

Theme Issue Article

Transcriptional and posttranscriptional regulation of the plasminogen activator system

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Summary

The core protein components of the plasminogen activator (PA) system are two plasminogen activators, two plasminogen activator inhibitors and a urokinase type plasminogen activator-specific cell surface receptor. Various types of biological regulation are exerted through the interplay of these components mutually and with extracellular matrix proteins and cell membrane proteins, with or without involving proteolytic activity. Reflecting these diverse biological roles, the level and activity of each component of the PA system is under the control of a variety of

regulatory mechanisms. The expression level of a protein reflects the level of the corresponding mRNA, which is essentially the net result of de novo synthesis, i.e. transcription, and degradation. Many recent studies have shown that the regulation of mRNA stability is dynamic and cell specific. Accordingly, we are learning that the mRNAs of the PA system are also the subject of diverse regulatory mechanisms. In this short review, we summarize current understanding of the transcriptional and mRNA-stability regulation of the PA system.

Keywords

Review, plasminogen activator, gene regulation, mRNA stability

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Introduction

Numerous secreted proteases exist that control various biological processes through activation or inactivation of target molecules. They usually exhibit limited substrate specificity with defined catalytic mechanisms. Many of these proteases constitute a functional entity and are aligned in a cascade of activation/inactivation reactions. Such an entity must be finely regulated for the organism to maintain the homeostasis of a particular biological state. The plasminogen activator (PA) system is one such system comprising two plasminogen activators (urokinase type, uPA, and tissue type, tPA), two PA-specific inhibitors (type 1, PAI-1, and type 2, PAI-2) and one uPA-specific cell surface receptor uPAR. Considering the PA system in a wider sense, additional components should also be included, i.e. plasminogen, the main substrate of uPA and tPA, α 2-antiplasmin, protease nexin (sometimes called PAI-3), and others. In this short review, however, we will only discuss gene expression regulation of the five basic components. The biological aspects of the PA system have been elaborated in reviews by us and others (1–6). Although the biochemical features of uPA and tPA are similar, i.e. the conversion (nicking) of ubiquitous zymogen plasminogen to active ser-

ine protease plasmin, their preferential sites of action are different. uPA works at the cell surface and tPA on fibrin clots. This suggests different biological roles and accordingly differing regulation of gene expression for the two molecules. Similarly, PAI-1 and PAI-2 are expected to be regulated differently because PAI-1 is a secreted protein, while PAI-2 is mainly expressed in the cytoplasm. The PA system exerts its biological roles not only through the activation of plasminogen into plasmin (classical role), but it also affects cellular activities such as cell migration and spreading, in a PA catalytic activity-independent manner through interplay between uPA/uPAR/PAI-1 and other extracellular matrix proteins or cell membrane-associated proteins. Reflecting the versatility of the system, expression of each component is controlled by different regulatory mechanisms, which may also vary with cell type.

The extent to which any given protein is expressed is determined mainly by the level of its corresponding mRNA. It has been assumed that transcriptional regulation plays a major role in regulating the level of mRNA. Indeed, a large number of transcription studies in the past have revealed various forms of transcriptional regulation of many genes, including those of the PA system. However, prompted by the finding that different mRNAs

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have different half-lives and that these vary under different cell culture conditions, the importance of mRNA stability regulation in the overall control of mRNA levels has been increasingly recognized and has attracted much research (7–12). In the following sections, we discuss both transcriptional and posttranscriptional (mRNA stability) regulation of genes in the PA system.

Regulation of gene transcription

The uPA gene

The uPA gene is expressed at low levels in a variety of cells. Its expression is also inducible by many different signals, such as growth factors, cytokines, peptide hormones, steroid hormones, genotoxic agents and cell morphology changes (1). The uPA gene minimal promoter contains a TATA-box, a GC-rich region of about 200 bases, one copy of the CAAT sequence immediately upstream and several hundred bases upstream of the transcription initiation site. The GGGCGG and CAAT sequences are recognized by the ubiquitous transcription factors SP1 and CTF, respectively, responsible for the basal low levels of uPA expression. Two mitogen-activated protein (MAP) kinase transduction pathways (Erk and JNK) have been shown to phosphorylate Sp1, activating the minimal promoter element and endogenous gene transcription (13). Transcription of the human uPA gene is strongly modulated by an inducible enhancer located at –2.0 kb (14). Enhancer activity requires the cooperation of an upstream composite Ets/AP1a (previously termed PEA3/AP1) and a downstream AP1b site. The two AP1 sites are separated by a 74-bp “cooperation mediator” (COM) region that contains the binding sites for several proteins known as urokinase enhancer factors (UEF). There is another composite Ets/AP1 site in the opposite orientation far upstream at –5.36 kb, –465 kb and –6.89 kb of the human, pig and mouse uPA genes. The two Ets/AP1 sites showed a strong synergism for induction by PMA and FGF-2 in transient transfection experiments in NIH3T3 cells (15) and PC3 human prostate cancer cells (Y.N. unpublished data). These elements are highly conserved in three mammalian uPA genes (human, mouse and porcine), suggesting their importance in uPA gene regulation (15).

Ets sites contain the minimum consensus GGAA sequence and are potentially recognizable by many Ets family members. However, only Ets-1 and Ets-2 have been shown to activate the uPA gene by transfection assays. In accordance, a high correlation between Ets-1 and uPA expression has been reported in several cell types (16–18), and expression of antisense Ets-1 oligonucleotides was shown to suppress uPA expression in endothelial cells (19, 20). The AP1 site was originally identified as a phorbol ester-responsive element. It is recognized by a transcription factor complex AP1 (21–23), which is either a homodimer of Jun or a heterodimer of Jun and Fos family members (23, 24). Additionally, Jun family members heterodimerize with ATF2 and ATF3, which belong to the (CRE-binding protein) CREB/activating transcription factor (ATF) family (25, 26). Transcription factors binding to Ets/AP1 sites are activated by members of the MAP kinase family. As these kinases are activated by various extracellular stimuli, such as growth factors, cytokines, osmotic stress, and DNA-damaging agents (27–30), the uPA promoter is potentially sensitive to a variety of signals. Ac-

cordingly, the Ets/AP1 sites in the uPA promoter are the final target elements of the signaling cascades induced by PMA (31, 32) (15), okadaic acid (33), cytoskeletal reorganization (CSR) (32, 34), growth factors such as FGF-2, HGF/SF and IGF-1 (35–38), oncogenes (35, 39, 40), genotoxic agents such as UV and MNNG (41, 42), and cytokines such as TNF α and macrophage colony-stimulating factor (CSF-1) (43–45). FGF-2 and HGF/SC utilize the Grb2/Sos1/Ha-Ras/c-Raf/MEK1/Erk2-dependent pathway to activate the uPA promoter (35–37). Similarly, CSR was shown to induce uPA gene transcription via the FAK/Src/Ras/Erk pathway. Moreover, using the small interfering RNA technique, Faisal et al. (46) showed that SchA adapter proteins play an active role in CSR-induced Erk activation and further demonstrated that CSR-induced ShcA tyrosine phosphorylation, its association with Grb2, Erk activation and uPA gene expression are all dependent on Rho, p38 MAP kinase and Src. Altogether, these reports indicate a prominent role for the Erk pathway in the induction of uPA transcription through the Ets/AP1 sites by multiple extracellular stimuli. In contrast, TNF α and genotoxic stresses such as UV and MNNG upregulate uPA gene transcription through the same enhancer element but via the JNK pathway (41, 42). Interestingly, the AP1 elements were also shown to mediate repression of the uPA gene by E2F1 (47). It should be noted that sequences of all three AP1 sites deviate from the consensus AP1 recognition sequence TGACTCA. This sequence divergence and the nature of the cooperation between Ets and AP1 sites and between two Ets/AP1 sites, suggests that regulation of the uPA promoter through the Ets/AP1 sites is broad, diverse and different from conventional AP1 site-dependent regulation.

