human umbilical vein (HUVEC). This induction of t-PA expression was found to be potentiated by increasing intracellular cAMP levels, although cAMP alone did not affect t-PA synthesis. In accordance with this, the previously observed stimulatory effect of thrombin (7-9) and histamine (9) on t-PA synthesis in endothelial cells could also be mediated by activation of PKC. These effectors induce a receptor-mediated activation of phospholipase C, generating a rise in endogenous diacylglycerols (DAG) (10): DAG are believed to be the physiological activators of PKC (11).

The present study was undertaken to further define the role of phospholipase C and PKC in the regulation of t-PA and PAI-1 synthesis in endothelial cells. It also addresses the question of whether endothelial cells from human aorta (HAEC), which have previously been shown to differ greatly from HUVEC in the amount of secreted t-PA and t-PA mRNA content (12), respond in the same way to activation of PKC. To learn more about the specificity of the PKC-dependent regulation of t-PA expression, parallel studies were performed on the regulation of platelet-derived growth factor (PDGF). The expression of PGDF in the endothelium is thought to be important in mediating vessel wall responses to injury via paracrine stimulation of neighbouring vascular cells (13,14). Cultured human renal microvascular endothelial cells have been reported to express two PDGF mRNAs, A and B, which are encoded by separate genes and the levels of which are increased by the stable PKC activator, 12-myristate-13-acetate (PMA) (15,16). Here we compare the induction of the t-PA, PAI-1 and PDGF mRNAs by PMA and cAMP.

We found that the induction of t-PA mRNA by PMA could be almost completely inhibited by the protein synthesis inhibitor cycloheximide, suggesting that it may be dependent on the synthesis of transcription factors. We have investigated therefore the effect of PMA and cAMP on the mRNA levels of c-jun and c-fos. The products of these two cellular proto-oncogenes have been shown to play a central role in regulating transcription from promoters responsive to PMA and cAMP (17), and could thus also be the mediators of the induced increase in t-PA expression.

MATERIALS AND METHODS

Materials

Phospholipase A2 (Lecithinase A; Phosphatidylcholine 2-acyl-hydrolase; EC 3.1.1.4) from porcine pancreas and Crotalus adamanteus venom, phospholipase C (Lecithinase C; phosphatidylcholine choline-phosphohydrolase; EC 3.1.4.3) from C.perfringens, 4β-phorbol-12-myristate-13-acetate (PMA), forskolin, and calcium ionophore A23187 were purchased from Sigma Chemical Co. (St. Louis, MO). 1-oleoyl-2-acetylglycerol was a generous gift from Prof. Y. Nishizuka, Kobe, Japan. Stock solutions of forskolin and PMA were prepared in ethanol. A23187 was dissolved in either ethanol or DMSO. OAG was suspended in 1% (v/v) DMSO, sonicated to prepare micelles, and then directly added to the cells.

t-PA (> 99% two-chain) was purified from Bowes-melanoma-cell culture medium as described by Kluft et al. (18). The activity is expressed as international units (i.u.), using the first international standard of the World Health Organisation, code 83/517, as a standard (19). Our t-PA preparation has a specific activity of 500,000 i.u./ml.

Enzyme immunoassay kits for determination of human t-PA antigen and PAI-1 antigen ("Imulyse") were obtained from Biopool (Umeå, Sweden). Other materials used in the methods described below have been specified in detail in the relating references.

Deoxycytidine 5[α-^{32}P] triphosphate was from Amersham International plc (Buckinghamshire, UK).

Cell culture experiments

Endothelial cells from human umbilical cord veins and human aorta were isolated using collagenase by techniques previously described (12,20). Cells were grown in fibronectin-coated dishes in M199 medium supplemented with 20 mM HEPES, 10% (v/v) human serum, 10% (v/v) newborn calf serum (heat-inactivated), 150 μg/ml ECGF (21), and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere, as described (12). Medium was replaced every 2-3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1:3.

For experiments, confluent cultures were used at second or third passage, and cells were always refed the day before the experiment with incubation medium, viz. M199 medium supplemented with 20 mM HEPES, 10% (v/v) human serum and penicillin/streptomycin. Conditioned media were obtained by incubating cells at 37°C for various times up to 24 h with incubation medium containing the appropriate concentration of the test compound or stock solvent (final concentration maximally 0.1% (v/v)). The media were centrifuged for 2 min in a Beckman Microfuge to remove cells and cellular debris, and samples were frozen at -20°C until use. Cells were washed three

times at 37 °C with phosphate-buffered saline (0.15 M-NaCl/10 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4) and used for isolation of RNA.

Assays

PAI-1 activity was determined by the method of Verheijen et al. (22): samples were titrated with increasing amounts of t-PA, followed by spectrophotometric measurement of the residual t-PA activity. One unit of PAI-1 is defined as the amount of inhibitor that neutralizes one i.u. of t-PA activity. t-PA antigen and PAI-1 antigen determinations were performed by commercially available enzyme immunoassay kits.

RNA hybridization

Total RNA was extracted from endothelial cells as described by Lizardi and Engelberg (23), with some minor modifications. In short, after washing the cells with warm (37 °C) PBS, lysis buffer (50 mM Tris.HCl, pH 7.4; 100 mM NaCl; 7.5 mM EDTA; 0.5% SDS; 150 μg/ml proteinase K) was added directly to the cells in the culture dish, followed by an incubation for 30 min at 37 °C. The proteinase K-SDS lysate was vigorously mixed in a Polytron PTA-7 blender (Kinematica GmbH, Luzern, Switzerland), and then subjected to phenol extraction. RNA was collected by ethanol precipitation and purified from contaminating DNA by precipitation in 3 M LiCl for 3 hours at 0 °C. After a second ethanol precipitation step, RNA samples were dissolved in H_2O. The RNA concentration in each sample was determined spectrophotometrically.

Equal amounts of total RNA from the different dishes were analyzed for their t-PA, PAI-1, PDGF-A, PDGF-B, c-jun, c-fos, c-myc and GAPDH mRNA content by Northern Blot hybridization. With Northern blotting, RNA samples were subjected to gel electrophoresis in formaldehyde agarose gels. After electrophoresis, RNA was transferred to Hybond N according to the instructions of the manufacturer. Pre-hybridization and hybridization were at 60 °C (1 mM EDTA; 7% SDS; 0.25 M NaCl; 0.25 M NaH_2PO_4-Na_2HPO_4; pH 7.2), essentially as described elsewhere (24). DNA fragments to be used as a probe were isolated from low melting point agarose (25). Hybridization was usually performed with 1 ng/ml of probe labelled by the random primer method (Multi-prime, Amersham, Houten, The Netherlands) to approximately 2×10^8 to 2×10^9 cpm/μg DNA.

The filters were washed at a stringency of 0.1 x SSC and 1% SDS for 2 x 15 min at 65 °C (25). The membranes were subsequently exposed to Amersham hyperfilm-MP with an intensifying screen at -80 °C. For the quantification of the relative amounts of mRNA, densitometry was used. In short, a scan of the bands was made on a CS 910 Shimadzu scanner and the areas under the peaks were integrated and plotted with the aid of a United Technology Packard data processor.

cDNA probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1.9 kb Bgl II fragment of the human t-PA cDNA (26); a 2.5 kb EcoRI fragment of a human PAI-1 cDNA of the 3.1 kb transcript (27); a 780 bp EcoRI-RsaI fragment of the human PDGF-A cDNA and a 890 bp PstI-NcoI fragment of the human PDGF-B cDNA, both provided by Dr. C. Betsholtz (28); a 1.9 kb c-jun cDNA fragment, a 2.6 kb c-fos cDNA fragment, and a 1.3 kb ClaI-EcoRI c-myc DNA fragment of a human genomic clone, spanning most of exon 3, provided by Drs. H. van Dam, R. Offringa and I. Laird Offringa; University of Leiden (29,30); and a 1.2 kb PstI fragment of a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (31) provided by Dr. R. Offringa, which is used as an internal standard probe (32).

RESULTS

Role of phospholipase C and PKC in regulation of t-PA and PAI-1 production

Addition of exogenous phospholipase C at high concentrations to several cell types has been shown to mimic activation of endogenous phospholipase C (33). As shown in Table 1, addition of 10^{-5} or 10^{-4} U/ml exogenous bacterial phospholipase C, but not phospholipase A_2, to endothelial cells induced a 2.5- to 3-fold enhancement of t-PA antigen, which was paralleled by a significant decrease in PAI-1 activity in the conditioned medium. Phospholipid hydrolysis by (endogenous) phospholipase C produces two important intracellular messengers, these being inositol 1,4,5-trisphosphate, which mobilizes intracellular calcium (34), and 1,2-diacylglycerol (DAG), which can activate PKC (11). Incubation of HUVEC with the synthetic DAG, 1-oleoyl-2-acetylglycerol (25 μM), or with the phorbol ester PMA (10 nM) caused a two- and eight-fold increase in t-PA accumulation, respectively. Elevation of intracellular calcium levels by the calcium ionophore A23187, however, only slightly enhanced t-PA (and PAI-1) production. These results point to an important role of PKC in regulating t-PA synthesis in endothelial cells. Similarly as seen with phospholipase C, PMA reduced PAI-1 activity in 24 hours conditioned medium. An increase in intracellular cAMP levels, as induced by the adenylate cyclase activator forskolin, led to a small decrease in the production of both t-PA protein and PAI-1 activity.

