Cell-type specific DNA–protein interactions at the
tissue-type plasminogen activator promoter in human
endothelial and HeLa cells in vivo and in vitro

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ABSTRACT

Tissue-type plasminogen activator (t-PA) gene expression in human endothelial cells and HeLa cells is stimulated by the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) at the level of transcription. To study the mechanism of transcriptional regulation, we have characterized a segment of the t-PA gene extending from –135 to +100 by in vivo footprinting analysis [dimethyl sulphate (DMS) method] and gel mobility shift assay. In vivo footprinting analysis revealed changes in cleavage pattern in five distinct promoter elements in both endothelial cells and HeLa cells, including a PMA-responsive element (TRE), a CTF/NF-1 binding site and three GC-boxes, and an altered cleavage pattern of the TRE and CTF/NF-1 element after PMA treatment of HeLa cells. Although endothelial cells and HeLa cells differed in the exact G residues protected by nuclear proteins, in vitro bandshift analysis showed that nuclear protein binding to the t-PA promoter was qualitatively and quantitatively very similar in both cell types, except for the TRE. Protein binding to the TRE under non-stimulated conditions was much higher in human endothelial cells than in HeLa cells, and this TRE-bound protein showed a lower dissociation rate in the endothelial cells than in HeLa cells. In endothelial cells, the proteins bound to the TRE consisted mainly of the AP-1 family members JunD and Fra-2, while in HeLa cells predominantly JunD, FosB and Fra-2 were bound. The proteins bound to the other protected promoter elements were identified as SP-1 (GC-box II and III) and CTF/NF-1 (CTF/NF-1 binding site). After PMA treatment of the cells, AP-1 and SP-1 binding was increased two-fold in endothelial cell nuclear extracts and >20-fold in HeLa nuclear extracts. In the endothelial cells, all Jun and Fos forms (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2) were part of the AP-1 complex after PMA induction. In HeLa cells, the complex consisted predominantly of c-Jun and the Fos family members FosB and Fra-2. In the light of previous studies involving mutational analysis of the human and murine t-PA promoter our results underline an important role of the five identified promoter regions in basal and PMA-stimulated t-PA gene expression in intact human endothelial cells and HeLa cells. The small differences in DMS protection pattern and differences in the individual AP-1 components bound in endothelial cells and HeLa cells point to subtle cell-type specific differences in t-PA gene regulation.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) plays a key role in the dissolution of the fibrin matrix of thrombi and haemostatic plugs (1). t-PA catalyzes the conversion of the zymogen plasminogen into the active serine proteinase plasin, the enzyme that digests fibrin. Gene targeting and gene transfer studies have confirmed the significant role of t-PA-mediated plasminogen activation in maintaining vascular patency (2). Regulation of t-PA expression, both in vitro and in vivo, has therefore been the focus of many studies (3).

t-PA in the circulation originates predominantly from the vascular endothelium (3). In vitro studies using cultured human endothelial cells have demonstrated that activation of protein kinase C (PKC) with vasoactive compounds such as thrombin or histamine, or with the phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA) stimulates t-PA expression (4–7). Based on nuclear run-on transcription assays, t-PA expression is modulated by PMA at the level of transcription (8). A very strong induction of t-PA gene transcription with PMA was found in HeLa cells (9). Transient transfection experiments in HeLa cells using deletion mutants of the t-PA gene promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene revealed that two regions in the t-PA promoter (between positions –102 to –115 and +60 to +74) are critical for basal and PMA-stimulated t-PA promoter activity (9). To pursue the physiological significance of these studies, we performed in vivo footprinting analysis in control and PMA-treated human endothelial cells and HeLa cells to reveal the pattern of protein–DNA interactions in the intact cell, where the nucleic acid is complexed with chromosomal proteins to form chromatin. In addition, such studies may reveal cell type-specific differences between primary human endothelial cells and the established human cervical carcinoma cell line, HeLa. Gel mobility

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shift assays were performed to identify the nuclear proteins which interact with the various binding sites in the promoter region of t-PA as revealed by in vivo footprinting.