The uPA gene has a functional NF κ B-like sequence, GGGAAAGATC, at –1583 bp in the human promoter. Two additional Rel-like binding sites are located in tandem repeat at –1865 and –1835 (48). The –1583 NF κ B sequence is conserved among the human, pig and mouse genes. The NF κ B sequence of the uPA gene promoter was shown to mediate PMA induction in HeLa and HepG2 cells via formation of two NF κ B heterodimeric complexes: one consisting of Rel/p65 and the other of p65/p50 (48). It is not clear how important this sequence for the mediation of PMA action, because its activity is only observable in the absence of the upstream sequence (48), and deletion of the Ets/AP1 site is enough to suppress PMA induction completely. In contrast, overexpression of uPA in pancreatic adenocarcinomas was shown to be regulated by constitutive activation of p65 (49). In addition, the NF κ B binding site is required for the integrin α v β 3/vitronectin-induced downregulation of the uPA promoter (50). Finally, a negative regulatory element, which modulates the enhancer activity, was identified at –600 bp (51). A cAMP-responsive enhancer was identified specifically in the porcine uPA promoter at –3.4 kb, which mediated induction of the uPA gene in LLC-PK₁ pig epithelial cells by the peptide hormone calcitonin or cAMP analogs (52, 53). This cAMP enhancer sequence, however, is not present in the corresponding regions of the uPA promoters in other mammalian species, suggesting that this particular type of hormonal regulation evolved much later. Other elements exist on the uPA promoter that mediate the expression of the gene in response to other stimuli or in certain cell types. For example, laminar shear stress induces uPA transcrip-

tion via a GATA-4 element located at -692 bp (54), while a CARG box was found at -4.9 kb in the murine promoter that mediated high uPA expression in skeletal myoblasts (55).

The positive relationship between uPA gene expression and the tumor cell metastatic potential deserves a special mention. uPA is secreted by many types of cancer cells, including breast, colon ovary, gastric, cervix, endometrium, bladder, kidney and brain tumor tissues, in higher amounts than corresponding normal tissues (56). As already mentioned, the NF κ B binding site in the uPA promoter mediates the direct induction of uPA gene expression by constitutively activated p65 (RelA) in pancreatic adenocarcinomas (49). In addition, β -catenin has been shown to induce uPA expression in colorectal tumors via two consensus TBE (TCF-binding elements) motifs (WWCAAAG) at positions -737 (TBE1) and -562 (TBE2) in the human uPA promoter (57). Important evidence has been obtained in recent years that uPA is differentially expressed in different cancer cell lines in relation to their invasive potential, and correlating with the methylation status of the uPA promoter. Accordingly, high uPA expression was shown to be correlated with hypomethylation of its promoter in the highly invasive hormone-insensitive human breast cancer cell line MDA-231 (58), while the silencing of uPA expression in the non-invasive hormone responsive MCF-7 cells was due to hypermethylation of the uPA promoter. In fact, reversal of the hypomethylation status of uPA promoter in MDA-231 cells was shown to block breast cancer growth and metastasis (59).

The tPA gene

tPA is produced by different cell types, in particular by endothelial cells, keratinocytes, osteoblasts and osteoclasts, melanocytes and neurons (60–62). Of neoplastic cells, tPA may be produced by melanoma, neuroblastoma, ovarian cancer, breast cancer and pancreatic cancer cells (63–65). Two major regulatory regions have been characterized in the human, mouse and rat tPA promoters. One region close to the transcription initiation site is highly conserved among all three promoters and constitutes the minimal promoter region required for basal and inducible expression. The other region is located far upstream in the human tPA promoter, making up a unique enhancer necessary for hormone induction. The minimal tPA promoter contains two copies of closely spaced GC boxes, which are binding sites for transcription factor Sp1. In deletion analysis of the tPA promoter in various cell lines, these GC boxes were shown to play an important role both for basal and induced transcription (66–69). Besides Sp1, a novel GC box-binding protein has been suggested to be responsible for neuronal-specific tPA gene expression (70). The tPA promoter (-119/+169) was activated by UV irradiation in HeLa cells and this induction was mediated by the binding of the transcription factor Sp1 to an AP-2-like sequence CCCCACCC in the tPA promoter (71).

Upstream of the GC boxes, all three mammalian tPA promoters contain a CCAAT-like element known to bind the NF1/CTF transcription factor. The significance of this NF1-binding site seems to be species specific. In the rat tPA promoter, the NF1-like sequence CTGGCGTCAAGCCAA at -145 to -158 is bound *in vitro* by NF1 and deletion of this sequence impairs FSH-induced transcription in rat granulosa cells and basal expression in neuroblastoma cells (68). Recently, this NF1

element was shown to function as a repressor of tPA transcription in rat small artery endothelial cells (72). In the mouse promoter, the NF1-like sequence at 162 to -172 can bind NF1, but deletion experiments in various tissues revealed that the site is not functional (70). Finally, *in vivo* genomic footprinting analysis using human umbilical vascular endothelial cells (HUVEC) and HeLa cells also revealed the occupancy of a CTF/NF1 binding site at position -202 to -187 with respect to the major transcription initiation site in the human tPA promoter. A CRE-like sequence is found in all three promoters upstream of the NF1-binding site. The CRE sequence in the rat tPA promoter is a perfect consensus CRE sequence TGACGTCA, while in both human and mouse promoters this element differs in one central nucleotide, TGACATCA, changing it to a sequence resembling an AP1-binding sequence TGACTCA (73). Corresponding to this difference, rat tPA is inducible in granulosa cells by gonadotropic hormones such as FSH and LH, which augment intracellular cAMP levels, via a consensus CRE site located at -184 to -178 (68). This cAMP-responsive element binds CRE-binding protein from both granulosa cells and neuroblastoma B103 cells *in vitro*. Both the CRE and NF1 sites contribute to the FSH responsiveness of the rat tPA gene in granulosa cells, whereas only the NF1 site is important for constitutive tPA expression in B103 cells. In contrast, the human and mouse tPA genes are unresponsive to these signals (74). Instead, the human uPA gene is induced by FSH in mouse granulosa cells by FSH via an AP-2-like site located at -72 and -29 (75). These results indicate functional interchangeability between uPA and tPA in certain situations. As expected, functional analysis of the human tPA promoter revealed that the AP-1-like sequence (at -222 to -214) and the GC box (at -50 to -36) are important for both constitutive and PMA-induced transcription in HeLa (67, 76) and HUVEC cells (69). In the mouse tPA promoter, the AP1-like sequence located at -175 to -168 and the two GC boxes mentioned above have been shown to be important for the retinoic acid/cAMP-mediated expression of tPA in F9 teratocarcinoma cells (77). The GC box-binding factor required for tPA induction during F9 cell differentiation was shown to be immunologically related to Sp1 (66). Interestingly, differential binding of CREB1 and ATF2 to the human AP1-like element appears to correlate with the differential regulation of tPA by phorbol esters in HT1080 and HeLa cells. PMA-mediated suppression of tPA expression in HT1080 cells involves a decrease in tPA transcription, with CREB1 as the major nuclear protein interacting with the tPA-CRE. In HeLa cells, ATF2 was the most active tPA-CRE-binding protein detected in both uninduced and PMA-induced cells. Since CREB1 can repress PMA-induced transcription of other target genes (including c-jun) (78), it was suggested that the mechanism for the transcriptional downregulation of tPA by PMA in HT1080 cells requires CREB1 binding to the tPA-CRE. ATF2, by associating with the same site, plays a role in PMA-mediated induction of tPA in HeLa cells. In a separate study, glucocorticoids have been shown to suppress the cAMP-stimulated tPA expression in rat mesangial cells via the CRE element located at -185 bp (79).