Effect of PMA and forskolin on t-PA and PAI-1 production in human umbilical vein and aorta endothelial cells

The role of PKC in t-PA expression was further evaluated, using the stable PKC activator, PMA. Treatment of HUVEC or HAEC for 24 hours with PMA showed a dose-dependent (range 0.01-10 nM) increase in t-PA production in the medium and a small

decrease in PAI-1 activity; at 100 nM PMA no further increase or only a slight decrease in t-PA stimulation was seen (results shown for HUVEC in Table 1). Further experiments were performed with optimal PMA concentrations only (i.e., 10 nM). PMA stimulation of t-PA synthesis was a time-dependent process, observable after a lag-period

Table 1. Effect of various agents on the production of t-PA and PAI-1 by endothelial cells.

		Production (% of control)	
Addition	Concentration	t-PA antigen	PAI-1 activity
None		100%	100%
Phospholipase C	10^{-6} U/ml	175 ± 12%	105 ± 12%
	10^{-5} U/ml	268 ± 14%	64 ± 17%
	10^{-4} U/ml	278 ± 8%	48 ± 10%
Phospholipase A2 (porcine pancreas)	10^{-6} U/ml	96 ± 8%	133 ± 10%
	10^{-5} U/ml	93 ± 2%	129 ± 9%
	10^{-4} U/ml	93 ± 4%	132 ± 1%
Phospholipase A2 (Crotalus adamanteus venom)	10^{-6} U/ml	95 ± 3%	133 ± 11%
	10^{-5} U/ml	98 ± 17%	117 ± 16%
	10^{-4} U/ml	125 ± 3%	120 ± 5%
A23187	0.3 μM	123 ± 14%	122 ± 15%
OAG	25 μM	194 ± 27%	101 ± 8%
PMA	0.01 nM	273 ± 42%	83 ± 11%
	0.1 nM	525 ± 81%	75 ± 7%
	1.0 nM	707 ± 63%	62 ± 10%
	10.0 nM	837 ± 91%	65 ± 9%
	100 nM	504 ± 112%	60 ± 14%
Forskolin	10 μM	79 ± 14%	75 ± 6%

Human umbilical vein endothelial cells were incubated for 24 h with various compounds at various concentrations and the CM were analyzed for t-PA antigen and PAI-1 activity as described in the Materials and Methods section. For A23187, OAG and PMA only those concentrations are shown, that gave maximal stimulation of t-PA production. Results are expressed as percentage values of controls and are means ± range of two independent experiments, with incubations in duplicate.

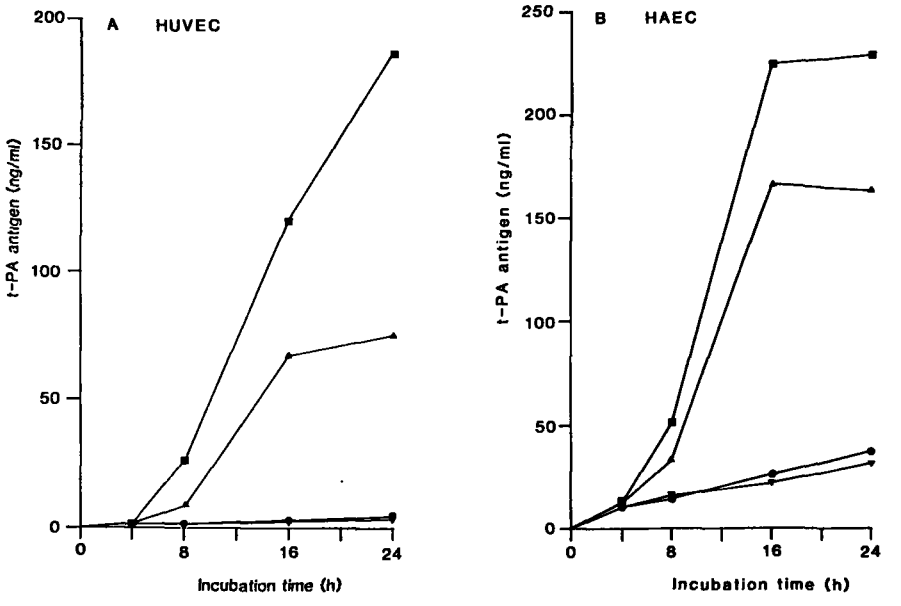


Figure 1. Time-course of t-PA antigen production in cultured endothelial cells incubated with PMA and/or forskolin. Endothelial cells from human umbilical vein (A) or human aorta (B) were incubated for various times up to 24 hours in the absence (●) or presence of 10 μM forskolin (▼), 10 nM PMA (▲), or 10 nM PMA and 10 μM forskolin (■), and the conditioned media were analysed for t-PA antigen. The results are representative of three separate, independent experiments.

of about 4 hours, and continuing up to at least 24 hours (Fig. 1A). This stimulatory effect of PMA could be further enhanced by the concomitant presence of the cAMP-raising agent forskolin (10 μM), although forskolin by itself slightly diminished t-PA production (Fig. 1; Table 1). HAEC showed a much higher basal t-PA expression, but reacted qualitatively in the same way as HUVEC towards PMA and forskolin (Fig. 1B). Concomitantly, the accumulation of PAI-1 antigen in the conditioned media of both types of endothelial cells was moderately enhanced with PMA, and somewhat decreased when the cells were incubated with forskolin (Figs. 2A and 2B). Measurement of PAI-1 activity essentially showed a similar picture (results not shown).

Effect of PMA and forskolin on t-PA, PAI-1, PDGF-A and PDGF-B mRNA levels

The cells used to study the effects of PMA and forskolin on t-PA and PAI-1 synthesis (Figs. 1 and 2) were examined for mRNA levels. Total cellular RNA from control cells and treated cells was analyzed by Northern blot hybridization using cDNA probes for t-PA, PAI-1, PDGF-A and PDGF-B. Fig. 3 illustrates the results obtained with HUVEC. Scanning of the autoradiographs of the Northern blots showed that a response on t-PA mRNA in the endothelial cells could be noted as early as 4 hours after addition of PMA

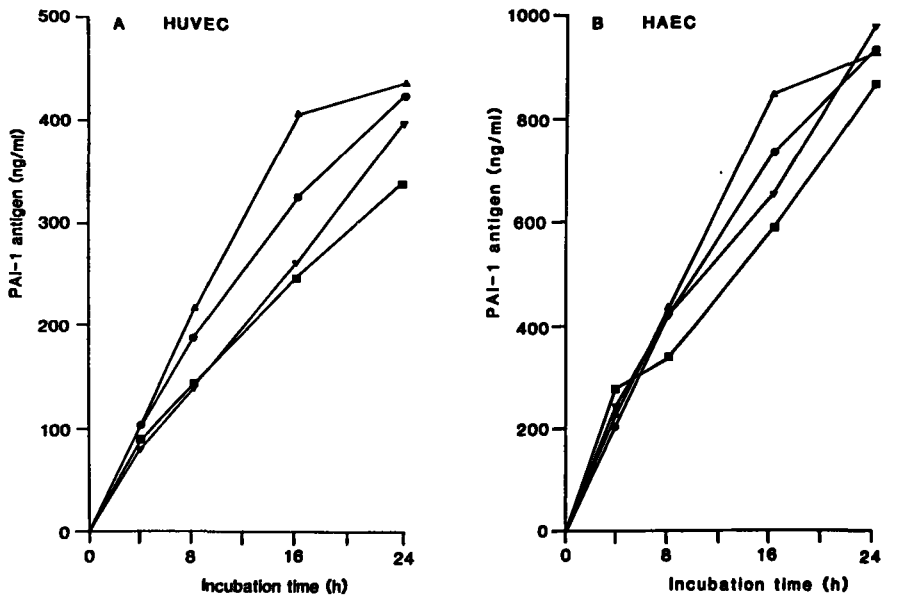


Figure 2. Time-course of PAI-1 antigen production in cultured endothelial cells incubated with PMA and/or forskolin. Endothelial cells from human umbilical vein (A) and human aorta (B) were incubated for various times up to 24 hours in the absence (●) or presence of 10 μM forskolin (▼), 10 nM PMA (▲) or 10 nM PMA and 10 μM forskolin (■), and the conditioned media were analysed for PAI-1 antigen. The results are representative of three separate, independent experiments.

(Fig. 3A). Stimulation was maximal at 8 hours, and subsided afterwards reaching control values after 24 hours. Peak levels of t-PA mRNA in HUVEC were at least 50-fold enhanced after PMA treatment, but an exact estimate of the stimulation factor is difficult because of the extreme low levels of t-PA mRNA levels in control cells. Forskolin alone showed no detectable effect on t-PA mRNA levels, but it further potentiated the PMA-induced increases in t-PA mRNA levels by about a factor two, without changing the time course of the induction. These results are in accordance with the data shown above for t-PA antigen production.