**MATERIALS AND METHODS**

**Materials**

Dimethyl sulphate (DMS) and piperridine were obtained from Fluka (Bornem, The Netherlands). Taq polymerase and RT-PCR were obtained from Pharmacia Biotech (Woerden, The Netherlands). -β-phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma (St Louis, MO). A stock solution of PMA (100 µM) was prepared in ethanol and kept at −20°C. The anti-c-Jun and anti-c-Fos rabbit polyclonal antibodies were a gift from Dr W. Oehler (Massachusetts Institute of Technology, Cambridge, MA) and Dr H.J. Rahmsdorf (Kernforschungszentrum, Karlsruhe, Germany), respectively. These antibodies preferentially recognize c-Jun and c-Fos, but also bind other members of the Jun and Fos protein family. Antibodies specific for the AP-1 proteins c-Jun, JunB, JunD, c-Fos and Fra-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CTF/NF-1 rabbit polyclonal antibodies were obtained from the Institut Pasteur, Paris. Anti-CTF/NF-1 rabbit polyclonal antibodies (10 µg) were obtained from Dr C. Pfarr and Dr M. Yaniv (Institut Pasteur, Paris). Anti-ELK rabbit polyclonal antibodies (1 µg) were obtained from Dr T. Oehler (Massachusetts Institute of Technology, Cambridge, MA) and Dr H.J. Rahmsdorf (Kernforschungszentrum, Karlsruhe, Germany). Anti-SP-1 and anti-AP-2 rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and had the following sequences: 5′-GGAGGATCCTTTTCTGTTG-3′ (random) sequence was used: 5′-GA-TCGAACTGACCGCCCGGCCCGT-3′; as a competitor oligodeoxynucleotide, the following sequences were used: 5′-GCCGTAGACCCCGGAGATCTGAAATC-3′ for the AP-1 promoter: to the primer-extended molecules was done

**DMS genomic footprinting**

Confluent cultures of HUVEC (486 cm²) or HeLa cells (162 cm²) were washed once with phosphate-buffered saline (PBS) (0.15 M NaCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) at room temperature, and then incubated with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were washed with ice-cold PBS and lysed in 10 mM Tris pH 7.7, 400 mM NaCl, 2 mM EDTA and 0.2% SDS. DNA was isolated by digestion with proteinase K (300 µg/ml, 37°C overnight), followed by phenol/chloroform extraction and ethanol precipitation (14). The DNA was dissolved in water to a final concentration of 1 µg/ml, and incubated with 10% piperidine for 30 min at 90°C. After ethanol precipitation, the samples were processed for ligation-mediated PCR (LMPCR) analysis. In vitro controls were obtained by the reaction of purified DNA with DMS as described by Maxam and Gilbert (15).

**LMPCR**

LMPCR was performed by the method described by Mueller and Wold (16). Elongation primer 1 (0.6 pmol, see Materials section) was annealed to 10 µg heat-denatured (3 min, 95°C) piperidine-cleaved DNA at 45°C for 30 min. Primer extension was then carried out with Sequenase version 2.0 for 15 min at 45°C by adding 8.8 µl of elongation mixture (20 mM MgCl₂, 20 mM DTT, 200 µM of dATP, dCTP, dGTP and dTTP, and 0.3 µl Sequenase version 2). The DNA polymerase was heat-inactivated by incubation at 67°C for 15 min. Ligation of the universal linker (100 pmol of annealed 25mer and 12mer oligodeoxynucleotide, see Materials section) to the primer-extended molecules was done.