The expression of the tPA gene is regulated by several hormones in different cell types. Studies by Bulens et al. (80) identified a multihormonal responsive region between -7.1 and -8.0 kb. This 0.9-kb DNA fragment acts as an enhancer that is acti-

vated by all classical steroid hormones (glucocorticoids, progesterone, androgens and mineralcorticoids) except estrogens. These responses are mediated through a retinoic acid response unit consisting of a direct repeat of the GGGTCA motif spaced by 5 nucleotides (DR5: -7319) and a hormone-responsive unit comprising four glucocorticoid-responsive elements (GRE: -7960, -7942, -7703, and -7501). In contrast, the tPA proximal promoter, which is primarily regulated through a cAMP-responsive element and two Sp1 binding sites, contains no hormone-responsive elements. Steroid hormones such as glucocorticoids and androgens, retinoids such as vitamin A and retinoic acid, and 1,25-dihydroxyvitamin D3 have been shown to increase tPA synthesis *in vivo* and *in vitro* (49, 80–88). Retinoic acid induces tPA expression in microvascular endothelial, oral squamous carcinoma and neuroblastoma cells (89, 90). The tPA/DR5 element interacted with the heterodimer composed of retinoic acid receptor α and retinoic X receptor *in vitro* and mediated regulation of tPA by retinoic acid (RA) in human fibrosarcoma, endothelial and neuroblastoma cells (86, 88). The multihormone responsive enhancer also contains a glucocorticoid-responsive unit (GRU) with four functional binding sites for the glucocorticoid receptor, located between -7,501 and -7,974 (80). Site-specific mutagenesis of the four GREs eliminated dexamethasone-mediated induction of the tPA multihormone-responsive enhancer. Therefore, the human tPA gene is a direct target for glucocorticoids, albeit through an unusually complex GRU composed of multiple binding sites for GR. In addition, 1,25-dihydroxyvitamin D3 was found to stimulate tPA expression in osteosarcoma cells via two newly identified vitamin D-responsive elements (VDRE) located at -7175 and -7315 in the multihormone-responsive enhancer of the tPA promoter (87). The VDRE located at -7315 is an inverted palindrome overlapping the previously identified RARE. Moreover, vitamin D3 treatment of primary osteoblasts derived from t-PA-LacZ transgenic mice containing 9 kb of the 5' sequence of the human tPA gene increased the number of lacZ-positive cells, reinforcing the model of enhancer function (87).

In 1981, tPA was demonstrated to be released at the neural growth cone (91). Morphological differentiation of neuroblastoma cells was accompanied by tPA induction, which also suggested that it may have a role in neural cell functions (64). Further studies unambiguously demonstrated the expression and role of tPA in the central nervous system (CNS), where it promotes events associated with synaptic plasticity and acts as a regulator of the permeability of the neurovascular unit. Most of these functions are independent of plasminogen (6), indicating the existence of an as-yet-unidentified substrate for tPA in the CNS. It has become clear that tPA is important in both the developing and mature CNS. Studies addressing the transcriptional regulation of the tPA gene have described crucial regulatory elements within the proximal region and also at far upstream locations, thereby highlighting the complex nature of the tPA promoter. Transgenic mice expressing 1.4 kb of the human tPA promoter fused to the LacZ reporter gene directed widespread expression to the CNS during embryonic development (92). Transgenic mice bearing 1.4, 3.0 and 9.5 kb of the human tPA promoter were used to study expression patterns directed by the human tPA promoter in the adult CNS. The 9.5-kb promoter fragment directed expression to the brain, most notably to the

dentate gyrus, superior colliculus, hippocampus, thalamus and piriform cortex (93). Staining was also observed in the retrosplenial and somatosensory cortex. The 3.0-kb promoter fragment directed generalized and poorly defined expression to the cortex and hippocampus, while the 1.4-kb tPA promoter directed expression selectively to the medial habenula. Intravenous administration of LPS into mice harboring the 9.5-kb tPA promoter resulted in an increase in reporter gene activity in the lateral orbital cortex and thalamus. Results of *in vitro* transfection experiments of NT2 human neuron-like teratocarcinoma cells with these series of tPA promoter deletion constructs confirmed the presence of regulatory elements throughout the 9.5-kb promoter region. A new NFAT recognition site has been described that may play a role in the selective expression of the 1.4-kb tPA promoter in the medial habenula (93). These results indicate that elements between -3.0 and -9.5 kb of the tPA promoter confer constitutive and inducible expression to specific regions of the CNS (93).

Finally, studies in knockout mice have confirmed that tPA participates in neuronal plasticity such as involvement in memory and learning activities (94). Interestingly, corticosteroids (also known as stress hormones) have a role in memory and learning processes (95), whereas mice deficient in tPA show higher stress tolerance and elevated and extended strength of corticosteroid levels after restraint stress (96). These results may be related to the ample responsiveness of the tPA gene to steroid hormones, via the multihormone enhancer element located at -7.1 kb. On the other hand, tPA (as well as PAI-1) plasma levels display a circadian rhythm for unknown reasons (97). Since cortisol also displays a circadian rhythm (98), and since tPA (and PAI-1) is regulated by glucocorticoids and contain GREs in its promoter, it is tempting to speculate that the steroid hormone regulation of tPA may underlay this circadian expression cycle.

The uPAR gene

uPAR is expressed in many different tissues and cell types, including peripheral blood monocytes, granulocytes, endothelial cells, B-lymphocytes, activated T-cells, neutrophils, fibroblasts, epithelial cells, normal bone marrow cells including megakaryocytes, keratinocytes and placental trophoblasts (99–103). uPAR expression can be regulated by various factors, including tumor promoters, cytokines, hormones and growth factors in a number of different cell types, indicating the potentially complex nature of uPAR gene regulation (5), consistent with a role for this molecule in diverse biological settings. There is a strong correlation between uPAR expression and the invasive cancer cell phenotype. Reflecting this, uPAR is expressed at elevated levels in almost all of the tumor tissues and cell lines, including breast, colon, gastric and certain lung carcinoma tissues (104, 105). However, there is disagreement between different laboratories as to which cells produce uPAR, whether it is produced by stromal cells such as macrophages or by cancer cells in breast and colon carcinoma tissues (106).