Analysis revealed roughly equal amounts of two PAI-1 mRNA species with lengths of 2.3 and 3.0 kb respectively (see Fig. 3B), the ratio of which did not markedly change during the various incubations. PAI-1 mRNA levels as shown in Fig. 3B represent the sums of both mRNA species. Similarly as seen for PAI-1 antigen, PMA addition to HUVEC induced a small increase at the mRNA level, whereas forskolin decreased PAI-1 mRNA concentrations in both the control and PMA-treated HUVEC.

Three transcripts of PDGF-A mRNA (2.8, 2.3, and 1.4 kb) and a single band of 3.7 kb PDGF-B mRNA were detected in the endothelial cells (Figs. 3C and 3D), which is consistent with the results of previous reports (35). The time course of stimulation of both

A and B chain message levels by PMA paralleled that for t-PA mRNA levels: an increase in PDGF mRNA level was already imminent after 4 hours, peaked at 8 hours (about 5-fold enhancement) and decreased gradually over the next 16 hours. Forskolin did not markedly affect PDGF-B mRNA levels, but it strongly suppressed PDGF-A mRNA levels, as had been seen with PAI-1 mRNA. In contrast to t-PA mRNA, an increase in cAMP levels did not significantly enhance the PMA-induced increases in PDGF-A and PDGF-B mRNA levels.

Two methods were used to ensure that the changes demonstrated were not due to unequal loading of mRNA onto the gel. Firstly, equal loading of total RNA was verified by visual inspection of the 28S and 18S ribosomal RNA on the gel illuminated by UV light. Secondly, after stripping, the blots were rehybridized with a labelled probe directed against GAPDH mRNA. As shown in Fig. 3E, no significant changes in GAPDH mRNA levels were observed, which also stresses the specificity of the effects described above.

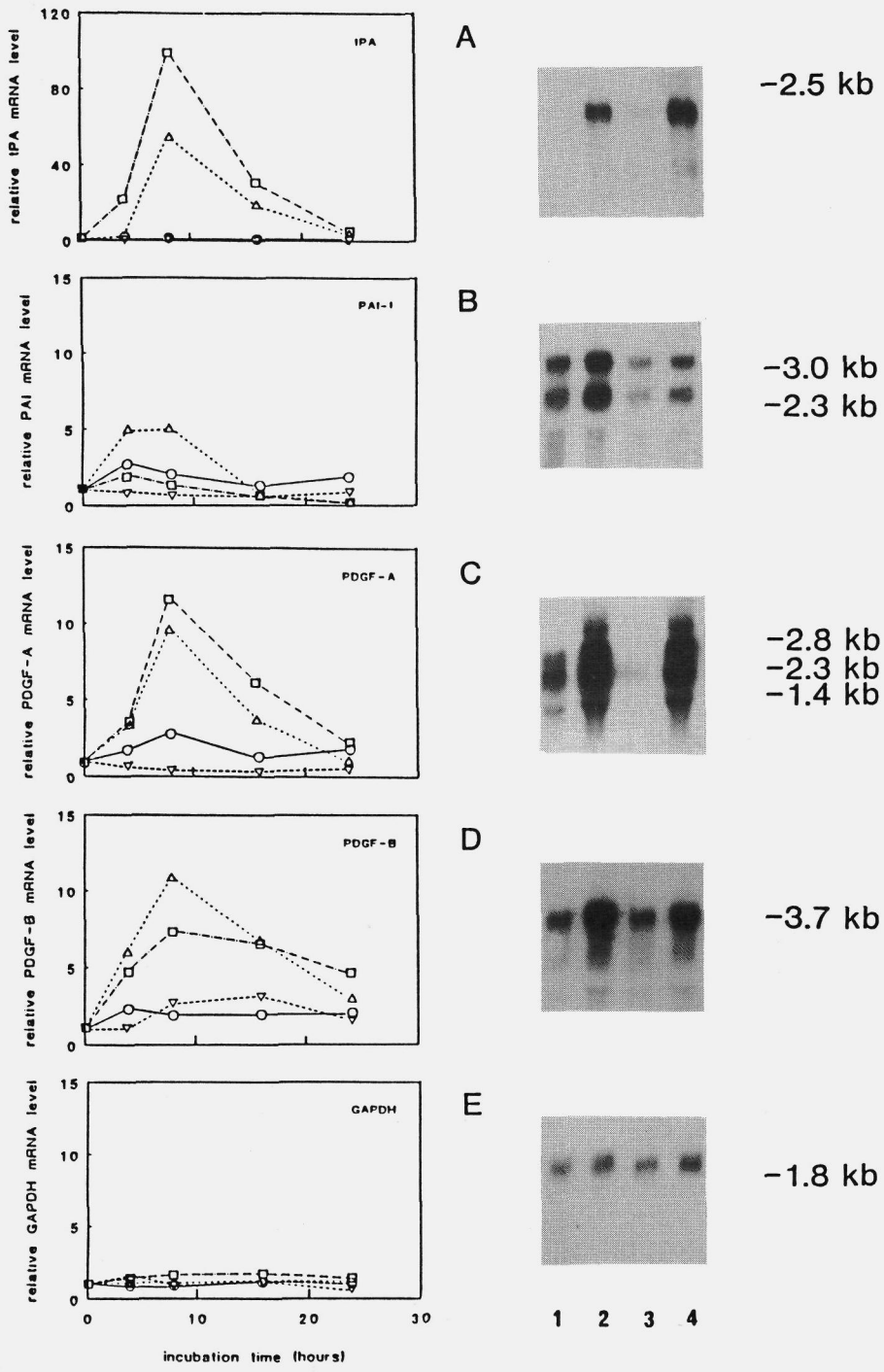
Results obtained with HAEC were qualitatively very similar to the data shown for HUVEC. Fig. 4 shows the mRNA levels of t-PA, PAI-1, PDGF-A, PDGF-B and GAPDH in HAEC at 8 hours only, when responses to PMA were maximal.

Activation of PKC results in induction of c-jun, c-fos and c-myc mRNA in endothelial cells

To determine whether PMA regulation of t-PA mRNA is a direct effect on gene expression or an indirect effect requiring the synthesis of another gene product, the protein synthesis inhibitor cycloheximide (CHX) was added to HUVEC 1 hour prior to PMA stimulation. Under these conditions, when protein synthesis was found to be inhibited for over 95%, the induction of t-PA (and PDGF) mRNA accumulation was almost completely suppressed, as analyzed by Northern blot hybridization (results not shown). This suggests that the induction of t-PA mRNA is dependent on the synthesis of transcription factors, which mediate the transcriptional response to activation of PKC. Such transcription factors recognize and bind to their specific cognate DNA sequences.

We have examined the 5'flanking DNA of the t-PA (36), PAI-1 (37), PDGF-A (38) and PDGF-B (39) genes for the presence of sequences similar to the consensus response

Figure 3. Effect of PMA and/or forskolin on t-PA, PAI-1, PDGF-A, PDGF-B and GAPDH mRNA content in HUVEC. Total RNA was isolated from confluent cultures of HUVEC incubated in the presence or absence of 10 nM PMA and/or 10 μM forskolin for the indicated time periods. mRNA levels at each time point were estimated by Northern blot analysis, followed by densitometric scanning of autographs (see Experimental Procedures). PAI-1 and PDGF-A mRNA levels shown represent the sums of the two and three transcripts, respectively. Results are expressed as relative amounts of mRNA. Except for t-PA, the mRNA level at time 0 has been set equal to 1, and the data at subsequent time points expressed as -fold induction. Because of the very low t-PA mRNA level at time 0, t-PA mRNA levels are expressed relative to the mRNA level in EC incubated for 8 hours with both PMA and forskolin, which level has been set equal to 100. ○, control; △, PMA; ▽, forskolin; □, PMA and forskolin. On the right of each panel, the corresponding autoradiographs at the 8 hours time point are shown. Lane 1: control; lane 2: PMA; lane 3: forskolin; lane 4: PMA and forskolin. The results are representative of three separate, independent experiments.