**Cell culture experiments**

Endothelial cells from human umbilical cord veins (HUVEC) were isolated by the method of Jaffe et al. (11), and cultured as previously described (12). HUVEC were grown on fibronectin-coated dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) (20 mM), newborn calf serum (heat-inactivated; 10% v/v), human serum (10% v/v), heparin (5 IU/ml), endothelial cell growth supplement (150 µg/ml) (13), l-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). HeLa cells were grown in DMEM supplemented with HEPES (20 mM), fetal bovine serum (heat-inactivated; 8% v/v), l-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Both cell types were grown at 37°C under a 5% CO₂/95% air atmosphere, and the medium was replaced every 2–3 days. Subcultures were obtained by trypsin/ethylenedinitro-tetracetic acid disodiumsalt-dihydrate (EDTA) treatment at a split ratio of 1:3 for HUVEC and of 1:10 for HeLa cells. HUVEC were cultured for maximally three passages.

For experiments, confluent cultures were used and the cells were always re-fed the day before the experiment with incubation medium, i.e. for HUVEC: DMEM supplemented with human serum (10%), l-glutamine, penicillin and streptomycin; and for HeLa cells: DMEM supplemented with l-glutamine, penicillin and streptomycin. After incubation of the cells with incubation medium containing the appropriate concentration of PMA (i.e. 10 nM for HUVEC and 162 nM for HeLa cells) or stock solvent, the cells were used for in vivo footprint analysis or the preparation of nuclear extracts.
overnight at 15°C by adding 20 µl of 17.5 mM MgCl₂, 42.3 mM DTT and 125 µg/ml bovine serum albumin (BSA) and 25 µl of ligation mix (10 mM MgCl₂, 20 mM DTT, 3 mM ATP, 50 µg/ml BSA and 0.4 U/µl T₄ DNA ligase). After ethanol precipitation, the pellets were dissolved in 60 µl water. PCR amplification was performed in 10 mM Tris (pH 8.8), 40 mM NaCl, 5 mM MgCl₂, 10 pmol primer 2, 10 pmol 25mer linker primer (see Materials section), 10 U Taq polymerase, 0.2 mM of dATP, dCTP, dGTP and dTTP and 0.01% (w/v) gelatin in a total volume of 100 µl on a Perkin Elmer Thermocycler 9600. Twenty cycles of PCR (1 min 95°C, 2 min 64°C and 3 min 75°C) were performed. Subsequently, linear PCR was done with 2 pmol end-labelled elongation primer 3 (see Materials section), 5 U Taq polymerase, 2 µl 2.5 mM dNTP-mix. One PCR cycle (2 min 95°C, 2 min 66°C and 10 min 75°C) was performed. The PCR-amplified fragments were extracted with phenol/chloroform, ethanol precipitated, and then separated on a 6% (w/v) denaturing polyacrylamide-gel (15). The sequence gel was dried on Whatman 3MM paper, and radiolabelled DNA fragments were visualized by autoradiography.

### Preparation of nuclear extracts

For gel shift experiments confluent cultures of HUVEC (324 cm²) or HeLa cells (162 cm²) were rinsed twice with ice-cold PBS and lysed in 2 ml of lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 1 mM DTT, 0.25 mM vanadate and 1 µg/ml of the protease inhibitors leupeptin, pepstatin and aprotinin). The lysates were homogenized in a Potter (20 strokes); nuclei were collected by centrifugation (5 min at 1000 g, 4°C), and washed once with lysis buffer. The nuclear pellet was resuspended in 150 µl of 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis(oxethylenenitro)tetraacetate acid (EGTA), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25 mM vanadate and 1 µg/ml of leupeptin, pepstatin and aprotinin. Suspensions were incubated for 15 min at 4°C while being continuously shaken, and then centrifuged at 10000 g, 4°C for 5 min. Supernatants were stored at −80°C until use. The protein concentrations in the nuclear extracts were determined using the Bradford protein assay.