The region for maximal promoter activity of the uPAR gene was defined in a 188-bp fragment between -141 and +47 relative to the transcription initiation site (107). This is a strong promoter. Accordingly, uPAR is overexpressed constitutively in invasive cancer cells and regulated by a number of stimuli. The promoter region of the human uPAR gene lacks conventional TATA and

CAAT boxes but contains a CpG-rich island and sequences related to consensus cis-acting elements for AP1 (one distal and one proximal), AP2, PEA3, NF κ B and Sp1 transcription factors that mediate the basal transcription of the gene. These cis-elements and the trans-acting factors involved in the regulation of uPAR gene expression have been characterized mainly in the laboratories of Drs. Blasi, Boyd and Wang. Soravia et al. (107) were the first to report a GC-rich region (-99/-70) bound with Sp1, which was required for basal expression of the gene. A separate sequence (-148/-124) was also identified that could bind Sp1/Sp3 and an AP2 α -related factor and was required, at least in part, for basal and PMA-inducible uPAR expression in colon cancer cells (108). In addition, tumor-specific transcription factor binding to the AP2/Sp1 site was found in gastrointestinal cancers (109). Nuclear substitutions that prevented the binding of the AP2 α -related factor and Sp1/Sp3 reduced uPAR promoter stimulation by PMA in GEO human colon cancer cells (108). In addition, two AP1 consensus motifs located at -70 and -183 have been identified and shown to be required for both constitutive and PMA-inducible expression of the uPAR gene in colon cancer cells (110, 111). Moreover, these and other studies showed that the PMA or Ras-dependent stimulation of uPAR gene expression involves a combination of downstream Erk and/or JNK kinase activation leading to translocation of cJun/JunD into the nucleus and AP1 transcriptional activation (111–113). The distal AP1 site at -183 in the uPAR promoter also mediated UVB-induced uPAR expression in human epidermal keratinocytes (114). Recently, the Kruppel-like KLF4 transcription factor has been identified as a novel regulator of uPAR expression in colonic crypt luminal surface epithelial cells, through the binding to multiple sites in the uPAR promoter (115). Colon cells from KLF4 null mice showed a dramatic reduction in uPAR protein compared with wild-type mice. Conversely, KLF4 expression in HCT116 colon cancer cells increased the amount of uPAR protein/mRNA. Transient transfection of KLF4 with a reporter driven by a 5'-deleted uPAR promoter fragment indicated that the proximal 200 bp is essential for optimal expression. Mobility-shifting experiments demonstrated binding of KLF4 to multiple regions of the uPAR promoter (-154/-128, -105/-71, and -51/-24), and chromatin immunoprecipitation assays confirmed the binding of KLF4 to the endogenous promoter. Deletion of the -144/-123 promoter region diminished but did not eliminate the ability of KLF4 to transactivate the uPAR promoter, suggesting cooperativity of these binding sites with respect to activation of gene expression (115). A recent study showed that Bcl-2 overexpression in breast carcinoma cells exposed to hypoxia induced uPAR expression via the Sp1 transcription factor and that the Erk1/2 signaling pathway plays a role in Sp1 transcriptional activity (116). Similarly, another report indicated that TGF β induced uPAR expression via Sp1 binding to the -70 bp sequence in the uPAR promoter in human monocyte-like U937 cells (117). In separate investigations, Hapke et al. identified a silencing motif consisting of a PEA3/Ets sequence at -248 bp (118), while Wang and colleagues identified an NF κ B site located at -45. The PEA3 element at -248 was shown to mediate the downregulation of uPAR expression by integrin β 3. Recent studies have indicated that the expression of uPAR is linked to the expression and ligation of $\alpha_v\beta_3$ (50). The

uPAR promoter (up to -1469 bp from the start site) that is normally constitutively active in CHO cells was downregulated by induced β 3-integrin expression. Deletion of the PEA3/Ets motif at -248 bp substantially impaired the ability of β 3-integrin to downregulate the uPAR promoter, suggesting that the PEA3 site acts as a silencing element (118). Thus, besides the physical interaction of β 3-integrin and uPAR at the cell surface, β 3-mediated outside-in signaling has been implicated in the regulation of uPAR gene transcription, suggesting a mutual regulation of adhesion and proteolysis receptors (50, 119, 120). Furthermore, overexpression of the intracellular integrin-binding protein β 3-endonexin decreased uPAR promoter (-398 bp) activity that is constitutive in endothelial cells (121). Mutation of the NF κ B promoter binding site at -45 in the human uPAR gene impaired the ability of β 3-endonexin to downregulate uPAR promoter activity. β 3-endonexin interacts directly with the p50/p65 transactivation complex and thereby inhibits binding of the κ B site to the NF κ B transcription factor. Thus, β 3-endonexin acts as a regulator of uPAR expression in β 3-integrin-mediated endothelial cell migration through direct interaction with p50/p65 (121). In an attempt to confirm these results obtained in cultured cells, studies analyzing tissue-specific expression of uPAR were performed in transgenic mice bearing a LacZ reporter gene with varying lengths (0.4, 1.5, and 8.5 kb) of uPAR promoter sequence. The results indicated that uPAR expression in different tissues is controlled at the transcriptional level (122). The 0.4-kb uPAR upstream sequence directed weak and strong LacZ expression in the placenta and epididymis, respectively; both of these tissues express endogenous uPAR. Conversely, transgene expression in the apical cells of colon, which are positive for endogenous uPAR protein, required 1.5 kb of upstream sequence for optimal expression. Furthermore, a putative regulatory region spanning -1295 to 1192 bp has been suggested to drive uPAR expression in colonic cells. Interestingly, placental transgene expression was augmented with the 8.5-kb upstream fragment compared with the shorter 1.5-kb fragment, indicating contributing elements between -1.5 and -8.5 kb. Thus, while 0.4 kb of upstream sequence directs uPAR expression in the epididymis, sequences located between 0.4 and 1.5 kb and between -1.5 and -8.5 kb are required for optimal tissue-specific expression in the colon and the placenta, respectively (122).

The PAI-1 gene

PAI-1 exerts its biological function not only through modulating the catalytic activity of uPA and tPA but also through modulating interaction between integrin-ECM and uPA/uPAR-ECM that do not necessarily involve catalytic activity of PA (3). The latter reaction is an important regulatory component of multicellular processes involving cell remodeling, such as angiogenesis, wound healing and possibly metastasis. PAI-1 is expressed in almost all cell types, prominent among which are adipocytes, hepatocytes and endothelial cells. It was first discovered as glucocorticoid-induced activity in rat hepatoma cells (123). Its expression is modulated by a variety of signals from mechanical or environmental stresses to autocrine and endocrine molecules, depending on cell type. Because the number of signals that affect PAI-1 gene regulation is vast, we restrict our discussion here to PAI-1 gene regulation by TGF β and hypoxia and regulation in

adipocytes. Other regulating signals are listed with the corresponding transcription factors and cis-acting elements in Table I, together with the literature references.

TGF β is an important regulator of epithelial cell motility and PAI-1 is one of most prominent and studied targets of TGF β (124). Hypoxia-dependent induction of PAI-1 gene may be relevant to its high expression in metastatic tumors (3). In recent years, the number of patients with type 2 diabetes has increased at an alarming rate (125). As type 2 diabetes is strongly correlated to obesity and the main cause of death of people suffering this disease is cardiovascular complication, it is very important to elucidate PAI-1 gene regulation in adipocytes, a major contributor organ of plasma PAI-1 in these disease conditions (126–128).