A
B
C
D
E
tPA
PAI-1
PDGF-A
PDGF-B
GAPDH
relative tPA mRNA level
relative PAI mRNA level
relative PDGF-A mRNA level
relative PDGF-B mRNA level
relative GAPDH mRNA level
incubation time (hours)
-2.5 kb
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-2.3 kb
-2.8 kb
-2.3 kb
-1.4 kb
-3.7 kb
-1.8 kb
1 2 3 4

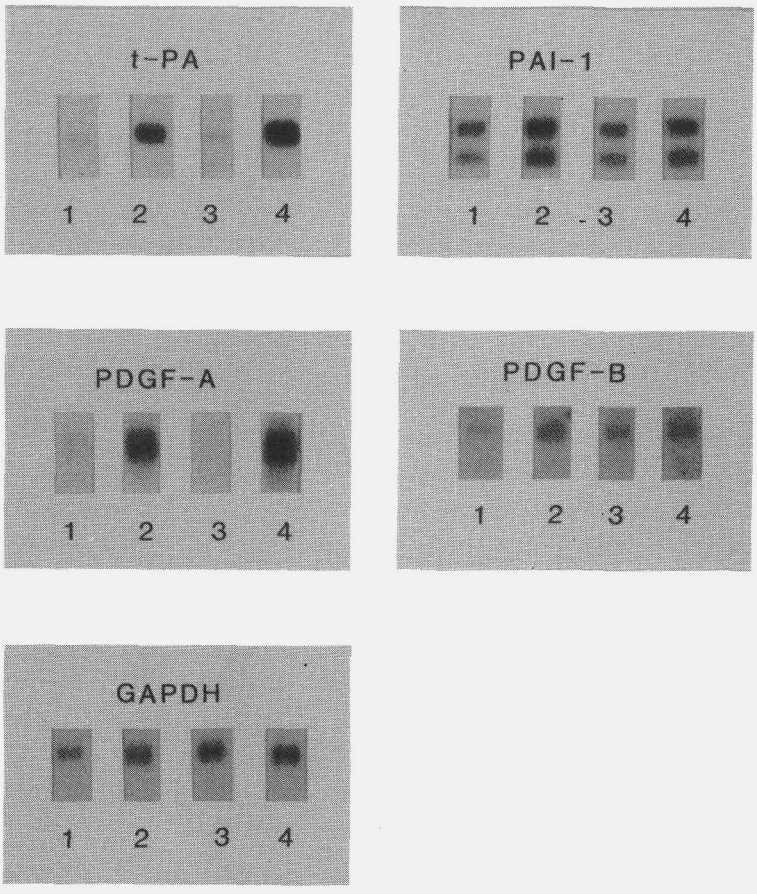

Figure 4. Effect of PMA and/or forskolin on t-PA, PAI-1, PDGF-A, PDGF-B and GAPDH mRNA content in HAEC. Total RNA was isolated from confluent cultures of HAEC, incubated in the presence or abscence of 10 nM PMA and/or 10 μM forskolin for 8 hours. mRNA levels were estimated by Northern blot analysis, and shown as autoradiographs.
Lane 1: control; lane 2: 10 nM PMA; lane 3: 10 μM forskolin; lane 4: 10 nM PMA and 10 μM forskolin.

elements for cAMP and PMA (40-43). A computer search revealed in the t-PA gene only a perfectly conserved Activator Protein 1 (AP-1) binding site at position -113 and two perfect PMA-response elements (TREs) at positions -3321 and -2316. No perfectly conserved cAMP and/or PMA response elements were found in the 5'-flanking DNA of the PAI-1, PDGF-A and PDGF-B genes. Both the AP-1 binding site and the TRE can bind the transcription factor AP-1, which is a homo- or heterodimer of the products of two cellular proto-oncogenes, c-jun and c-fos (44). We therefore evaluated the effects of PMA on c-jun and c-fos mRNA levels in HUVEC and HAEC. Activation of PKC by PMA resulted in both types of endothelial cells in a marked and transient increase in the

mRNA levels of c-jun and c-fos, as illustrated in Fig. 5 for HUVEC. The detailed time course showed that c-jun mRNA was elevated as early as 15 min after the addition of PMA, reaching its maximum (about five-fold increase) at around 1 hour. Thereafter, c-jun mRNA decreased to the pre-induction level within 3 hours. Two different c-jun mRNA forms were detected, which may be accounted for by the different positions of the poly (A) addition signals (45). The larger species was much less abundant at basal levels, reached its maximum induction somewhat later, and remained elevated for a longer time than the small species, which resulted in about equal levels of the two mRNA forms after 4 hours. In contrast to c-jun, c-fos mRNA was hardly detectable in unstimulated HUVEC or HAEC. Upon addition of PMA, c-fos mRNA levels rapidly and strongly increased, reaching peak levels after about 45 min, followed by a rapid decline to undetectable levels after about 2 hours (shown in Fig. 5 for HUVEC). Fig. 5 also demonstrates that c-myc mRNA, another "early response" gene, increased more slowly than c-jun and c-fos mRNA upon stimulation, with a peak after about 2 hours. Since cAMP was found to potentiate the PMA-induced t-PA expression, it seemed of interest to determine if cAMP could also affect c-jun and c-fos expression. Indeed, the simultaneous presence of forskolin strongly enhanced the PMA-induced c-fos mRNA accumulation in both HUVEC and HAEC, without altering the time profile of induction. This stimulating effect of forskolin on c-fos expression was at least 3-fold in HUVEC (results not shown) and about at least 10-fold in the experiment with HAEC, shown in Fig. 6. In contrast to c-fos, c-jun levels remained unaltered by the extra addition of forskolin (results not shown).

DISCUSSION

The experiments described above demonstrate for both human umbilical vein and aorta endothelial cells that activation of PKC, either directly via DAG or PMA, or indirectly via exogenous phospholipase C, leads to increased t-PA and PDGF (A and B) expression, without substantially affecting PAI-1 synthesis. The expression of t-PA mRNA differs in fine-tuning (with cAMP) from that of PDGF-A and PDGF-B, and is preceded by the induction of the proto-oncogenes c-jun and c-fos.

A rise in endogenous DAG is a common feature of many signal transduction systems as a result of receptor-mediated activation of phospholipase C upon exposure of cells to specific effectors. Although exogenous phospholipase C will not necessarily reach the same intracellular sites nor act on the same substrates as endogenous phospholipase C, our studies indicate that phospholipase C from C. perfringens might imitate the physiological role of intracellular phospholipase C as demonstrated by an approximately three-fold stimulation of t-PA production (Table 1). The specificity of the effect is

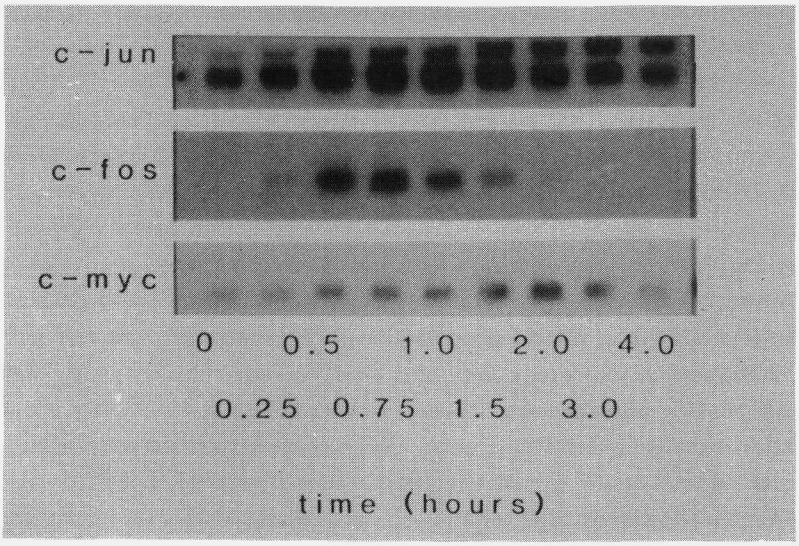

Figure 5. Proto-oncogene inducton by PMA in cultured endothelial cells.
Total RNA was isolated from confluent cultures of HUVEC incubated with 10 nM PMA for the indicated time periods. mRNA levels for c-jun, c-fos and c-myc were estimated by Northern blot analysis.

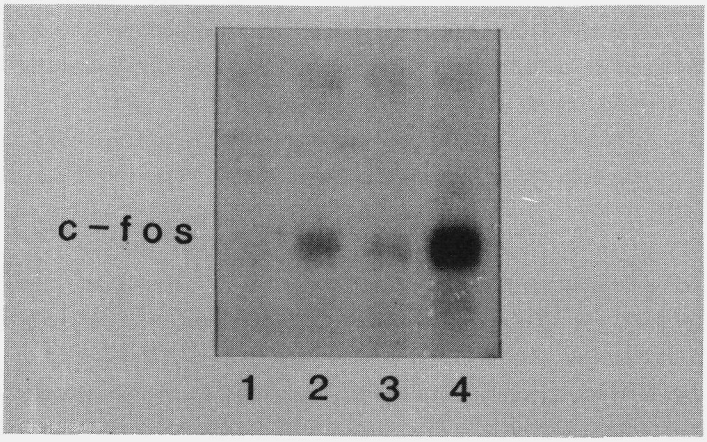

Figure 6. c-fos mRNA induction by PMA and/or forskolin in cultured endothelial cells.
Total RNA was isolated from confluent cultures of HAEC incubated for 1 hour in the absence (1) or presence of 10 nM PMA (2), 10 μM forskolin (3), or 10 nM PMA and 10 μM forskolin (4). mRNA levels for c-fos were estimated by Northern blot analysis.

emphasized by the lack of effect of exogenous bacterial phospholipase A2. Activation of phospholipase C also leads to mobilization of intracellular calcium, but the observed increase in t-PA synthesis is likely to be due solely to the generation of DAG, since only

the addition of synthetic DAG, and not the addition of the calcium ionophore A23187, caused an increase in t-PA synthesis (Table 1).

