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Involvement of Sp1 in basal and retinoic acid induced transcription of the human tissue-type plasminogen activator gene

Pascal Merchiers, Frank Bulens, Astrid De Vriese, Désiré Collen, Alexandra Belayew*

Center for Molecular and Vascular Biology, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium

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Abstract Transcription of the human tissue-type plasminogen activator (t-PA) gene is regulated by a multi-hormonal responsive enhancer at −7 kb. Transient co-transfections of *Drosophila* SL2 and human HT1080 fibrosarcoma cells with *t-PA* reporter constructs showed that Sp1 and Sp3 activate the *t-PA* promoter. Moreover Sp1 (but not Sp3) binding to the promoter is involved in induction by retinoic acid (RA), a response mediated through the enhancer. The role of Sp1 is specific, since mutation of the *CRE* element in the promoter did not affect response to RA. In contrast, the glucocorticoid induction mediated by the enhancer is independent of these *Sp1* and *CRE* elements.

Key words: Tissue-type plasminogen activator; Promoter-enhancer; Sp1; Retinoic acid receptor

1. Introduction

The conversion of plasminogen to plasmin was initially identified as the key event in fibrinolysis, a process that maintains vascular patency [1]. Since then, the plasminogen-plasmin system has also been implicated in other (patho)physiological functions, like cancer, restenosis, embryonic development, ovulation and certain brain processes [2]. The system is activated via the serine proteinases urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), which is the main activator during fibrinolysis [1]. Studies with t-PA deficient mice underscored its involvement in brain processes like long term potentiation and excitotoxin induced neuronal cell death in the hippocampus [3,4].

The human *t-PA* gene promoter contains two major transcription start sites, which are dependent on a *TATAA* box and an initiator element, respectively [5,6]. Transcription of the human *t-PA* gene is regulated through a cAMP responsive like element (*CRE* like), which mediates induction by protein kinase C (PKC) [6]. An in vivo footprinting analysis of the human *t-PA* promoter in HeLa cervix carcinoma cells and in human umbilical vein endothelial cells revealed the presence of two *Sp1* binding sites (*Sp1c*: bp −72 to −66; *Sp1d*: bp −48 to −39), just upstream from the initiator element, a situation which is often associated with TATAA less promoters [7]. Sp1 binding to these sites is assumed to attract the RNA polymerase II pre-initiation complex through a tethering factor or through specific interactions with the TATAA box binding protein (TBP) or TBP associated factors [8,9]. The Sp1-related transcription factor Sp3 also interacts with GC or GT boxes and depending on the promoter context, can either repress or activate transcription, or even cooperate with Sp1 [10].

The hormonal regulation of the human *t-PA* gene transcription is mediated through an enhancer located at −7.2 kb from the initiator element. This enhancer confers a response to all steroid hormones, except estrogens, and to retinoic acid (RA) [11,12]. The steroid hormone response is mediated through a hormone responsive unit consisting of four glucocorticoid responsive elements (*GRE*) located at bp −7960, −7942, −7703 and −7501. In contrast, only one retinoic acid responsive element, which comprises a *DR5* element located at bp −7319, mediates the induction of the human *t-PA* gene by RA.

An important issue concerning enhancer action is enhancer/promoter selectivity which allows enhancers to discriminate between different promoters [13]. In order to confer the hormonal stimulus, the far upstream *t-PA* enhancer must be able to induce the *t-PA* promoter activity in a specific way. This might be achieved through interactions between transcription factors bound to the *t-PA* enhancer and to the *t-PA* promoter. In the present study, we have investigated whether the induction by RA and dexamethasone (DEX), a synthetic analogue of glucocorticoids, both mediated through the enhancer, required the Sp1 binding sites previously characterized in the human *t-PA* promoter.

It is shown that Sp1 and Sp3 bind to the *t-PA* promoter and induce promoter activity in SL2 *Drosophila* cells, devoid of endogenous Sp1 and Sp3. Transient transfection studies in these SL2 cells and in HT1080 fibrosarcoma cells showed that the RA but not the DEX induction was dependent on the two *Sp1* sites present in the human *t-PA* promoter.

2. Materials and methods

2.1. Reagents

Human HT1080 fibrosarcoma and SL2 *Drosophila* cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and from Dr. W. Wahli (Institut de Biologie Animale, Université de Lausanne, Switzerland) respectively. *t-PA520CREmut-CAT* was a gift from Dr. R. Medcalf (Department of Medicine, Box Hill Hospital, Victoria, Australia). The polyclonal antibody against Sp3, the insect cell expression vectors for Sp1 (*pPacSp1*), Sp3 (*pPacSp3*), and GR (*pPacGR*), the reporter constructs *(GRE)$_6$Adh-CAT* and *pPadhβGal* and the bacterial expression vector for the Sp1 DNA binding domain (*pET3b/Sp1BamHI*) were obtained from Dr. G. Süske (Institut für Molekularbiologie und Tumorforschung, Philips Universität Marburg, Marburg, Germany). Insect cell expression vectors for RAR (*pPacRAR*) and RXR (*pPacRXR*) were a gift from Dr. N. Gill (Division of Endocrinology and Metabolism, University of California, San Diego, CA, USA).