TGF β

Functional analysis of the PAI-1 promoter for TGF β induction has located a major regulatory region between –800 to –500 with respect to the transcription initiation site. This region contains several cis-elements, but the involvement of these elements for TGF β induction varies slightly with cell type and the context of the template in which these elements are characterized. In HepG2 cells, abutting sequences with high homology to consensus binding sites for the CCAAT-binding transcription factor nuclear factor I (CTF/NF-I) (TGGCTGCATGCC: 560 to –548) and the ubiquitous factor (USF) (E-box, CACGTG: –568 to –563) are responsive to TGF β induction in the natural promoter

context with 800 nt 5' flanking region and in isolation linked to SV40 promoter (129). Dennler et al. (130) showed in the same cells that three TGF β -responsive elements {AG(C/A)CAGACA}, termed "CAGA boxes", are located at –740 to –732 (AGC-CAGACA), –590 to –582 (AGACAGACA) and –286 to –278 (AGACAGACA), and that they cooperatively mediate TGF β induction through interacting with Smad3/Smad4 heterodimers. Song et al. (131) showed in Hep3B cells that a 12-bp element (AGACAAGGTTGT) at –736 to –725, termed "TGF β -responsive sequence (TRS)", partial overlaps a CAGA-box and can mediate strong transcriptional activation by TGF β when multiple copies are located upstream of a heterologous promoter. Interestingly, TRS mutants in which the AGAC sequence remained intact also failed to mediate TGF β induction and to compete with the wild-type TRS for Smad3/Smad4 binding, suggesting an important role in TGF β induction for TRS sequences other than AGAC. Hua et al (132) showed in HT 1080-derived cells that the transcription factor E-box-binding protein TFE3 synergizes with Smad3/Smad4 complex to mediate TGF β induction in a manner dependent on Smad3 phosphorylation, with the former binding to an E-box (–568 to –563) and the latter to a sequence immediately upstream containing CAGA boxes (–590 to –572). Furthermore, interaction between the transcriptional adaptor p300 or CBP with Smad3 is induced by TGF β treatment, suggesting that p300 (or CBP) acts as a coactivator in bridging the Smad3/Smad4 complex and the general transcriptional machinery (133). Datta et al. (134) have shown a novel form of

Table I: PAI-1 gene regulators.

Regulators	Cells ^a	Cis-elements ^b	Trans-factors	Features	Reference
wound (scrape)	rat keratinocytes			Erk-dependent	(248)
oxidative stress	GH4 (rat pituitary)	–61: TGAGTTCA	AP-1		(249)
Ca ²⁺	HepG2	–195: CACGTACA	HIF-1	via HIF-1 α induction	(250)
DNA alkylating agents	NIH3T3 (mouse fibroblast)	–160 : ACACATGCCTCAG-CAAGTCC	p53		(251)
TPA	HT1080, HeLa, HepG2	–82 to –65	AP-1, AP-2, SPI-like		(252,253)
fatty acid	HepG2	72 bp: –599 to –528	SPI-like	PKC dependent	(137)
IL1 / oncostatin M	HepG2, cortical astrocytes	–61 : TGAGTTCA	c-fos/c-jun	via induction of c-fos	(254,255)
hyperglycemia	rat primary GMC	SPI sites –76 : GGTGG and –44 : CTGCC	SPI	SPI glycosylation involved	(135,136,256)
glucocorticoid	HTC, trophoblast, adipocytes,	rat –1212 : TGAAAGCACA-CACTGTTCTTGACAGA			(156,257,258)
retinoic acid	VSMC			dependent on genistein-sensitive tyrosine kinase	(259)
thrombin	HUVEC, HK2 (kidney PTEC)			JNK/API pathway: PKC/src dependent	(260,261)
Angiotensin II	rat primary VSMC, rat primary mesangial cells	rat –89 to –50 (=human –91 to –50)	SPI & API	MEKK1-dependent	(262), (138)
fibrin fragment	rat primary lung fibroblast	–59: TGAGTTCA	AP-1 (c-fos/junD)	conserved cis element	(263)
circadian regulation	BAEC, HUVEC	two E-boxes CACGTG at –684 and –565 (human gene)	CLOCK/BMAL, CLOCK /CLIF		(264), (265)
cytochalasin D/ colchicine	rat VSMC (R22)			MEK-dependent, sensitive to genistein and herbimycin A	(266)
ethanol	HUVEC	–800 to –549		inhibition seen at 0.02 %	(267)

BAEC, Bovine aortic endothelial cells; GMC, glomerular mesangial cells; HTC, rat hepatoma cells; HUVEC, human umbilical endothelial cells; PTEC, proximal tubular epithelial cell; VSMC, vascular smooth muscle cells

^a unless otherwise mentioned, cells are human ^b unless otherwise mentioned, numbering is on the human PAI-1 gene promoter.

For TGF β , TNF α , hypoxia and insulin, see text.

TGF β regulation in PAI-1 gene regulation in which TGF β induces the interaction between Smad3 and SP1, thereby enhancing PAI-1 gene transcription via the two SP1-binding sites in the proximal region of the PAI-1 promoter: (GGGTGGGG) at -77 to -70 and (CCTGCCC) at -46 to -40. Importantly, induction of the endogenous PAI-1 gene by TGF β was efficiently suppressed by the SP1 inhibitor mithramycin, while reporter gene induction by TGF β from a Smads-responsive promoter that contained multiple CAGA copies was not affected by the same inhibitor. It should be mentioned that the sequence around this promoter proximal regulatory region is well conserved between human and mouse, while the sequence around the two upstream E-boxes is not conserved in mouse. SP1 sites may, therefore, be more important than upstream elements in TGF β regulation. Actually, SP1 sites have also been shown to mediate PAI-1 gene induction by glucose/glucosamine (135, 136), fatty acid (137), and angiotensin II (138).

Hypoxia

Hypoxia, a condition where oxygen supply is reduced, occurs in various pathological situations, such as sepsis, atherosclerotic lesions leading to myocardial infarction and deep vein thrombosis (139). Hypoxia is also conspicuous in solid tumors, hampering radio therapy (140). Hypoxia induces the expression of various genes by increasing the levels of the transcription factor HIF-1, which involves hypoxia-induced suppression of ubiquitin-dependent HIF α subunit degradation. (141). Since the first report that PAI-1 mRNA could be strongly induced by hypoxic conditions in human endothelial cells (142), several different mechanisms by which the PAI-1 gene is upregulated have been reported. Kietzmann et al. (139) identified in rat hepatoma cells putative hypoxia-responsive elements HRE1 (-175 to -168) and HRE2 (-165 to -158) and found that HRE2 was most critical for induction by mild hypoxia (8% O₂) and HIF-1 binding. Later, the same group showed that upstream stimulatory factor-2a (USF-2a) interacts with HRE1 and downregulates the PAI-1 promoter, thus suggesting balancing activities of HIF-1 and USF-2a in PAI-1 gene regulation in many clinical conditions (143). Fink et al. (144) identified in human HepG2 cells an HRE (CACGTA-CA) at -195 to -188 that mediated the effect of severe hypoxia (1~2 % O₂) by interacting with HIF-1. This site corresponds to and is identical with the USF-2a-binding HRE1 site in the rat promoter.

Accumulation of HIF1 α in freshly biopsied keloid tissues has been reported, suggesting that a local state of hypoxia exists in keloids (145). Based on this observation, Zhang et al. (146) examined the effect of hypoxia (1% O₂) on PAI-1 gene regulation in freshly prepared human keloid-derived fibroblasts and found that the PAI-1 mRNA is strongly induced by the elevation of HIF-1 protein levels. Interestingly, PAI-1 mRNA induction and activation of the PAI-1 promoter was strongly attenuated by LY294002 and Wortmannin, which are inhibitors of the phosphatidylinositol-3-kinase(PI3-K)/protein kinase B (PKB) pathway, and genistein, an inhibitor of tyrosine kinases. Furthermore, Erk MAP kinase pathway inhibitor PD98059 suppressed PAI-1 mRNA induction without affecting HIF-1 levels. In HepG2 cells, hypoxia-mediated HIF-1 accumulation and PAI-1 mRNA induction were suppressed by inhibitors of p38 MAP kinase and PI3-K

but not of Erk MAP kinase (147). It appears that several signaling pathways are involved in hypoxia-induced regulation of HIF-1 activity and PAI-1 mRNA induction at different levels.