The effective dose range for t-PA induction by PMA in HUVEC and HAEC (this study) and other cell types such as HeLa cells (46,47) and melanoma cells (48), is in the nanomolar range. This is comparable to the apparent dissociation constant of 8 nM determined by Nishizuka (49) for the complex between PKC and a related phorbol ester. It seems likely therefore, that the induction of t-PA is a function of the amount of existing PMA-PKC complex in the cell. The phorbol ester PMA was found to be much more potent in stimulating t-PA synthesis than DAG (Table 1). Several explanations can be offered for discrepancies between PMA and DAG effects (50), including failure of DAG to enter cells efficiently, rapid metabolism of DAG, inability of DAG to reach all intracellular sites accessible to PMA, and qualitative differences in the effects of PMA and DAG on PKC.

In agreement with other studies (3-5) we found that the enhancement of t-PA synthesis by PMA in HUVEC and HAEC was accompanied by a transient rise in t-PA mRNA and could be potentiated by elevation of intracellular cAMP levels (Figs. 3 and 4). To learn whether such a potentiating phenomenon is a general characteristic in endothelial cells, we also studied the expression of PDGF mRNA in these cells. Previous work has demonstrated that in PMA-stimulated renal microvascular EC the PDGF B-chain and, to a much lesser extent, the PDGF A-chain mRNA levels are increased (15,16). In this paper we show that in HUVEC, PMA stimulates the expression of the two genes to the same extent, whereas in HAEC the induction of PDGF-A seems more pronounced (Figs. 3 and 4). The time profile of induction is very similar to that seen with t-PA. In contrast to t-PA expression, however, cAMP did not further enhance the PMA-induced increases in PDGF-A and PDGF-B mRNA levels. This suggests a complex interplay between the PKC and the protein kinase A pathways in regulating endothelial expression of t-PA, PDGF-A and PDGF-B.

The strong, transient induction of elevated t-PA gene expression by PMA in endothelial cells was shown to be dependent on protein synthesis. Together with the presence of AP-1 binding sequences in the 5' upstream region of the t-PA gene, this prompted us to study the expression of the c-jun and c-fos genes, the protein products of which are associated as a heterodimer to form the transcription factor AP-1. As shown in Figs. 5 and 6, PMA addition to HUVEC or HAEC resulted in a rapid and strong increase of both c-jun and c-fos mRNA, with c-fos mRNA levels even further increased by the addition of forskolin. This induction precedes that of t-PA, suggesting that accumulation of jun and fos may be necessary for the subsequent increase in t-PA gene expression. Also, PMA enhances c-jun and c-fos expression only for a very short period of time which would explain the transient character of the increase in t-PA mRNA levels. However, to prove a role of the AP-1 binding sequences in mediating an enhanced t-PA

gene expression will at least require transient expression analysis in endothelial cells employing deletion mutants of the t-PA gene promoter fused to a reporter gene like chloramphenicol acetyltransferase. Such studies should also consider other specific cAMP- and PMA-responsive elements in the t-PA gene: Medcalf et al. (51) recently described that the AP-1 site at position -113 and an exon-located AP-2 binding site at position +60 in the t-PA gene cooperate in basal expression and convey activation by PMA and cAMP in HeLa cells.

The presence of a given motif in the t-PA gene is merely a first step in unravelling the complex gene regulation, and may not be sufficient to constitute a transcriptionally active response element. This is strikingly illustrated by our studies on PAI-1 gene expression: PMA hardly affects PAI-1 gene expression in HUVEC or HAEC, as shown in Figs. 2-4. On the other hand, PMA strongly stimulates PAI-1 gene transcription in human rhabdomyosarcoma cells (52) and in the human hepatoma cell line Hep G2 (53). This suggests that besides the presence of regulatory sequences, the availability of specific transcription factors such as AP-1 or AP-2, and cell-specific components should be considered for responsiveness of a gene in a certain cell-type. Similarly PAI-1 expression can be regulated at the nuclear post-transcriptional level by the c-myc oncogene (54,55). A rise in c-myc mRNA in endothelial cells did not, however, have a corresponding effect on PAI-1 mRNA levels (Fig. 5).

Histological studies on biopsy and autopsy specimens of human arteries and veins (56) as well as studies on cultured human endothelial cells from different vascular origins strongly suggest that regional differences may exist in the t-PA production by the endothelium (12). In accordance with this we found that confluent cultures of HAEC produced about 5- to 10-times more t-PA than HUVEC under basal conditions. An important finding of this study is that the regulation of t-PA expression via PKC seems to be qualitatively very similar in both types of endothelial cells, despite the large difference in basal expression. It is, moreover, of importance for the design of pharmaca that can modulate endogenous fibrinolysis, that the effect of activated PKC on t-PA synthesis, but not PDGF synthesis, could be potentiated by forskolin. This may allow fine-tuning of the desired endothelial response.

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CHAPTER 8

GENERAL DISCUSSION

Introduction

The studies reported in this thesis were aimed at elucidating some aspects of the regulation of the human plasminogen activator inhibitor 1 (PAI-1) gene expression.

At the start of this project, several studies had suggested that PAI-1 synthesis is highly regulated. In most of these studies, only PAI inhibitory activity had been measured in assays based on titration of PAI with t-PA or u-PA. Interpretation of these assays was complicated by the fact that increased PAI activity can be caused by either increased PAI protein or decreased PA levels. Furthermore, in the medium of cultured cells PAI-1 exists partly in an inactive (latent) form; a change in the ratio of active over inactive PAI-1 can then wrongly be interpreted as a change in PAI-1 synthesis. Since then (from 1986) PAI-1 immuno-assays and PAI-1 cDNA became available. This allowed the study of PAI-1 expression at the protein and mRNA level, and largely confirmed and extended earlier work. A picture emerged of a highly regulated gene: for instance, PAI-1 synthesis and mRNA levels are increased by growth factors like TGF-β and EGF, by acute phase reactants like LPS, TNF and IL-1, by the protein kinase C activating phorbol ester PMA, and by hormones like insulin, testosterone and dexamethasone (for a review see Andreasen et al., 1990).

The work in this thesis is an onset to a better understanding of this complex regulation of PAI-1 gene expression. It shows that the synthesis of a functional transcript of PAI-1 is regulated at several levels. Important aspects that are dealt with are: the significance of consensus sequences in transcriptional regulation; transcription factors and cell specificity; and the origin of PAI-1 in plasma. Below, the present knowledge and uncertainties with respect to these aspects of PAI-1 gene expression will be discussed in a broader context.

Significance of consensus sequences in transcriptional regulation

The transcription of eukaryotic genes is regulated by cis-acting DNA elements, which exert their effects on transcription through interaction with trans-acting protein factors (transcription factors). These cis-acting elements consist of short, about 6 to 20 bp, specific sequences that are recognized by specific transcription factors. Generally speaking, the cis-acting elements function independent on location and orientation (see for a recent review Mitchell and Tjian, 1989). Alignment of these specific DNA sequences from different genes led to the definition of so-called consensus sequences: a consensus sequence is derived from nucleotides conserved with a frequency of 50% or

more (Evans, 1988). For a large number of specific binding sites a consensus sequence has been determined (for a compilation see Wingender, 1988). We searched the PAI-1 gene and flanking regions for the presence of DNA sequences with a homology of 80% or more to these consensus sequences. As is shown in Table 1, a computer-aided search revealed the presence of many putative regulatory sequence elements upstream of the human PAI-1 gene. Among these are putative binding sites for AP-1 and AP-2 (mediating the response to phorbol ester; Angel et al., 1987; Roesler et al., 1988), for NF-κB (mediating the response to cytokines; Lenardo et al., 1989), and for NF-1 (mediating the response to growth factors; Rossi et al.,1988). In addition we found a large region with sequence identity in the upstream regions of the human PAI-1 and t-PA

Table 1. Putative transcription regulatory elements upstream of the human PAI-1 gene

Element	Putative role in gene regulation	References
TATAA	initiation of transcription	22,24
CCAAT	enhancement of transcription	22,24
SP-1	enhancement of transcription	10,22,24
AP-1	serum and/or PMA response	3,9
AP-2	PMA and/or cAMP response	33
ERE	oestrogen response	4,17
GRE/PRE *	glucocorticoid response and progesterone response	4,37
NF-1	growth factor response	34
NF-κB	acute phase response	21
Z-DNA	acute phase response	8
Acute phase signal	acute phase response	1,12
PAI-1/t-PA element	unknown	5

* Only the most conserved hexanucleotide of the consensus sequence is present.
Numbering position according to sequence human PAI-1 gene (Bosma et al., 1988).

gene (Chapter 2 of this thesis). Although the presence of these putative regulatory elements in the PAI-1 gene would nicely explain the observed regulation of PAI-1 gene expression by, for instance, PMA, IL-1, TNF, and TGF-β, caution should be taken in drawing such conclusions.