Dulbecco's modified Eagle's medium (DMEM) and all medium

*Corresponding author. Fax: (32) (16) 34 59 90.
E-mail: alexandra.belayew@med.kuleuven.ac.be

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supplements were purchased from Life Technologies, Inc. (Gent, Belgium), tissue culture recipients from Corning, Inc. (New York, USA) and Becton Dickinson (Franklin Lakes, NJ, USA), retinoic acid (RA), dexamethasone (DEX) and chloramphenicol from Sigma (St. Louis, MO, USA). DNA purification columns from Qiagen (Chatsworth, CA, USA), acetyl-CoA and [^{3}H]acetyl-CoA from ICN Biomedicals (Costa Mesa, CA, USA), Lipoluma from Lumac-LSC (Olen, Belgium), luciferin substrate, the full length Sp1 protein and the Erase a Base system from Promega (Madison, WI, USA). Bradford reagent was purchased from BioRad (Hercules, CA, USA), Galacto Light system from Tropix, Inc. (Bedford, MA, USA), oligonucleotides and poly(dIdC)·poly(dIdC) from Amersham Pharmacia Biotech (Gent, Belgium). Polyclonal antibodies directed against the Sp1 protein was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Reporter constructs

Isolation of *t-PA* upstream sequences and their incorporation in chloramphenicol acetyltransferase (CAT) reporter plasmids has been described previously [11]. All genomic sequences analyzed in this study are numbered relative to the transcription start site of Henderson and Sleigh, 1992 [5]. All reporter constructs have a modified *pBLCAT3* backbone obtained by deletion of the cryptic *AP1* site, present in the latter backbone, by a *Hin*dIII-*Aat*II digest, resulting in *pBLCAT3/ΔAP1*. For the *t-PA632-CAT/ΔAP1* construct, fusion to the *CAT* gene was done at bp +197 of the *t-PA* gene and the 5′ endpoint coordinate is −632. Introduction of mutations in reporter constructs was based on the polymerase chain reaction (PCR). The mutations in the *Sp1c* and/or *Sp1d* sites in *t-PA632-CAT/ΔAP1* were introduced with the *Sp1c/dmut*, *Sp1cmut* or *Sp1dmut* primers. Sequences of the oligonucleotides used in the mutagenesis are listed in Table 1. The 5′ progressive deletion mutants of the *t-PA* promoter were obtained by 5′ exonuclease III digestion of *t-PA632-CAT/ΔAP1* (Erase a Base system). In these constructs the human *t-PA* promoter is fused at bp +197 to the *CAT* gene, and the 5′ deletion endpoints are as indicated: *t-PA490-CAT/ΔAP1*, *t-PA242-CAT/ΔAP1*, *t-PA205-CAT/ΔAP1*, *t-PA163-CAT/ΔAP1*, *t-PA110-CAT/ΔAP1*, *t-PA39-CAT/ΔAP1*. The *t-PA* enhancer (*t-PA2.4*; bp −9578 to −7144) was cloned as a *Hin*dIII fragment in front of t-*PA632-CAT/ΔAP1*, *t-PA632Sp1c/dmut-CAT/ΔAP1* and *t-PA520CREmut-CAT/ΔAP1*.

All mutations were confirmed by cycle sequencing with the ABI Prism 310 Genetic Analyzer (Perkin Elmer, Zaventem, Belgium). Generated sequences were analyzed with the GCG software at the Belgian EMBNET Node (BEN).

2.3. Cell culture and transfection

HT1080 cells were maintained in supplemented DMEM, containing glutamine (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat inactivated fetal calf serum. For transfection, cells were seeded at a density of $2–4\times10^4$ cells/cm^2 and grown overnight at 37°C in a humidified 95% air, 5% CO_2 atmosphere in medium with 5% heat inactivated charcoal treated fetal calf serum. The calcium phosphate co-precipitation method was applied to a 6-well dish using a DNA mixture of 20–60 μg of reporter plasmid and where mentioned, with 1 μg of *pRSVluc*. Cells were treated with the indicated hormone 12 h after the glycerol shock. SL2 *Drosophila* cells were maintained in M3 medium supplemented with nystatin (10 U/ml), glutamine (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat inactivated insect cell qualified fetal calf serum at 28°C. For transfection, SL2 cells were seeded at a density of 2.5×10^5 cells/cm^2 in M3 medium supplemented as described above but with 5% heat inactivated insect cell qualified fetal calf serum. The calcium phosphate co-precipitation method was used with 16 μg of reporter construct, 1 μg of each of the pPac expression vectors and 1 μg of pPadhβGal for three wells of a 6-well plate. This DNA mixture was adjusted to 20 μg with empty pPac expression vector. Cells were treated with the indicated hormones 12 h after transfection. DEX was dissolved in ethanol at a concentration of 10^{-3} M and stored at −80°C. RA was dissolved in dimethylsulfoxide at a concentration of 10^{-2} M and stored at −80°C. The appropriate concentration was added to the medium in a volume corresponding to 0.1% of the culture medium. Control medium contained an equal amount of excipient.