### Electromobility shift assay

Oligodeoxynucleotides were end-labelled using T₄-kinase and subsequently purified by phenol/chloroform extraction and ethanol precipitation. For the electromobility shift assay (EMSA), 25 fmol (~10⁴ c.p.m.) of labelled double-stranded oligodeoxynucleotide was mixed with nuclear extract (5 µg protein) in a total volume of 20 µl of 20 mM HEPES (pH 7.9), 20 mM KCl, 2 mM MgCl₂, 20% glycerol, 2.5 mM EDTA, 2 mM spermidine, 1 µg poly(dI-dC), 1 µg BSA and 1 mM PMSF. The mixture was incubated at 4°C for 30 min. All bandshifts were performed in the presence of a 100-fold excess of unlabelled nonhomologous DNA in order to prevent specific probe/protein interactions. Furthermore, all DNA–protein complexes were checked for the sequence specificity of the binding reaction by adding a 100-fold excess of the same, unlabelled, double-stranded oligodeoxynucleotide (100× competitor). For generation of supershifted complexes, the nuclear extracts were preincubated for 1 h at 4°C with the appropriate antiserum prior to the binding reaction. The antibodies against Fos and Jun were previously shown not to interfere with nuclear protein binding to the jun1 TRE and the SV-40 enhancer, respectively (17,18). The antibodies against SP-1 and CTF/NF-1 did not inhibit nuclear protein binding to the human t-PA GC-box I and to the t-PA TRE, respectively (data not shown). DNA–protein complexes were separated from the non-bound oligodeoxynucleotide by electrophoresis on a 5% polyacrylamide gel in 0.25TBE buffer (22.5 mM Tris-borate, 0.5 mM EDTA) (14). Electrophoresis was carried out at room temperature at 150 V for 70 min, using 0.25xTBE as running buffer. The gel was dried on Whatman 3MM paper, and DNA–protein complexes were visualized by autoradiography.

## RESULTS

### Genomic DMS footprinting of the t-PA promoter in HUVEC and HeLa cells

To study the interactions between nuclear proteins and the human t-PA promoter sequence in vivo in intact HUVEC and HeLa cells, we performed genomic DMS footprinting. Using the appropriate primers, the DNA–protein interaction sites of the t-PA promoter region between −130 and +100 were mapped. As shown in Figure 1 and summarized in Figure 2, only nine out of the 16 affected residues coincide in HUVEC and HeLa cells. However, all affected residues are clustered around the same five consensus sites for transcription factor binding. These five boxes consist of a PMA responsive element (TRE) between positions −112 and −104; a consensus site for the family of CCAAT-binding transcription factors, also referred to as nuclear factor 1 (CTF/NF-1) binding site, between positions −92 and −77; and three GC-boxes between positions −43 and −34, +39 and +45, and +62 and +68, which have homology to SP-1 and activator protein-2 (AP-2) binding sites. Although the affected residues are clustered around the same five boxes in HUVEC and HeLa cells, the observed differences in the pattern of protection suggest that the proteins bound in HeLa and HUVEC may be similar but are not necessarily identical.

PMA treatment of the cells did not markedly alter the DMS footprint pattern, except for that of the TRE-like region in HeLa cells (Fig. 1B). Thirty minutes of PMA treatment slightly induced protection of the G residue at −113 and strongly increased protection of the G residue at −104. After 3 h the protection of the G residue at −107 was further induced, and then remained unchanged up to 6 h. In contrast to HeLa cells, in HUVEC these residues were already contacted by protein under non-stimulated conditions, and PMA treatment had no marked effect on their methylation and subsequent cleavage. Finally, in HeLa cells PMA also affected binding in the CTF/NF-1-like region, reflected by an increase in hypersensitivity of the G residue at position −91 (Fig. 1B).