Firstly, not all sequence elements shown in Table 1 perfectly match the consensus sequence. Although substitution of a nucleotide in a consensus sequence may have a functional meaning, for instance in rendering cell specificity, it may also prevent binding of the transcription factor to this site. Secondly, evidence is now accumulating that neighbouring sequences can be of importance for the function of a binding site. Thirdly, the presence of transcription factors that can interact with these consensus sequences is required for their role in gene regulation (see below). Finally, other, still unidentified sequence elements may also play a role. To illustrate this point, expression studies have established the DNA region of PAI-1 mediating the response of human HT-1080 cells and rat FTO2B cells to the glucocorticoid, dexamethasone (Riccio et al., 1988; Van Zonneveld et al., 1988). The found region did not contain a consensus glucocorticoid responsive element (GRE). On the contrary, the putative partial GRE did not seem to be involved in the dexamethasone response (Van Zonneveld et al., 1988). Also, the regions of the PAI-1 gene mediating the response to the growth factor TGF-β did not contain a consensus NF-1 binding site, although a sequence element similar to an NF-1 site in the α_2(I) collagen gene was identified (Westerhausen et al., 1991).

In conclusion, identification of sequences by comparative analysis alone is not sufficient to establish a role in gene regulation. Expression and footprinting studies are required for functional identification of regulatory sequences upstream of the PAI-1 gene. The nucleotide sequence of the PAI-1 gene provides information needed to design these experiments. Furthermore, since our knowledge on the relation between transcriptional factors and their binding sites is growing rapidly, sequence information in itself may become more informative.

Transcription factors and cell specificity

As discussed above, the mere presence of a consensus sequence is not sufficient for its role in gene regulation. In addition, the presence of transcription factors that can interact with such sequences is required. This thesis shows several examples in which the presence of transcription factors could play a decisive role in the regulation of PAI-1 gene expression.

1) PAI-1 gene contains a binding site for AP-1 (Table 1). Induction of AP-1 by serum or PMA leads to an increase of PAI-1 gene transcription in Hep G2 cells.
2) The PAI-1 gene contains binding sites for AP-2 (Table 1). AP-2 can act as a basal enhancer and is absent in Hep G2 cells (Imagawa et al., 1987). This might explain

why the expression of the -114 to +75 PAI-1/ CAT construct is much lower in Hep G2 cells than in HT-1080 and endothelial cells (appendix Chapter 3).

Even if consensus sequences and transcription factors are present, other (possibly cell- or tissue-specific) factors may be required for optimal gene transcription. E.g., similarly to Hep G2, PMA induces AP-1 in endothelial cells. This induction of AP-1 is possibly involved in increased t-PA expression in endothelial cells. The question arises as to why an increase in AP-1 leads to a strongly increased PAI-1 expression in Hep G2, but not in endothelial cells. Whether this means that AP-1 is not involved in regulation of PAI-1 expression in endothelial and Hep G2 cells or if another (cell- or tissue-specific) factor is required, can not be deduced from our data.

In conclusion, besides the presence of regulatory sequences and the availability of specific transcription factors, other, cell-specific components should be considered for responsiveness of a gene in a certain cell type.

Post-transcriptional regulation

Our studies show that regulation of PAI-1 gene expression can occur at a post-transcriptional level: in the nucleus and in the cytoplasm (Chapter 6). Prendergast et al. have also provided evidence that in immortalized primary rodent fibroblasts and in 3T3 cells, PAI-1 expression can be regulated at a nuclear post-transcriptional level (Prendergast et al., 1989,1990). Besides the transcriptional regulatory elements described above, the human PAI-1 gene contains several sequences that could be involved in this post-transcriptional regulation (Table 2): several polyadenylation sites and a sequence possibly involved in mRNA stability were identified.

Correct 3'-end processing at a polyadenylation site requires a highly conserved hexanucleotide, AAUAAA (Proudfoot and Brownlee, 1976), with just downstream a less conserved U-rich or GU-rich sequence (Gil and Proudfoot, 1987). At the 3'-end of the human PAI-1 gene several consensus polyadenylation signals are present, that render a 3.2 kb mRNA species (Chapter 2). About 900 bp upstream of the 3'-end another polyadenylation site is present (Ny et al., 1986). This site, AAUAAU followed by a GU rich region, only exists in humans and higher primates (Cicila et al., 1988). Alternative polyadenylation at this site would explain the occurrence of a second PAI-1 mRNA species of about 2.4 kb in human cells but not in non-primate cells such as bovine endothelial cells and rat hepatoma cells. Inasmuch a change in the use of these two alternative polyadenylation sites contributes to the observed shift in the ratio between the two mRNA species in the nuclei of Hep G2 cells treated with PMA, cannot be deduced from our work. Such a shift in the use of alternative polyadenylation sites is not without precedent: it has also been observed for the mouse dihydrofolate reductase gene during cell growth (Kaufman and Sharp, 1983). Other post-transcriptional nuclear

Table 2. Sequences in 3'-untranslated region of human PAI-1 gene involved in post-transcriptional regulation

Element		Position	Putative role in post-transcriptional regulation	References
poly-adenylation signal	AAUAAA	12095 to 12100 12125 to 12130 12130 to 12135 12134 to 12139	poly-adenylation site	29,41
	AAUAAU	11172 to 11177		41
AU rich region		11791 to 11877	mRNA stability	6,35
conserved region:				
human gene		11486 to 11636	unknown	5
human cDNA		2307 to 2448		25
rat cDNA		2387 to 2532		43
murine cDNA		2448 to 2593		28

Numbering position according to sequence human PAI-1 gene (Bosma et al., 1988).

processes may also affect the ratio between the two PAI-1 mRNAs. In this respect the induction of c-myc mRNA by PMA in Hep G2 is interesting as c-myc protein may affect PAI-1 gene expression at the level of RNA processing, nuclear RNA turnover, or RNA export (Prendergast et al., 1989,1990).

In the 3'untranslated region of the larger PAI-1 mRNA in human an AU rich sequence is present. Similar sequences in the 3'untranslated ends of lymphokine and proto-oncogene mRNAs render a cycloheximide-reversible instability to these messengers (Shaw and Kamen, 1986; Caput et al., 1986). Cycloheximide (CHX) also has a stabilizing effect on the large PAI-1 mRNA in human endothelial-, rhabdomyosarcoma-, and T-CAR1 cells (Van den Berg et al., 1988, Mayer et al., 1988, Georg et al., 1989). In Hep G2 cells no effect of CHX on PAI-1 mRNA levels is seen (Chapter 6). However, CHX prevents the induction of a factor by PMA which destabilizes the 3.2 kb PAI-1 mRNA in these cells. Whether such a destabilizing factor is already present in unstimulated endothelial, rhabdomyosarcoma, and T-CAR1 cells is not clear, but it might explain the effect of CHX on PAI-1 mRNA in these cells.

In-vitro translation studies showed that the 3'-untranslated region of the human PAI-1 gene does not affect the translational capacity of the messenger.

The 3'untranslated region of the PAI-1 gene of rat and mouse does not contain an AU rich sequence, although U rich stretches are found (Zeheb and Gelehter, 1988; Prendergast, 1990). Whether the absence of such a sequence results in a more stable PAI-1 mRNA in these species is presently unknown. It is noteworthy that in the 3'untranslated region, which forms a highly variable part of the gene, a region of about 100 bp long is strictly conserved between the PAI-1 genes of human, rat and mouse (Table 2). The significance of this conserved region, if any, is not known.

In conclusion, the human PAI-1 gene contains sequences that can play a role in its post-transcriptional regulation at several levels. This post-transcriptional regulation can differ per cell type, indicating that cell-specific protein factors are involved. Confirmation of the functionality of the alternative polyadenylation signal in human PAI-1 gene requires further studies, like nuclease mapping or primer extension experiments.

Origin of PAI-1

The origin of PAI-1 in blood plasma has not yet been established. Of course, the endothelial cell is a good candidate: the acute phase behaviour of PAI-1 in vivo can be mimicked in cultured endothelial cells. There is more controversy about the role of other cell types. In man, a good correlation exists between PAI-1 and insulin levels in plasma. Insulin does not affect PAI-1 synthesis in endothelial cells under standard incubation conditions, but does increase PAI-1 expression in the human hepatoma cell line Hep G2 and primary cultures of human hepatocytes, suggesting that these cells may contribute to plasma PAI-1 levels. On the other hand, high insulin levels in mice do not affect PAI-1 plasma levels (Emeis, unpublished observation) and rat hepatocytes in vivo do not synthesize PAI-1 (Quax et al., 1990; Konkle et al., 1990; Podor et al., 1990). Similarly, insulin does not induce PAI-1 synthesis in cultured rat hepatocytes (Kooistra, unpublished observation). Thus, a contribution of hepatocytes to PAI-1 levels in plasma may depend on species.