After transfection, cell extracts were prepared by three freeze-thaw cycles in 100 mM Tris-HCl pH 7.8, 5 mM EDTA. CAT activity was determined by the liquid scintillation method [14]. Luciferase or β-galactosidase activity, used as an indicator for the transfection efficiency, were measured in a Berthold luminometer after addition of the luciferin or the Galacton substrate respectively. All data shown represent values obtained from at least two independent experiments, each performed in triplicate ($n=6$ or 9) and for which at least two different plasmid preparations were used. All data are depicted as mean ± S.E.M.

2.4. Electrophoretic mobility shift assay

HT1080 nuclear extract was prepared as described [12]. The Sp1 DBD was expressed into BL21DELysS bacteria from the *pET3b/Sp1BamHI* expression vector, and purified as described [15].

Sequences of the oligonucleotides used in electrophoretic mobility shift assay are listed in Table 1. Twenty pmol of DNA oligonucleotides were labeled by the T4 polynucleotide kinase in the presence of [γ^{32}P]-ATP and purified after electrophoresis on a 20% polyacrylamide gel. HT1080 cell nuclear extract and Sp1 DBD were incubated for 25 min at room temperature in the binding buffer (10 mM Tris pH 8, 1 mM dithiotreitol, 500 μM EDTA, 80 mM KCl, 0.1% Triton X-100, 1μg BSA, 0.5 μg poly(dIdC), 12% glycerol) containing 20 000 cpm of the labeled oligonucleotide. HT1080 nuclear extract was pre-incubated with polyclonal antibodies directed against Sp1 or Sp3 for 10 min at room temperature. All competitor oligonucleotides were added simultaneously with the labeled oligonucleotide in the indicated molar excess.

The samples were loaded on a 4% polyacrylamide gel and separated by electrophoresis at 4°C for 90 min in 0.5×Tris-borate buffer. Gels were dried and the bands were visualized by overnight autoradiography at −80°C.

3. Results

3.1. Interaction of Sp1 and Sp3 with sequences in the human t-PA promoter

RA and DEX were previously shown to induce *t-PA* gene transcription in HT1080 fibrosarcoma cells [11]. In order to evaluate a role of the proximal promoter in these responses, we first investigated whether nuclear extracts from these cells contained proteins binding the two *Sp1* sites (*Sp1c* and *Sp1d*) previously identified in the t-PA promoter [7].

As a control, electrophoretic mobility shift assay (EMSA) was performed with the bacterially expressed Sp1 DNA binding domain (Sp1DBD) and a radiolabeled oligonucleotide comprising the *Sp1d* sequence (Fig. 1A, lanes 2–6). The complex formed was specific since it was competed by an oligonucleotide comprising the Sp1 consensus sequence, but not by an oligonucleotide comprising the unrelated NF-I sequence (lanes 3, 4 and 5, 6). Several protein-DNA complexes were observed when a similar experiment was performed with HT1080 nuclear extracts (Fig. 1A, lanes 7–13). The presence of Sp1 and Sp3 in the two slower migrating complexes was

Table 1
Oligonucleotides

Name	Sequence
Sp1c	ACAGAAA**CCCGCCC**AGCCGGGG
Sp1cmut	ACAGAAA<u>A</u>CGC<u>A</u>CAGCCGGGG
Sp1d	CCACCGAC**CCCACCC**CCTGCCTG
Sp1dmut	CCACCGAC<u>ACT</u>A<u>GT</u>CCCTGCCTG
Sp1c/d	ACAGAAA**CCCGCCC**AGCCGGGGCCACCGAC**CC-CACCC**CCTGCCTG
Sp1c/dmut	ACAGAAA<u>A</u>CGC<u>A</u>CAGCCGGGGCCACCGAC<u>ACT</u>A<u>GT</u>-CCCTGCCTG
Sp1cons	ATTCGATCGGGGCGGGGCGAGC

Sequences of the oligonucleotides used in the electrophoretic mobility shift assays or to introduce site specific mutations in *t-PA632-CAT/ΔAP1*.
Putative *cis*-elements are represented in bold in the wild-type sequences. Introduced mutations are underlined.