### Identification of nuclear proteins that bind to the t-PA promoter

To identify the nature of the proteins bound to the t-PA promoter, the DNA–protein interactions were studied in vitro by using the electromobility shift assay (EMSA). All five regions identified with the in vivo footprinting assay bound nuclear protein, and protein binding was qualitatively and quantitatively comparable in HUVEC and HeLa cells, except for the TRE-like binding site (Figs 3–7). The specific DNA–protein complex formed with the TRE-like sequence was far more abundant with nuclear extracts from HUVEC than from HeLa cells (Fig. 3), which is in...
Figure 1. In vivo DMS footprinting of the −136 to +100 5′-flanking region of the t-PA promoter in HUVEC and HeLa cells. HUVEC (A) or HeLa cells (B) were incubated with PMA for various time periods (as indicated) and used for in vivo footprint analysis, as described in the Methods section. Regions containing hypersensitive and protected G residues, as compared to the naked DNA control (N), are indicated with arrows (↑ and ↓, respectively). Nucleotide positions of the t-PA promoter sequence are shown by the numbers to the left.

agreement with the higher protection of this region in HUVEC in the in vivo footprint analysis (Fig. 1). Dissociation experiments showed that the association of protein to the TRE-like site was stable over a 20 min period when using nuclear extracts from HUVEC, while with nuclear extracts from HeLa cells the protein rapidly dissociated from the DNA (Fig. 4). PMA strongly induced protein binding with nuclear extracts from HeLa cells (>20-fold) but hardly further increased protein binding with nuclear extracts from HUVEC (~2-fold) (Fig. 3). This is consistent with the in vivo footprint data, which showed a stronger induction of protection in PMA-treated HeLa cells than in PMA-treated HUVEC. PMA did not alter the protein dissociation rate with nuclear extracts of either cell type (Fig. 4), indicating that the strong induction of protein binding activity in the nuclear extracts from PMA-treated HeLa cells is probably the result of an increase in protein levels.

TRE-like consensus sites bind transcription factors belonging to the families of the activator protein-1 (AP-1) or the cAMP responsive element binding (CREB) proteins, and also heterodimers formed between these two families like c-Jun/ATF-2 (17). We found that protein binding to the TRE-like region of the t-PA promoter in both HUVEC and HeLa nuclear extracts was strongly inhibited with polyclonal antibodies directed against the AP-1 family members Jun and Fos (Fig. 3). Apparently, this region of the t-PA promoter is bound by Jun/Fos heterodimers. Similar results were obtained with nuclear extracts of PMA-treated HUVEC (Fig. 3). The protein–DNA complex formed with nuclear extracts from PMA-treated HeLa cells was fully inhibited with anti-Fos polyclonal antibody, but only partially (about 50%) with anti-Jun polyclonal antibody (Fig. 3). To further characterize the composition of the AP-1 complexes bound to the t-PA TRE, we performed bandshift experiments with antibodies specifically recognizing the different AP-1 family members (Fig. 5). With nuclear extract from non-stimulated endothelial cells a marked decrease in AP-1 binding was observed after pre-incubation of the nuclear extract with anti-JunD and anti-Fra-2 antibodies, suggesting that complex formation of the antibody with JunD or Fra-2-containing dimers mostly interfered with DNA binding but hardly yielded the formation of ‘supershifted’ complexes. A slight but significant decrease was seen with anti-c-Jun, anti-JunB, anti-c-Fos, anti-FosB and anti-Fra1 antibodies (Fig. 5). After PMA treatment of the HUVEC, all AP-1 family members appear to contribute to a similar extent to the increase in AP-1 binding activity. The DNA binding of the AP-1 proteins present in untreated HeLa nuclear extracts could be reduced predominantly by anti-JunD, anti-FosB, and anti-Fra-2 antibodies, and, to a minor extent, by anti-c-Fos and...
mobility than DNA-bound AP-1 as shown in Figure 5 was found. The minor DNA–protein complex with a slightly lower mobility than DNA-bound AP-1, as shown in Figure 5 was found to contain c-Jun, JunD and ATF-2 in non-stimulated endothelial cells, and JunD and ATF-2 in non-stimulated HeLa cells. After incubation of both cell-types with PMA, a very weak binding of c-Jun/ATF-2 was found (data not shown). To learn whether the TRE-element of the t-PA promoter is indeed not efficiently bound by c-Jun/ATF-2, we performed parallel binding experiments with the two TREs present in the c-jun promoter. These two TREs have core sequences identical or very similar respectively to the TRE in the t-PA promoter, and have been shown previously to bind c-Jun/AP-2 effectively (17). The c-jun promoter TREs showed an over 30-fold stronger c-Jun/AP-2 binding activity with HeLa nuclear extracts than the t-PA TRE, indicating that the t-PA TRE has a relatively poor affinity for c-Jun/AP-2, possibly due to the influence of flanking sequences.