As discussed in Chapter 1, many other cell types produce PAI-1. Among these, smooth muscle cells are of particular interest. Smooth muscle cells synthesize PAI-1 both in-vitro and in-vivo (Knudsen et al.1987; Laug et al., 1989; Padró et al. unpublished observations). As smooth muscle cells account for a significant amount of total body weight their contribution may be significant. Furthermore, smooth muscle cells respond to several mediators known to increase plasma PAI-1 levels, such as LPS (see Quax, 1991). However, so far now there is no direct evidence for their contribution to PAI-1 plasma levels. In normal human blood, in addition to the PAI-1 plasma pool a PAI-1 pool is present in platelets, but the contribution of PAI-1 in platelets to basal plasma PAI-1 levels can only be marginal (Sprengers et al., 1986).

In conclusion, endothelial cells, hepatocytes and smooth muscle cells may contribute to plasma PAI-1 levels. The contribution of these cell types to plasma PAI-1 may vary

among species: for example in man, but probably not in rat or mouse, liver hepatocytes may synthesize PAI-1.

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SUMMARY

In Chapter 1, plasminogen activator inhibitor 1 (PAI-1) is introduced. PAI-1 is a specific inhibitor of plasminogen activator activity, and thus could play a key role in biologically important systems, such as fibrinolysis, inflammation, cancer, and tissue remodelling. High PAI-1 levels are associated with an increased risk of thromboembolic disease. As might be expected for such an important protein, the expression of PAI-1 is highly regulated by a variety of factors, including acute phase mediators, hormones, growth factors and activators of protein kinase C.

As a first step in studying the regulation of the PAI-1 gene at the level of transcription, the human PAI-1 gene and flanking DNA regions have been isolated and sequenced (Chapter 2). The human PAI-1 gene is 12.1 kilo base pairs (kb) long. It consists of 9 exons, coding for two PAI-1 mRNA species, intervened by 8 introns. The PAI-1 gene contains 12 copies of a common repeat element (Alu), 5 of which are associated with a new repetitive element (Pur). The significance of these repeat elements, if any, for PAI-1 expression is unclear. Computer aided analysis also showed the presence of several putative regulatory elements upstream of the PAI-1 gene, which could function as specific binding sites for specific proteins regulating gene transcription rate (transcription factors). A major finding was the presence of a large region of 520 bp in the 5'-flanking DNA of the human PAI-1 gene showing about 80% homology with a region in the 5'-flanking DNA of human tissue-type plasminogen activator (t-PA).

In Chapter 3, this PAI-1/t-PA homologous region was further characterized. We identified 3 more copies of this sequence in genbank, and showed by southern blotting that this sequence is repeated manifold in the total human genome, and therefore is a new repetitive element. Expression studies in human endothelial-, hepatoma- and fibrosarcoma- cell lines indicated that this region does not play a role in the basal expression of the PAI-1 gene in these cells.

In man, plasma PAI-1 levels are highly correlated to plasma insulin levels. Chapter 4 describes that insulin enhances PAI-1 synthesis in the human hepatoma cell line, Hep G2, and in primary cultures of human hepatocytes. In both cases this increase in PAI-1 protein synthesis is accompanied by elevated PAI-1 mRNA levels. These results suggest that Hep G2 is a suitable model to study regulation of PAI-1 synthesis in human hepatocytes.

In Hep G2 cells PAI-1 expression can be stimulated by various serum growth factors, many of which could act via activation of protein kinase C (PKC). To study this further, we have compared the effect of serum and a specific, stable activator of PKC, phorbol-12-myristate-13-acetate (PMA) on PAI-1 gene expression (Chapter 5). Both serum and PMA strongly and transiently increased PAI-1 mRNA levels, with the response to PMA

being larger but shorter in time than that to serum. With both inducers, the increase in mRNA levels was preceded by a parallel increase in gene transcription rate. The induced increase in PAI-1 gene transcription was dependent on ongoing protein synthesis, suggesting that it is mediated through a newly-synthesized transcription factor. It is hypothesized that this transcription factor could be Activator Protein 1 (AP-1), for which a putative binding site is present upstream of the PAI-1 gene. AP-1 is a homo- or heterodimer of two oncogene products, Jun and Fos, the mRNAs of which were shown to be transiently induced by serum and PMA prior to the activation of PAI-1 gene transcription. Similar to PAI-1, the induction of these two genes by PMA was larger and shorter in time than that by serum.

In man, two PAI-1 mRNA species, of 3.2 kb and 2.4 kb, are found. These two forms differ only in the length of their 3'-untranslated end, possibly as a result of alternative polyadenylation (see Chapter 2). During the studies with PMA as described in Chapter 5 it was found that the two PAI-1 mRNAs could vary in relative concentration. This observation was elaborated in Chapter 6. Upon PMA addition to Hep G2 cells, the two mRNA species were differently induced, with a different time profile and to a different extent. At least two post-transcriptional processes may contribute to the different induction profile of the two PAI-1 mRNA species. Firstly, it was shown that PMA induced a change in post-transcriptional nuclear processing of the PAI-1 gene transcript, resulting in a shift of the relative amounts of the two mRNAs produced. Secondly, PMA induced a rapid turnover of the large mRNA: after PMA addition we found a half-life of maximal 0.85 h for the 3.2 kb mRNA species and of 2.5 h for the 2.4 kb form. The rapid decay of the larger mRNA species required ongoing translation and transcription, indicating that a labile protein factor coded for by a labile messenger may mediate this rapid decay. In vitro-translation experiments in a rabbit reticulocyte lysate showed that the two PAI-1 mRNAs have the same translational capacity.

In Chapter 7 we have examined the effect of PKC activation on the expression of PAI-1 and t-PA in endothelial cells from human umbilical vein and human aorta. In contrast to Hep G2 cells, PAI-1 expression is hardly enhanced by PMA in endothelial cells, although these cells were responsive to PMA, as shown by a strongly increased expression of t-PA and platelet-derived growth factor -A and -B. The induction of t-PA expression could be further stimulated by simultaneous addition of the adenylate cyclase activator, forskolin. This induction of t-PA was shown to require protein synthesis, and was preceded by a transient increase in c-jun and c-fos mRNAs. Also, the induction of c-fos mRNA was further potentiated by forskolin. These observations and the presence of an AP-1 binding site in the t-PA promoter region would be in line with a role of the transcription factor AP-1 in the induction of the t-PA gene by PMA. The cell-specific increase in PAI-1 gene expression by PMA in Hep G2 but not in endothelial cells is discussed.

In the final chapter (Chapter 8), some important aspects dealt with in this thesis are discussed in a broader context, including the significance of consensus sequences in the PAI-1 gene as regulatory elements, and post-transcriptional processes such as mRNA processing, transport, and stability. In addition we comment upon cell specific regulation and the origin of PAI-1 found in plasma.

SAMENVATTING

Hoofdstuk 1 introduceert het eiwit dat centraal staat in dit proefschrift: de plasminogeen activator remmer 1 (PAI-1). Als een specifieke remmer van zowel weefsel-type plasminogeen activator (t-PA) als urokinase-type plasminogeen activator (u-PA), kan PAI-1 een belangrijke rol spelen bij de regulatie van een aantal (patho)fysiologische processen waarbij het plasminogeen activatie systeem betrokken is zoals fibrinolyse, ontstekings-reacties, ovulatie, metastase en wondherstel. Zo zijn verhoogde PAI-1 niveaus in plasma onder andere geassocieerd met een verhoogd risico op hart- en vaatziekten. Het spreekt dan ook vanzelf, dat de expressie van zo'n belangrijke remmer gereguleerd wordt door een groot aantal fysiologische factoren, zoals ontstekingsmediatoren, hormonen, groeifactoren en activatoren van intracellulaire regulatie-enzymen zoals protein kinase C.

Om de regulatie van PAI-1 synthese op gen niveau te kunnen bestuderen, is het humane PAI-1 gen met zijn flankerende DNA gebieden geïsoleerd en is de base volgorde in dit DNA bepaald (Hoofdstuk 2). Het humane PAI-1 gen is ongeveer 12,1 kilo basen (kb) lang, en bestaat uit 9 exonen (onderbroken door 8 intronen) die coderen voor twee PAI-1 mRNA-vormen met een lengte van respectievelijk 2,4 kb en 3,2 kb. In het PAI-1 gen zijn 12 copieën van een repetitieve sequentie, het zogenaamde Alu-element, aanwezig. Vijf van deze Alu-elementen blijken gekoppeld aan een nieuwe repetitieve sequentie, door ons Pur-element genoemd. Het is niet bekend of deze repetitieve sequenties betekenis hebben voor de regulatie van het PAI-1 gen. Verder is het promoter gebied onderzocht op de aanwezigheid van potentiële regulatie-elementen. Deze regulatie-elementen zijn korte, specifieke sequenties die als bindingsplaats fungeren voor specifieke eiwitten, transcriptie-factoren genaamd. Op basis van de regulatie-sequenties van verschillende genen zijn de zogenaamde consensus-sequenties gedefinieerd: een consensus-sequentie is afgeleid van de nucleotiden die met een frequentie van tenminste 50% geconserveerd zijn. Uit een computer-onderzoek blijkt dat het PAI-1 promoter gebied naast algemene consensus-sequenties zoals een TATAA box, ook sequenties bevat die een rol kunnen spelen bij de regulatie van PAI-1 door ontstekings-mediatoren, hormonen, groeifactoren en activatoren van intracellulaire regulatie-enzymen. Opvallend is dat in het PAI-1 promoter gebied een stuk van 520 bp lang aanwezig is, waarvan de sequentie voor meer dan 80% homoloog is aan een sequentie in de t-PA promoter.