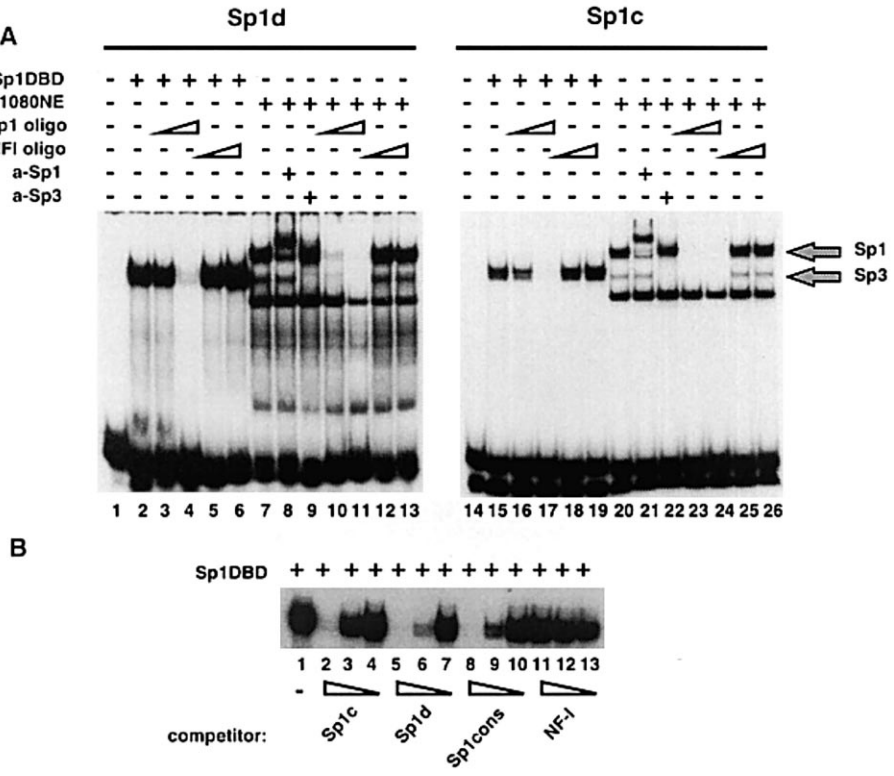

Fig. 1. A: Binding of Sp1 and Sp3 to the *Sp1c* and *Sp1d* oligonucleotides. EMSA was performed with the indicated ^{32}P-labeled oligonucleotides and with the Sp1 DBD (lanes 2–6, 15–19) or HT1080 nuclear cell extract (lanes 7–13, 20–26) as described in Section 2. The Sp3- and Sp1-DNA complexes are indicated by arrows. Competition experiments were performed with 500-fold (lanes 4, 6, 11, 13, 17, 19, 24, 26) or 100-fold (lanes 3, 5, 10, 12, 16, 18, 23, 25) molar excess of unlabeled oligonucleotide. B: Comparison of Sp1 affinity for *Sp1c* and *Sp1d*. Binding of Sp1DBD to the oligonucleotide comprising the *Sp1* consensus site was competed with non-labeled oligonucleotides comprising the *Sp1c*, the *Sp1d*, the *Sp1 cons* (consensus) and the unrelated *NF-I* sequence. A 500-fold (lanes 2, 5, 8, 11), 100-fold (lanes 3, 6, 9, 12) or 10-fold (lanes 4, 7, 10, 13) molar excess of competitor was used. DNA-protein complexes are shown.

shown by co-incubation with specific antibodies (Fig. 1A, lanes 8, 9). Specificity of these interactions was shown by competition as above (Fig. 1A, lanes 10–13). Similar results were obtained for the radiolabeled *Sp1c* oligonucleotide (Fig. 1A, lanes 15–26), although the complexes observed were weaker. Binding of the Sp1DBD protein to the *Sp1* consensus oligonucleotide was challenged by competition with unlabeled *Sp1c* or *Sp1d* oligonucleotides in EMSA (Fig. 1B). Addition of a 100-fold excess of *Sp1c* (Fig. 1B, lane 3) was less efficient than addition of *Sp1d* (Fig. 1B, lane 6), suggesting a higher affinity of Sp1 for *Sp1d* than for *Sp1c*.

In conclusion, Sp1 and Sp3 present in HT1080 nuclear extracts interact in vitro with two sequences (*Sp1c* and *Sp1d*) located just upstream from the initiator element in the *t-PA* promoter.

3.2. Involvement of Sp1 and Sp3 in human t-PA promoter basal activity

To assess the importance of Sp1 and Sp3 for basal *t-PA* promoter activity, SL2 *Drosophila* cells which are devoid of these transcription factors, were transiently transfected with a reporter construct containing the *t-PA* promoter (*t-PA632-CAT/ΔAP1*) and expression vectors encoding Sp1 and Sp3. In the absence of Sp1 or Sp3, no *t-PA* promoter activity was detected in SL2 cells. A 16 ± 1.2-fold induction of CAT activity was observed in the presence of Sp1 compared to its absence, while co-expression of Sp3 yielded a 5 ± 0.1-fold activation (Fig. 2A). Simultaneous expression of Sp1 and Sp3 led to a synergistic activation of *t-PA632-CAT/ΔAP1* (40.5 ± 1.5-fold).