The CTF/NF-1-like binding site showed one DNA–protein complex with both HUVEC and HeLa nuclear extracts which was not altered after PMA treatment of the cells (Fig. 6). This complex could be competed for >80% with an anti-CTF/NF-1 polyclonal antibody and therefore apparently consists of DNA-bound CTF/NF-1 protein family members (Fig. 6).

Of the three GC-boxes identified, boxes II and III bound SP-1 (Fig. 7B and C) and box I was occupied by an unidentified protein using nuclear extracts from HUVEC and HeLa cells (Fig. 7A). Two SP-1 containing complexes were formed, except for GC-box II which, when incubated with nuclear extracts from HeLa cells, formed one SP-1 containing complex and one complex containing an unidentified protein. Since all DNA–protein complexes are partially inhibited with the anti-SP-1 antibody, binding of other nuclear proteins cannot be excluded. However, none of the GC-boxes bound any AP-2 protein although both HUVEC and HeLa cells expressed AP-2 (as assessed by EMSA with a consensus AP-2 binding site, data not shown). The protein–DNA complexes formed with all three GC-boxes were strongly induced when using nuclear extracts from PMA-treated HeLa cells, but hardly induced (up to 2-fold) with nuclear extracts from PMA-treated HUVEC (Fig. 7).
DISCUSSION

Previous investigations of the regulation of the human t-PA gene by transient transfection assays in HeLa cells employing deletion mutants of the t-PA gene promoter demonstrated that the DNA elements which regulate constitutive and PMA-stimulated expression are encoded by sequences downstream of position –115 of the t-PA gene (9). In this study, we further characterized the –135 to +100 region of the human t-PA promoter for persistent and PMA-inducible DNA–protein interactions in cultured vascular endothelial cells and HeLa cells. In vivo genomic footprinting analysis revealed five distinct protein binding elements in both endothelial cells and HeLa cells, corresponding to a PMA responsive element (TRE; –112 to –104), a CTF/NF-1 binding site (–92 to –77) and three GC-boxes (–43 to –34, +39 to +45, and +62 to +68). After PMA treatment of HeLa cells, the G residues of the TRE consensus sequence (–113, –107 and –104) were less susceptible to methylation, reflecting enhanced protein binding. In contrast to HeLa cells, these residues were already fully occupied by protein under non-stimulated conditions, and PMA treatment had no marked effect on their methylation and subsequent cleavage. The subtle differences between endothelial and HeLa cells in the DMS footprint pattern of the TRE consensus sequence are reflected in subtle differences in the composition of the bound protein complexes. In human endothelial cells, the proteins bound to the TRE consisted mainly of the AP-1 family members JunD and Fra-2, while in HeLa cells predominantly JunD, FosB and Fra-2 were bound. Also, the t-PA TRE sequence was bound much more efficiently by the AP-1 complexes from endothelial cells than from HeLa cells.

After PMA treatment of the endothelial cells, all Jun and Fos forms (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2) contributed to the AP-1 complex, while in HeLa cells, the AP-1 complex consisted predominantly of c-Jun and the Fos family members FosB and Fra2. Since the various members of the Jun and Fos families of transcription factors differ in their transactivation potential (19), these differences may be involved in cell-type specific differences in t-PA expression. Our results show also subtle differences in binding of nuclear proteins distinct from AP-1 to the t-PA promoter in human endothelial and HeLa cells, both in respect to the DMS protection pattern and to the transcription factors bound. The identified transcription factors bound to the CTF/NF-1 site and GC-boxes II and III included CTF/NF-1 and SP-1, respectively, in both cell types. Like for the AP-1 binding site, the presence of subtle differences in the DMS footprint pattern of the CTF/NF-1 binding site may point at cell-type specific differences in the CTF/NF-1 family members bound, since the CTF/NF-1 family exists of a large group of related transcription factors (20).