Regulation in adipocytes

In obese and insulin-resistant conditions, elevated levels of PAI-1 expression and of insulin, TNF α and TGF β have been reported (148-150), suggesting a causal relationship between these hormones/cytokines and PAI-1 expression, especially in adipocytes. Transcriptional effects of TGF β on the PAI-1 promoter have been discussed above. As TGF β was strongly induced by TNF α in 3T3-L1 adipocytes, it was suggested that TNF α induces PAI-1 via, at least partly, TGF β induction (150). However, a search for TNF α -responsive elements within 3 kb of the PAI-1 promoter by transfection assays did not clearly identify such elements (151, 52). Recently, Hou et al. (153) characterized the endogenous PAI-1 gene by cross-species sequence homology analysis as well as DNase I-hypersensitive site analysis and identified a functional NF κ B binding site TGGAATTTCT located at about -15 kb that could mediate the response to TNF α . The analysis, however, was performed in primary bovine aortic endothelial cells and it remains to be seen whether the same regulatory site is also functional in adipocytes.

Adipocytes are prominent targets of insulin action (>200,000 receptors per cell) (154) and one of the main sources of PAI-1 in obese and type 2 diabetes (127, 128, 155). Although insulin was shown to induce PAI-1 gene in adipocytes (156), TNF α and TGF β were considered to be the major inducers of the PAI-1 gene in adipocytes under insulin-resistant conditions (149, 157, 158). Subsequently, important results indicated that glucose transport and PAI-1 gene expression are mediated by different insulin signaling pathways in insulin-resistant ob/ob mice and in insulin-resistant 3T3-L1 adipocytes (159). Ligand-bound insulin receptor triggers activation of two different signaling pathways, one involving Erk MAP kinase and the other involving PI3 kinase (PI3-K) (160). The mitogenic Erk signaling pathway activates the PAI-1 gene (159) by activating the transcription factor AP1 (161). It was also reported that the PI3-K pathway was compromised in skeletal muscles from type 2 diabetes patients (162, 163) or from insulin-resistant db/db mice (164) but that insulin-induced Erk activation was not affected. These results strongly suggest that insulin resistance selectively interferes with PI3-K activation. Because under insulin-resistant conditions (at least at an early stage) the level of circulating insulin concentration in the body is markedly augmented (hyperinsulinemia) (165), it may follow that the PAI-1 gene receives more positive signals in insulin-resistant conditions. Furthermore, it seems that suppression of the PI3 kinase pathway in insulin-resistant conditions reinforces the positive signals from the Erk pathway. It has been reported in various types of cells that the PAI-1 gene is negatively regulated by free E2F (47, 166). E2F is a cell cycle-regulating transcription factor whose activity is checked by forming a complex with a pocket-binding protein such as retinoblastoma protein (pRB). Its activation involves hyperphosphorylation of pRB. In T lymphocytes, it was shown that activation of the PI3-K pathway leads to E2F activation (167). Thus, insulin receptors generate both positive and negative signals on the PAI-1 gene through

the Erk and PI3 kinase signaling pathways, respectively, and insulin-resistant conditions leave only the positive signal. This scenario has recently been confirmed by Venugopal et al. (168) in caveolar dysfunction-induced insulin-resistant 3T3-L1 adipocytes. They show that caveolar dysfunction induced by cholesterol depletion impairs only the PI3-K signaling pathway and that PAI-1 mRNA induction by insulin is enhanced by caveolar dysfunction. They further show that a pharmacological agent that disrupts the E2F-pRB complex counteracts the effect of caveolar dysfunction on PAI-1 gene expression. A further twist in this context is that elevated PAI-1 expression is not only the consequence of insulin resistance but may also be its cause (169, 170). Disruption of the PAI-1 gene in insulin-resistant ob/ob mice restored low levels of insulin, glucose and TNF β in circulation (169). Obesity and insulin resistance developing in wild-type mice on a high fat/high carbohydrate diet were completely prevented in mice lacking the PAI-1 gene (170). Lopez-Aleman et al. (171) showed that PAI-1 treatment of NIH3T3 cells suppressed insulin-induced PKB activation but not Erk activation in a manner dependent on vitronectin. These observations taken together raise the interesting possibility that PAI-1 is a positive feedback regulator of its own gene through modulation of the activity of its negative regulator E2F. Details of the underlying mechanism, especially events taking place at the insulin receptor level in the presence of PAI-1, remain to be elucidated.

The PAI-2 gene

Various agents controlling PAI-2 gene expression have been reported, including growth factors (TGF β , EGF, M-CSF and GM-CSF), hormones (retinoic acid, dexamethasone and vitamin D3), cytokines (TNF α , IL-1 and IL-2), vasoactive peptides (angiotensin II), toxins (dioxin and endotoxin) and tumor promoters (phorbol esters and okadaic acid) (172). Of these regulators, the most investigated are tumor promoters and TNF α . In the promoter proximal region there are two AP1-like elements, AP1a (TGAATCA: -103 to -97) and AP1b (TGAGTAA: -114 to -108), and one CRE-like element (TGACCTCA: -187 to -182) (173). It was shown in human HT1080 fibrosarcoma cells that AP1a and CRE-like sites are required for both basal and PMA-induced PAI-2 gene activation (173). It seems that c-Jun and JunD are the major components binding to the AP1a element under both basal and PMA-treated conditions (174). Interestingly, basal and PMA-induced transcription of the PAI-2 gene in HT1080 and U937 cells was significantly greater with a -219-bp than with a -1100-bp promoter construct, suggesting the presence of a repressor site between -219 and -1100 (174). However, identification of the exact sequence within this region and transacting factors responsible for this activity has not yet been reported.

The PAI-2 gene is one of most TNF α -responsive genes in several cell types. Deletion analysis of the PAI-2 promoter in HT1080 cells showed strong TNF α inducibility only after the 5' end point of the reporter genes was shortened to -219 (175). The region between -259 and -219 had a selective repressing activity on TNF β induction; reporter inducibility by PMA and okadaic was not affected by the presence of this region (175). Although, proteins binding to this site and a site immediately 3' have been suggested by gel shift assays, their identification and role in

TNF α induction still remain to be worked out. In the same study, another distal region between -1859 and -1100 was suggested to harbor a repressor activity on induction by PMA, okadaic acid as well as TNF α . Antalis et al. (176) characterized 5.1 kb of 5' flanking region in U937 cells by deletion analysis and found a silencer between -1977 and -1675 that acts in an orientation- and position-independent but not cell-specific manner. The silencer activity was localized to a 28-bp sequence containing a 12-bp palindrome centered at an Xba I restriction site at position -1832, CTCTCTAGAGAG, which was termed PAI-2-upstream silencer element-1 (PAUSE-1). Later analysis defined the minimal functional PAUSE-1 element as TCTN_xAGAN₃T₄, where x=0, 2 or 4 (177). UV-crosslinking analyses determined that the PAUSE-1 binding protein was ~67 kDa, but its identity remains unknown. In their study, PAUSE-1 was not characterized in the context of TNF α induction. It would be interesting to see whether PAUSE-1 is the element that selectively repressed TNF α inducibility shown by (175).