Site specific mutations of *Sp1c* and *Sp1d* (*Sp1c/dmut*) completely abolished Sp1 binding as shown when EMSA was performed with a radiolabeled oligonucleotide encompassing the two sites and purified Sp1 (Fig. 2B). These mutations were introduced in the reporter vector (*t-PA632Sp1c/dmut-CAT/ΔAP1*) leading to a 2-fold reduction of the activation by Sp1, but not affecting response to Sp3 (Fig. 2A). The synergistic induction by Sp1 and Sp3 was strongly affected indicating that the *Sp1c* and *Sp1d* sites were involved. The reporter vectors carrying either or both *Sp1c* and *Sp1d* mutations were then evaluated in transient transfection of HT1080 cells revealing a 60–70% reduction in basal t-PA promoter activity (Fig. 2C).

Since mutation of the *Sp1c* and *Sp1d* sites did not result in complete suppression of the Sp1 activation in the SL2 cells, progressive deletion mutants of the *t-PA* promoter were used to search for additional Sp1 responsive elements (Fig. 3). As expected, Sp1 activation of *t-PA-CAT* reporter constructs was strongly affected by deletion of the *Sp1c* and *Sp1d* sites (*t-PA39-CAT/ΔAP1* versus *t-PA110-CAT/ΔAP1*). In addition, a 3- to 4-fold decrease in Sp1 activation was observed upon deletion of bp −242 to −205 (*t-PA242-CAT/ΔAP1* versus *t-PA205-CAT/ΔAP1*), a region encompassing the previously identified *CRE* like element. Several complexes were formed in EMSA between HT1080 nuclear extract and an oligonucleotide covering this region, however they did not include

Sp1, nor could purified Sp1 bind to this sequence (data not shown). Further studies are required to elucidate this phenomenon.

In conclusion, these data demonstrate that Sp1 and Sp3 activate *t-PA* promoter activity in SL2 insect cells both individually and synergistically. Mutations of the *Sp1c* and *Sp1d* sites reduce the activation by Sp1 in SL2 cells and the basal promoter activity in HT1080 cells.

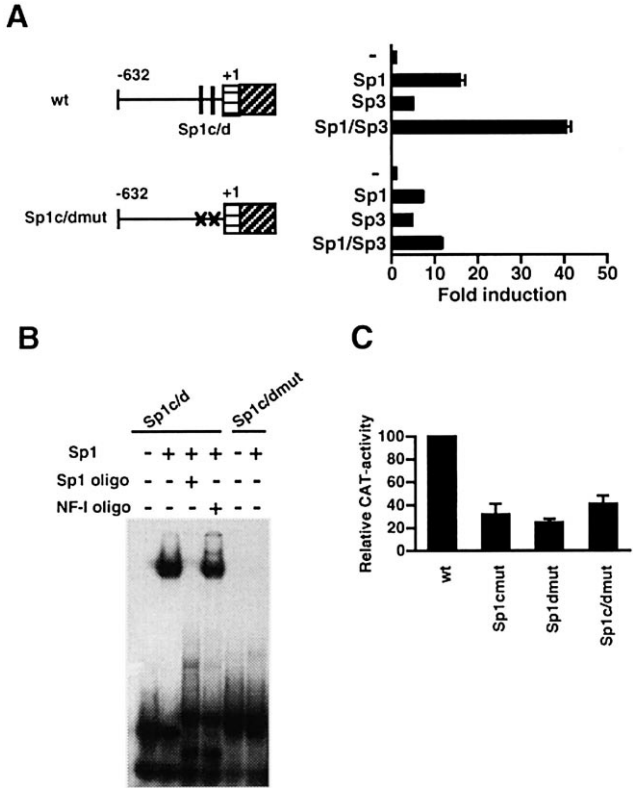

Fig. 2. Activation of the human *t-PA* promoter by Sp1 and Sp3. A: Right panel: schematic representation of the *t-PA* reporter constructs. The *Sp1c/d* sites and mutations are indicated with a bold bar and crosses respectively. Left panel: SL2 cells were co-transfected with 4 μg of either *t-PA632-CAT/ΔAP1* (wt) or *t-PA632Sp1c/dmut-CAT/ΔAP1* (Sp1c/dmut) reporter vector, and with 250 ng of expression vector either without insert (−) or with the Sp1 or Sp3 cDNA as indicated. For the synergy study, 125 ng of Sp1 and Sp3 expression vector were added simultaneously (Sp1/Sp3). In all cases, 250 ng of pPadhβgal were added, allowing correction for transfection efficiency. Cells were harvested after 24 h and CAT and β-galactosidase activities were determined. The fold induction of CAT activity in the presence of Sp1 and/or Sp3 compared to the CAT activity in their absence is represented. B: Evaluation of the *Sp1c/dmut* sequence for Sp1 binding. EMSA was performed with ^{32}P-labeled *Sp1c/*>it>d and *Sp1c/dmut* oligonucleotides and the full length purified Sp1 protein (Promega) as described in Section 2. Competition was performed with 500-fold molar excess of competitor (Sp1 consensus or NF-I oligonucleotides). C: Involvement of the *Sp1c* and *Sp1d* sites in *t-PA* promoter activity in human cells. HT1080 cells were transiently transfected with the *t-PA632-CAT/ΔAP1* reporter vector either wild-type (wt) or carrying mutations in the *Sp1c* or *Sp1d* site, or both sites as indicated. In all cases, *pRSVluc.* was added, allowing correction for transfection efficiency. Cells were harvested after 36 h and CAT and luciferase activities were determined. Data correspond to CAT activities relative to the wild-type construct. Details are provided in Section 2.