In Hoofdstuk 3 is dit PAI-1/t-PA element verder gekarakteriseerd. Een onderzoek naar het voorkomen van deze sequentie in alle thans gepubliceerde gen-sequenties laat zien dat deze sequentie in tenminste 3 andere genen aanwezig is. Experimentele analyse door middel van zogenaamde "southern blot" hybridisatie geeft aan dat het PAI-1/t-PA element mogelijk zo'n 100 keer voorkomt in het complete humane genoom, en dus een

nieuw repetitief element is. Expressie-studies in humane endotheel-, hepatoma- en fibrosarcoma-cellen wekken de indruk dat dit PAI-1/t-PA element geen rol speelt in de basale expressie van het PAI-1 gen in deze cellen.
Bij de mens bestaat er een sterke correlatie tussen plasma niveaus van PAI-1 en insuline. Hoofdstuk 4 beschrijft dat insuline de PAI-1 synthese verhoogt in de humane hepatoma cellijn Hep G2 en in primaire cultures van humane hepatocyten. In zowel Hep G2 als in humane hepatocyten gaat deze toename van PAI-1 synthese gepaard met verhoogde PAI-1 mRNA niveaus. Deze resultaten suggereren dat Hep G2 cellen een geschikt model systeem vormen om de regulatie van PAI-1 synthese in humane hepatocyten te bestuderen.
In Hep G2 cellen kan de PAI-1 expressie gestimuleerd worden door verschillende groeifactoren aanwezig in serum, mogelijk via activatie van protein kinase C. Om de rol van protein kinase C in PAI-1 inductie door serum verder te bestuderen, hebben we in Hoofdstuk 5 het effect van serum vergeleken met het effect van een stabiele activator van protein kinase C, phorbol-12-myristaat-13-acetaat (PMA). Zowel serum als PMA induceren een sterke, kortstondige verhoging van de PAI-1 mRNA niveaus, waarbij het effect van PMA wel sterker is, maar korter duurt dan het effect van serum. Bij beide stimulatoren wordt de verhoging van de mRNA spiegels voorafgegaan door een verhoging van de gen-transcriptie snelheid. Deze verhoging in PAI-1 transcriptie is afhankelijk van eiwit-synthese, hetgeen suggereert dat hiervoor een transcriptie-factor moet worden gesynthetiseerd. Een mogelijke kandidaat is het zogenaamde Activator Proteïne 1 (AP-1), waarvoor een potentiële bindingsplaats aanwezig is in het PAI-1 promoter gebied. AP-1 is een homo- of heterodimeer van twee oncogen producten, Jun en Fos, waarvan de mRNA niveaus voorafgaand aan de inductie van PAI-1 gen transcriptie door serum en PMA kortstondig verhoogd zijn. In overeenstemming met de inductie van PAI-1 is de inductie van deze twee genen door PMA ook sterker, maar korter van duur dan de inductie door serum.
Bij de mens zijn twee PAI-1 mRNA-vormen aanwezig, met een lengte van respectievelijk 2,4 kb en 3,2 kb. Deze 2 mRNA's verschillen alleen in de lengte van het niet-coderende deel aan het 3'-uiteinde, en zijn waarschijnlijk het gevolg van alternatieve polyadenylering (Hoofdstuk 2). Gedurende de experimenten met PMA bleek dat de onderlinge verhouding in de concentratie van deze twee mRNA's kan variëren. Deze observatie is opgevolgd in Hoofdstuk 6. Na toevoeging van PMA aan Hep G2 cellen blijken de twee mRNA-vormen verschillend te worden geïnduceerd, zowel wat betreft het tijdsprofiel ans het inductie-niveau. Tenminste twee post-transcriptionele processen kunnen hierbij een rol spelen. Ten eerste induceert PMA een verandering in de post-transcriptionele nucleaire processing, waardoor de verhouding waarin beide mRNA's geproduceerd worden, verschuift. Ten tweede induceert PMA een versnelde afbraak van de lange mRNA: na toevoeging van PMA wordt een halfwaardetijd gevonden van maximaal 0,85

uur voor de 3,2 kb mRNA-vorm en van 2,5 uur voor de 2,4 kb mRNA-vorm. Deze snelle afbraak van de lange mRNA is afhankelijk van zowel translatie als transcriptie, wat suggereert dat hierbij een labiel eiwit betrokken is dat wordt gecodeerd door een labiele mRNA. Uit in vitro translatie-experimenten in een konijne-reticulocyten-lysaat blijkt dat het extra deel van de 3,2 kb-vorm geen invloed heeft op de translatie-capaciteit.
In Hoofdstuk 7 is het effect van PMA op PAI-1 en t-PA expressie in menselijke endotheelcellen uit navelstreng en aorta beschreven. In tegenstelling tot wat er gebeurt in Hep G2 cellen, heeft PMA nauwelijks effect op PAI-1 synthese in endotheelcellen, hoewel deze cellen wel gevoelig zijn voor PMA, zoals o.a. blijkt uit de verhoogde synthese van t-PA en van platelet-derived growth factor -A en -B. De inductie van t-PA expressie door PMA kan verder verhoogd worden door toevoeging van forskoline, een activator van adenylaat cyclase. De inductie van t-PA is afhankelijk van eiwit synthese en wordt voorafgegaan door een kortstondige verhoging van Jun en Fos mRNA's. Bovendien wordt de inductie van Fos verder verhoogd door forskoline. Deze waarnemingen en de aanwezigheid van een AP-1 bindingsplaats in de t-PA promoter geven aan dat AP-1 een rol zou kunnen spelen in de inductie van t-PA door PMA. De cel-specifieke verhoging van PAI-1 expressie door PMA in Hep G2 maar niet in endotheelcellen wordt bediscussieerd.
In het laatste hoofdstuk (Hoofdstuk 8) wordt een aantal aspecten van PAI-1 expressie, zoals die in dit proefschrift aan de orde komen, in een bredere context besproken. Hierbij wordt achtereenvolgens ingegaan op de betekenis van de aanwezige consensus sequenties in het PAI-1 gen voor de regulatie van PAI-1 expressie, de rol van post-transcriptionele regulatie en cel-specifieke factoren bij PAI-1 expressie, en de herkomst van PAI-1 in plasma.

ABBREVIATIONS

AP	activator protein
bp	base pair(s)
cAMP	cyclic adenosine 5'-monophosphate
cDNA	copy DNA
CHX	cycloheximide
DMEM	Dulbecco's modification of Eagle's medium
EDTA	ethylene diamine tetraacetate
EGF	epidermal growth factor
ERE	estrogen responsive element
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GRE	glucocorticoid responsive element
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL	interleukin
kb	kilo base (pairs)
kD	kilo Dalton
LPS	lipopolysaccharide
min	minute(s)
mRNA	messenger RNA
NF	nuclear factor
nt	nucleotide(s)
PA(s)	plasminogen activator(s)
PAI(s)	plasminogen activator inhibitor(s)
PDGF	platelet derived growth factor
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PRE	progesteron responsive element
s	second(s)
SSC	standard saline citrate
TGF	transforming growth factor
t-PA	tissue-type plasminogen activator
Tris	tris-(hydroxymethyl)aminomethane
TNF	tumor necrosis factor
u-PA	urokinase-type plasminogen activator

NAWOORD

Veel mensen hebben op hun eigen, soms onnavolgbare wijze bijgedragen aan de totstandkoming van dit proefschrift. Deze laatste bladzijde wil ik graag gebruiken om een aantal met name te noemen:
Dr. Teake Kooistra, die het mij mogelijk maakte dit onderzoek uit te voeren, mij op een deskundige wijze heeft begeleid en zich als co-promotor aan dit proefschrift heeft verbonden;
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CURRICULUM VITAE

26 november 1958	geboren te Engwierum
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Van november 1984 tot augustus 1990 was ik werkzaam op het Gaubius Instituut TNO te Leiden. De eerste anderhalf jaar heb ik in het kader van de vervangende dienstplicht onder leiding van dr. D.C. Rijken onder andere de structuurfunctie van t-PA onderzocht. Vanaf augustus 1986 heb ik als wetenschappelijk assistent onder leiding van dr. T. Kooistra onderzoek verricht naar de regulatie van PAI-1 expressie. De resultaten van dit werk zijn in dit proefschrift beschreven. Per 1 april 1991 ben ik als toegevoegd onderzoeker aangesteld bij de vakgroep Inwendige Geneeskunde van de Faculteit der Geneeskunde van de Universiteit van Amsterdam.