The lower dissociation rate of the DNA-bound AP-1 complex in human endothelial cells as compared to HeLa cells may be due to ...
to differences in posttranslational modification (i.e. phosphorylation) of the AP-1 proteins (19) or to a different activity of the AP-1 inhibitory protein IP-1 (21). IP-1 function is blocked by protein kinase A (PKA) activation, which also further enhances t-PA transcriptional induction by PMA (22,23). We observed, however, no increase in AP-1 binding activity after treatment of both cell-types with the cAMP-raising compound forskolin (Arts et al., unpublished data), which argues against a role of IP-1 in the regulation of AP-1 binding activity. However, at present the existence of other cellular proteins modulating the DNA binding activity of AP-1 which are not regulated by PKA-dependent signalling cannot be excluded.

Two of the five protein binding sites, the TRE and GC-box III, were also reported by Medcalf et al. (9) to be essential for basal and PMA-induced t-PA promoter activity in HeLa cells on the basis of mutational analysis. We found, using gel-shift assays, that nuclear protein binding to each of these sites was strongly induced in PMA-treated HeLa cells (~20-fold) and ~2-fold in human endothelial cells, which parallels the difference in transcriptional induction of t-PA by PMA in HeLa cells and HUVEC (6,9). In contrast to the increase in nuclear protein binding to the TRE, the enhanced nuclear protein binding to GC-box III was not reflected in a change in the cleavage pattern of the G residues of this region in the in vivo footprint, possibly because these G residues were already optimally accessible for methylation, or because of a high rate of exchange of bound proteins to this sequence.

Our observation that GC-boxes II and III bind SP-1 protein is in line with previous reports that human t-PA transcription predominantly initiates from a TATA-less promoter at position +110 (24). Such TATA-less promoters depend on SP-1 for the recruitment of the transcription initiation complex (25). Additional evidence for an important role of SP-1 in t-PA transcription is provided by the study of Medcalf et al. (9) who reported a strict correlation between nuclear protein binding to GC-box III (i.e. SP-1) and t-PA expression in different cell-types: a high nuclear protein binding and t-PA expression in Bowes melanoma cells, intermediate in HeLa cells and hardly detectable nuclear protein binding and no t-PA expression in HepG2 cells (9).

Our finding that the GC-box at +60 in the human t-PA promoter binds SP-1, is in contrast to reports suggesting that GC-box III is an AP-2 binding site. This suggestion, however, was based on experiments which showed competition of GC-box III nuclear protein binding by a consensus AP-2 binding site (9). Since a consensus AP-2 binding site is also capable of binding SP-1 protein (Arts and Kooistra, unpublished data), these experiments are not directly indicative of AP-2 binding. Similarly, the GC-boxes in the murine t-PA promoter have also been reported to lack affinity for AP-2 (26).

Our structural analysis of the human t-PA promoter extends previous studies by the identification of three additional protein binding sites (CTF/NF-1 and GC-boxes I and II), which were previously not detected by transfection and in vitro footprinting techniques (9,27,28). The exact role of these elements in human t-PA transcription remains unclear at present and needs to be established by mutational analysis and transfection experiments. They are likely to be important in t-PA expression, however, since deletion and/or mutation of these sites in the very homologous murine t-PA promoter hampered t-PA transcription (20,29–31).

In conclusion, our studies on the identification of DNA–protein interactions at the t-PA promoter in intact human cells did not only confirm the in vivo presence of interactions previously detected in vitro, but also identified three additional protein binding sites. In addition, our experiments showed the existence of subtle differences in DNA–protein interactions between HUVEC and HeLa cells, which may be essential for appropriate transcriptional control of t-PA in different physiological contexts.

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