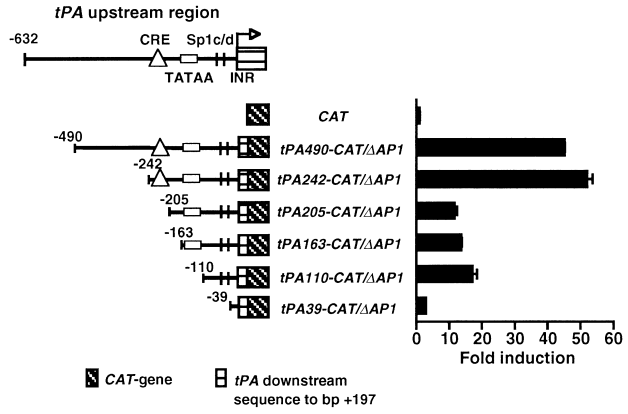

Fig. 3. Delineation of the regions involved in the Sp1 activation of the *t-PA* promoter. Left panels: Schematic representation of the reporter constructs, with the *cis*-elements *Sp1c*, *Sp1d* and *CRE* indicated. SL2 cells were transfected with 4 μg of the indicated reporter constructs, 250 ng of Sp1 expression vector and 250 ng of pPadhβ gal. After 24 h cells were harvested and CAT and β-galactosidase activities were determined. Fold induction of the CAT activity in the presence versus absence of Sp1 is represented. Correction for transfection efficiency was performed via the β-galactosidase activity.

3.3. Involvement of Sp1c and Sp1d in the hormonal response of the t-PA enhancer

We then evaluated whether the *Sp1c* and *Sp1d* sites in the *t-PA* promoter played a role in the hormonal inductions medi-

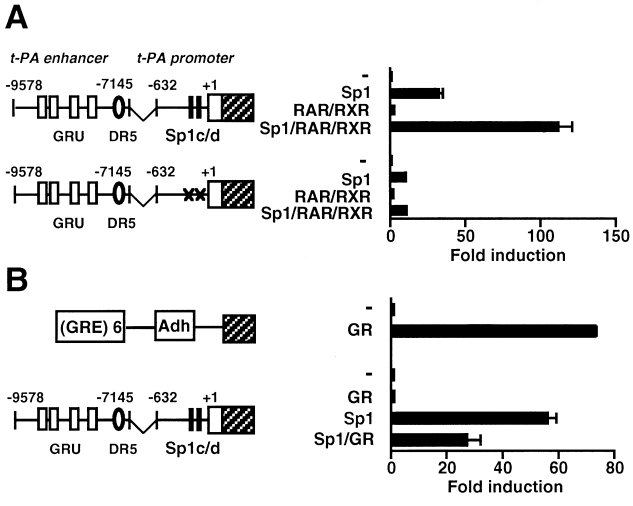

Fig. 4. Role of Sp1 binding sites in the *t-PA* promoter for hormonal induction mediated by the *t-PA* enhancer. Left panels: Schematic representation of the reporter constructs, with the *cis*-elements *Sp1c*, *Sp1d*, *DR5* the glucocorticoid responsive unit (*GRU*) and mutations are indicated by vertical lines, an oval, squares and crosses. SL2 cells were transfected with 4 μg of either *t-PA2.4-632-CAT/ΔAP1* or *t-PA2.4-632Sp1c/dmut-CAT/ΔAP1* (panel A), and either *$(GRE)_6$-Adh-CAT* or *t-PA2.4-632-CAT/ΔAP1* (panel B). Co-transfection was performed with 250 ng of pPadhβgal and 250 ng of the indicated expression vectors. The total amount of expression vector was adjusted to 750 ng with empty expression vector for each experiment. The following day the cells were stimulated for 24 h with 10^{-7} M RA (panel A) or with 10^{-7} M DEX (panel B). Cells were harvested and CAT and β-galactosidase activities were determined. The data represent the fold induction in the presence versus absence of the indicated expression vectors. Correction for transfection efficiency was performed with β-galactosidase activity values.