Regulation of mRNA stability

uPA mRNA

uPA mRNA stability has been shown to be modulated either positively or negatively by various means, including protein synthesis inhibitors, Ca²⁺, α_v integrin, PKC downregulation, glucocorticoids, shear stresses, TNF α and dioxin (54, 178–184).

The most widely studied mRNA instability elements are adenylate/uridylate (AU)-rich (185, 186) and are generally referred to as AREs. The AREs are usually composed of pentameric AUUUA or nonameric UUAUUUUAUU (187, 188) sequences and are grouped into three classes (I, II, III) depending on the absence or presence of the consensus AUUUA motifs and how these motifs are arranged within the ARE (189). The 3'-UTR of uPA contains several highly homologous regions but only the AU-rich element (~50 nt) has been assigned a specific function. Functional dissection of the 3'-UTR of uPA mRNA in LLC-PK₁ cells using a hybrid globin mRNA reporter system defined at least two independently acting mRNA instability-determining regions, one of which was the ARE. The ARE of uPA mRNA is highly conserved amongst a number of species. It is a class I ARE containing two separate AUUUA and AUUUUUA motifs (190). Changes in the post-transcriptional control of uPA have been linked to differential uPA expression in cancer cells. Indeed, in cells where uPA mRNA expression is low, uPA mRNA is unstable with a half-life of about 1 h (178, 191). However, in metastatic breast cancer cell lines where the stationary level of uPA mRNA is very high, uPA mRNA is extremely stable with a half-life exceeding 17 h (192, 193), due partly to impairment of ARE-mediated degradation mechanisms (192). It has been shown that the heterogeneous ribonuclear protein C (hnRNP C) binds to the ARE in the 3'-UTR of uPA mRNA (192) and that levels of ARE-binding activity in cytoplasmic extracts from MDA-MB-231 cells are higher than in LLC-PK₁ or HeLa cells, where uPA mRNA is less stable. It was subsequently found that the p38 MAP kinase-MAPKAP kinase (MK2) pathway is constitutively activated in these cells and is responsible for the augmented uPA mRNA stability (194–196). Further analysis showed that MK2 activation enhances cytoplasmic distribution of the nuclear pro-

tein HuR, which may promote HuR interaction with ARE-mRNA resulting in its stabilization (194). In this context, it is noteworthy that TNF α treatment of LLC-PK₁ cells activates both p38 and Erk MAP kinases and at the same time stabilizes uPA mRNA. Inhibition of both p38 and Erk MAP kinases is necessary to suppress the stabilization of uPA mRNA (L. Montero and Y. Nagamine, unpublished data). Tran et al. (197) recently identified three uPA ARE binding proteins in HeLa nuclear extracts. Two are known ARE-binding proteins, HuR and NFAR, and the third was a putative DExH RNA helicase RHAU. RHAU physically interacts with the deadenylase PARN, and the exosome and enhances the deadenylation and decay of reporter constructs containing the uPA ARE (ARE^{uPA}). RHAU does not bind to ARE but interacts with NFAR1 and HuR in a manner dependent on RNA, most likely the ARE (197). It should be noted that RHAU does not enhance the decay of uPAR mRNA, notwithstanding the fact that it also harbors a class I ARE. In accordance with this, NFAR1 does not bind to ARE^{uPAR}, suggesting the importance of NFAR1 in target specificity for RHAU (N. Akimitsu and Y. Nagamine, unpublished data). It is not known yet whether RHAU is dynamically regulated by extracellular signals. Besides the RNA helicase core region that is highly conserved among DExH/D family members, RHAU contains unique amino- and carboxyl-terminal regions that may serve as docking sites for regulatory co-factors (198, 199). It would be interesting to determine whether hnRNP C, HuR, NFAR1 and RHAU functionally and physically interact on the ARE of uPA mRNA.

tPA mRNA

tPA expression is also subject to post-transcriptional regulation. Initial studies implicated a role for the 5'-UTR in the post-transcriptional regulation of the tPA gene (200) as deletion of the 5'-UTR from tPA mRNA resulted in an increase in tPA mRNA stability in transfected COS cells. The influence of the 5'-UTR is interesting because the tPA gene has two transcription initiation sites (TIS) located 110 bp apart. The experiments described in (200) were based on the upstream TIS, which produces a 5'-UTR of 209 nt. In at least two other human cells systems, the second TIS in fact is preferred (201, 202), creating a 5'-UTR of only 99 nt and it is possible that the two tPA transcripts have different inherent decay rates due to the different lengths of the 5'-UTRs. The 5'-UTR is more often associated with changes in translation, although a role for the 5'-UTR in the control of mRNA instability is not unprecedented (203). The 3'-UTR of tPA contains a number of novel features. Although it lacks a classical ARE, deletion of the entire 3'-UTR causes a threefold increase in tPA mRNA stability in transfected COS cells with a concomitant increase in translation (204) but the mechanisms behind this change in tPA mRNA stability are unknown.

Studies on the developmental changes in gene expression in mouse oocytes have highlighted a role for translational silencing in the regulation of tPA expression. In oocytes, tPA mRNA is dormant but becomes translated at a certain stage in development. This occurs via translational silencing linked to cytoplasmic polyadenylation (205, 206). In young mouse oocytes, tPA mRNA is polyadenylated in the nucleus but is deadenylated to form a short poly(A) tail in the cytoplasm and stored until maturation (207). Translational activation of tPA mRNA during

meiotic maturation of mouse oocytes is associated with poly(A) tail elongation (206, 208). An AU-rich element within the 3'-UTR of tPA mRNA has been shown to be associated with adenylation and deadenylation. This element has been termed the adenylation control element (ACE) (207). Cytoplasmic polyadenylation requires both the polyadenylation signal (AAUAAA) and the ACE but deadenylation only requires the ACE (207, 208). The ACE and the AAUAAA are masked by antisense oligodeoxynucleotides in primary mouse oocytes and this region becomes unmasked during maturation and with the first stages of adenylation before translation (209). A 80-kD protein ("ACEB") interacts with the ACE in primary oocytes and is suggested to mediate translational silencing (210). A recent report has also demonstrated a possible role for ACE in the translational regulation of a GFP reporter gene within the central nervous system of transgenic mice (211).

uPAR mRNA

The first line of evidence to link post-transcriptional events with uPAR gene expression was seen in A459 cells in which the stability of uPAR mRNA significantly increased after treatment with PMA and TGF- β (212). Actinomycin-D block experiments using human mesothelioma (MS-1) showed that uPAR mRNA half-life increased after treatment with PMA or cycloheximide (213). Subsequently, a 50-kDa uPAR mRNA binding protein (uPARmRNABp) was identified that selectively recognized a 51-nt fragment within the uPAR coding region. This fragment was also able to destabilize the stable β -globin mRNA (213). Recent observations suggested that tyrosine kinases are involved in the post-transcriptional regulation of uPAR expression. To this end, PMA mediated induction of uPAR expression was correlated with tyrosine phosphorylation of the uPARmRNABp. Furthermore, this phosphorylation inhibits the interaction between uPAR mRNA-uPARmRNABp and stabilizes the uPAR transcript (214). The 50-kDa uPARmRNABp was purified from human bronchial epithelial (Beas2B) cells and identified as phosphoglycerate kinase (PGK) (215). Overexpression of PGK in H157 lung carcinoma cells results in a decrease in cytoplasmic uPAR mRNA and cell surface uPAR protein expression, thus confirming that PGK regulates uPAR expression at the post-transcriptional level (215).