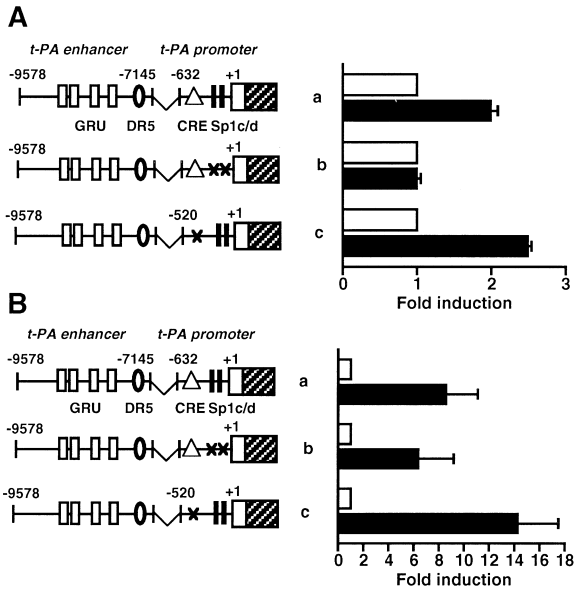

Fig. 5. Role of Sp1 elements present in the *t-PA* promoter for induction by RA and DEX mediated by the *t-PA* enhancer in human cells. Left panels: Schematic representation of the reporter constructs. Right panels: HT1080 cells were transfected with reporter vectors *t-PA2.4-632-CAT/ΔAP1* (a), *t-PA2.4-632Sp1c/dmut-CAT/ΔAP1* (b) and *t-PA2.4-520-CAT/ΔAP1* (c). Cells were grown for 24 h with either control medium, 10^{-7} M RA (panel A) or 10^{-7} M DEX (panel B). Cells were harvested and CAT activity was determined. Results are represented as fold induction of the CAT activity in hormone treated versus control cells.

ated by the *t-PA* enhancer. A reporter construct containing the *t-PA* enhancer linked to the *t-PA* promoter (*t-PA2.4-632-CAT/ΔAP1*) was evaluated in SL2 cells: as expected, co-transfection with an expression vector encoding Sp1, led to a 32.5 ± 2.7 basal activation (Fig. 4A). Co-expression of retinoid receptors RAR and RXR and addition of 10^{-7} M RA did not affect *t-PA2.4-632-CAT/ΔAP1* activity. However, further addition of an Sp1 expression vector allowed induction of the CAT activity 111.9 ± 9.3-fold, showing that the induction by RA was completely dependent on the presence of Sp1. Mutation of the *Sp1c/d* sites in the *t-PA* promoter reduced the basal activation by Sp1, as already shown (Fig. 2A), but also completely eliminated induction by RA (Fig. 4A), underscoring the role of *Sp1c* and *Sp1d*. In contrast, co-expression of Sp3, RAR and RXR only showed a 5.8 ± 0.1-fold induction after RA treatment compared to a 4.5 ± 0.15-fold induction for co-expression of Sp3 alone, indicating that binding of Sp3 to the human *t-PA* promoter is not sufficient for the RA induction mediated by the *t-PA* enhancer.

A similar analysis was performed to evaluate a role of Sp1 in response to glucocorticoids (DEX). No induction by 10^{-7} M DEX, but a repression of the activation by Sp1 was observed for *t-PA2.4-632-CAT/ΔAP1* upon co-expression of Sp1 and the glucocorticoid receptor (GR) (Fig. 4B). The positive control *$(GRE)_6$-Adh-CAT*, which contains six glucocorticoid response elements in front of the insect *Adh* promoter, was strongly induced in the same conditions, indicating the functionality of the GR expression vector.

The impact of the *Sp1c/d* mutations on the RA responsiveness of the *t-PA* reporter construct was then evaluated in human HT1080 cells. Transient transfection of the HT1080 cells with *t-PA2.4-632-CAT/ΔAP1* led to a 2 ± 0.09-fold induction upon treatment with 10^{-6} M RA (Fig. 5A, a). In contrast, no effect of RA could be observed on transient expression of *t-PA632Sp1c/dmut-CAT/ΔAP1* (Fig. 5A, b). The Sp1 requirement for the induction by RA was specific since mutation of the cyclic AMP response element (*CRE*) in the *t-PA* promoter (*t-PA2.4-520CREmut-CAT/ΔAP1*) did not affect the RA response (2.5 ± 0.04-fold). In contrast, the *Sp1c/d* mutations did not affect the induction by DEX of *t-PA2.4-632-CAT/ΔAP1* (Fig. 5B).

In conclusion, transient transfection analysis in SL2 and HT1080 cells showed that the *Sp1c* and *Sp1d* sites are required for the induction by RA, but not by DEX, although both responses are mediated through the *t-PA* enhancer. In contrast, a functional *t-PA CRE* is not required for the RA induction in HT1080 cells.