The 3'-UTR of uPAR mRNA harbors an ARE of approximately 50-nt in length comprising a class I ARE nonameric motif (UUAUUUUAUU) overlapping a potential AU-rich sequence, UUAUUUUUAUA. The uPAR ARE confers instability to the stable β -globin mRNA in both Jurkat T and HeLa cells (216). Although this ARE is highly unstable, in human Jurkat T cells this instability is overcome by engagement of the β 2-integrin LFA-1 (216). In addition, HuR specifically interacts with the uPAR ARE and overexpression of HuR stabilizes the uPAR mRNA in HeLa cells (194). In uPAR-transfected kidney cells, uPA was found to increase uPAR expression at a post-transcriptional level, by increasing the activity of a novel cellular factor that binds the coding region instability determinant of uPAR mRNA, presumably acting to stabilize the transcript (217). A similar effect was also observed in cells obtained from patients affected by non-small cell lung carcinoma (218).

PAI-1 mRNA

PAI-1 mRNA exists as two transcripts in humans (3.2 kb and 2.2 kb), but only the longer transcript is present in lower animals. In humans, the two transcripts encode an identical protein; the only difference being that the longer transcript possesses an extended 3'-UTR as a consequence of an alternate polyadenylation site in the PAI-1 gene (219–221). The alternate selection of the polyadenylation sites is based on sequence elements within the human PAI-1 3'-UTR rather than being a consequence of the species-specific distribution of a regulatory protein (222).

The longest PAI-1 transcript contains 3–6 copies of the AUUUA pentamer, depending on the species (219, 221, 223). The 3.2-kb variant is less stable (half-life 51–56 min) than its shorter counterpart, most likely due to the presence of a copy of the pentamer (AUUUA), which is absent from the more stable 2.2-kb form (half-life 2.5–2.8 h) (220). Growth factors, cytokines and hormones have been implicated in the modulation of PAI-1 mRNA stability. In human HepG2 hepatoma cells, TGF β and insulin increase the half-life of 3.2-kb PAI-1 mRNA but not the 2.2-kb form (124). On the contrary, insulin-like growth factor (IGF-1) stabilizes both species of PAI-1 mRNA (124, 224). Hence, the two transcripts are differentially regulated at the level of mRNA stability. What this means physiologically is uncertain but it may be that the selective use of one particular transcript influences the level of PAI-1 synthesis by imposing a different mRNA decay rate.

8-Bromo-cAMP, a cyclic nucleotide analogue causes a decrease in PAI-1 mRNA in HTC rat hepatoma cells (225) while the PAI-1 3'-UTR can confer cAMP-dependent instability to β -globin mRNA. The active region within the PAI-1 3'-UTR responsible for the cAMP effect is located within a 134-nt region known as the cAMP responsive sequence (PAI-CRS) (226, 227). The protein binding to this element have been isolated and termed PAI-RNA binding protein 1 (PAI-RBP1) (227, 228) but the role of PAI-RBP1 in PAI-1 regulation has yet to be defined. A number of other agents have been shown to influence PAI-1 mRNA turnover, including osteogenic protein-1 (229), angiotensin II (230, 231), and *Rickettsia rickettsii* infection (232).

PAI-2 mRNA

The PAI-2 is one of the most highly regulated genes known, at least in terms of the magnitude by which it is induced by growth factors, hormones, cytokines (233, 234) and tumor promoters (235, 236). Although PAI-2 induction involves substantial changes at the level of transcription, post-transcriptional events are also important in modulating its expression. This was first revealed over a decade ago, when it was shown that the increase in PAI-2 mRNA after synergetic stimulation by PMA and TNF α (1000– to 1500-fold) cannot be accounted for by increase in PAI-2 transcription rate alone (50-fold), suggesting that post-transcriptional processes influence PAI-2 gene expression (235). PAI-2 mRNA contains a functional nonameric (UUAUUUUAU) ARE element in its 3' untranslated region (237). Mutagenesis of this element partially stabilized the normally unstable PAI-2 mRNA, hence revealing a functional role for this motif (237, 238). The element also provides binding sites for several ARE binding proteins, including the stabilizing protein HuR (238)

and the mRNA destabilizing protein tristetraprolin (TTP), the latter identified from a yeast three hybrid screen (239). HuR is a member of the Hu family of mRNA binding proteins and has been associated with promotion of mRNA stability (240–242). TTP, on the other hand, is a potent mRNA destabilizing protein that associates with ARE elements in cytokine transcripts, including TNF α (243, 244) and IL-3 (245). Overexpression of TTP in HEK 293 cells transfected with a constitutively active PAI-2 expression vector resulted in loss of PAI-2 mRNA, suggesting that TTP can indeed regulate PAI-2 expression. However, more detailed studies are needed to determine whether TTP influences endogenous PAI-2 expression and whether TTP and HuR interact during this process. Other cytoplasmic and nuclear proteins also bind to the ARE with the PAI-2 3'-UTR (237, 238) but these are yet to be identified. The PAI-2 transcript also possesses another instability determinant located within exon 4 of the PAI-2 coding region (246). UV-crosslinking studies have identified two RNA-binding proteins (approximately 50–52 kDa) that specifically interact with this sequence (246). Taken together, the data published to date suggest that PAI-2 mRNA stability is influenced by elements located within both the coding region and the 3'-UTR. It remains to be determined whether these instability elements in the coding region and the 3'-UTR act in a coordinated fashion to control PAI-2 mRNA stability.

Concluding remarks and perspective

In the course of analyzing the transcriptional regulation of the PA system genes, most studies searching for particular cis-elements have been performed on limited lengths of promoters, using transient or stable transfection of reporter gene constructs. This approach most likely misses regulatory elements located far away from the transcription initiation site or within introns. Although DNAase I hypersensitive site analysis has been employed less and less in recent years, it still remains a powerful tool for the initial step of transcriptional analysis, as is evident from the discovery using this strategy of a distal TNF α -responsive site in the PAI-1 gene, located 15 kb upstream of the transcription initiation site (153). Many cis-elements still remain to be identified for various inducers of the genes of the PA system and we do not know for sure whether some of those already identified are the sole regulatory elements responsible for a particular induction, or whether they offer a partial view of a more complex regulatory network. It would be worthwhile to consider employing this older method for initial analysis of the promoters, implemented by new and potent technical strategies (i.e., chromatin immunoprecipitation analysis at target gene sites).

So far, we have discussed the regulation of the PA system at only two levels, gene transcription and mRNA stability. Many more processes must be involved as sites of regulation for controlling the final level of active proteins, such as translation, localization of proteins expressed, and stability of proteins. Especially with the emergence of microRNA in the horizon of gene regulation, study of translational regulation of the PA system may provide unexpected novel forms of regulation. It has been suggested that at least 10% of protein-coding genes are the target of microRNAs and, interestingly, PAI-1 mRNA-binding protein is one of them (247).

Finally, it is worth noting that the complexity emerging from studies of the regulation of PA system gene components is compatible with mounting evidence for the involvement of these “old” proteins in non-classical fibrinolytic functions, including brain plasticity, metabolic and hormonal disorders, and aging.

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