4. Discussion

In the present study we first showed that binding of Sp1 and/or Sp3 to the *Sp1c* and *Sp1d* sites in the proximal *t-PA* promoter was required for basal activity in *Drosophila* SL2 cells. EMSA competition experiments with the *Sp1* consensus element and the two *t-PA Sp1* sites revealed that the most upstream sequence (*Sp1c*) has an approximately 10-fold lower affinity for Sp1 than the downstream site (*Sp1d*). Mutation of either site severely hampered the basal activity of the *t-PA* promoter in HT1080 cells, indicating that both sites were as important. Some basal activity in HT1080 cells, as well as some activation by Sp1 in SL2 cells, were still observed with the *t-PA* promoter carrying the double mutation, suggesting that additional *cis*-elements were involved. Indeed, evaluation of progressive deletion mutants of the *t-PA* promoter in the SL2 cells, delineated a second region important for activation by Sp1 which coincides with the *CRE* element. However, no direct interaction with Sp1 was observed, suggesting an alternative mechanism for this Sp1 activation.

Simultaneous expression of Sp1 and Sp3 in SL2 cells stimulated *t-PA* promoter activity in a synergistic way compared to Sp1 or Sp3 alone. In analogy to the *t-PA* promoter, Sp3 also activates the leukocyte integrin gene CD11c through two binding sites [16], indicating that Sp3 does not always act as a repressor. Intriguingly, if the mutations disrupting the *Sp1c* and *Sp1d* sites strongly affected the activation by Sp1, they only had a slight effect on the activation by Sp3 suggesting that Sp3 could induce the *t-PA* promoter through alternative *cis*-elements.

Previous studies have shown that the enhancer, which is located at −7.2 kb upstream from the human *t-PA* gene, mediates induction by RA and DEX [11,12]. The present study revealed a unique role for the *Sp1c* and *Sp1d* sites of the proximal promoter in the hormonal response mediated by this enhancer. The induction of the *t-PA* gene by RA could only be reconstituted in SL2 cells upon co-expression of Sp1, but not of Sp3, in addition to RAR/RXR and *t-PA2.4-632-CAT/ΔAP1*. Mutation of the *Sp1c* and *Sp1d* sites in this reporter construct completely suppressed induction by RA. In contrast, the induction by DEX could not be reproduced upon co-transfection of the SL2 cells with *t-PA2.4-632-CAT/ΔAP1* and expression vectors for Sp1 and GR, suggesting that additional factors were required for this hormonal response.

The role of Sp1 in the induction by RA was confirmed in

mammalian cells. Indeed, no RA induction was observed upon transient transfection of HT1080 cells with *t-PA2.4-632Sp1c/dmut-CAT/ΔAP1* treated with 10^{-6} M RA. To investigate whether the loss of induction by RA was not a consequence of the lower basal activity of the *t-PA* promoter mutated in the *Sp1* sites, another mutation known to decrease the basal level was evaluated in HT1080 cells: mutation of the *CRE* did not affect the induction by RA, indicating a specific Sp1 requirement. RA receptors have been shown to regulate gene transcription by interaction with other transcription factors, such as AP2 [17]. The specific Sp1 requirement for the induction by RA, but not by DEX, might indicate a direct or indirect interaction between RAR/RXR and Sp1. Direct protein-protein interactions between Sp1 and a number of nuclear receptors, like COUP-TF and ER, have been reported [18,19]. Moreover, a multi-protein complex comprising Sp1, CBP and the progesterone receptor regulates transcription of the $p21^{WAF1}$ cyclin dependent kinase inhibitor gene in breast cancer cells [20], while Sp1, PPAR and RXR synergistically induce the transcription of the acyl co-enzyme A oxidase promoter [21]. Alternatively, an indirect interaction might be mediated by the multi-subunit cofactor required for Sp1 activation (CRSP) that was recently identified and contains the nuclear hormone receptor co-activator TRIP2/PBP previously shown to interact with RXR among others [22]. Whether a direct or indirect protein-protein interaction between Sp1 and RAR/RXR is responsible for the observed induction by RA of the human *t-PA* gene remains to be investigated. Intriguingly, Sp3 driven *t-PA* promoter activity could not support the RA induction, identifying new divergent functions between the Sp1 and Sp3 proteins. Indeed, a transcriptional inhibitory domain has been identified in Sp3 and is lacking in Sp1. Further studies should evaluate whether this domain prevents support of RA induction of the *t-PA* gene, and whether this phenomenon has a physiological relevance for t-PA expression in tissues with different ratios of the two factors.

The need for Sp1 binding to the *t-PA* promoter for induction by RA mediated through the DR5 element in the *t-PA* enhancer could also reflect some kind of promoter/enhancer selectivity [13,23]. Recently it was shown that the AE1 enhancer from the *Drosophila* Antennapedia gene complex preferentially activates a TATAA box containing promoter [24]. A similar situation could be envisioned for the *t-PA* gene: the RAR/RXR heterodimer bound to the *t-PA* enhancer might be targeted only to the *t-PA* promoter because of its need for the *Sp1* sites.

In conclusion, Sp1 and Sp3 were shown to activate the *t-PA* promoter, by interacting with the two *Sp1* sites upstream from the initiator element. Binding of Sp1, but not of Sp3, to these elements was a prerequisite for induction by RA, but not by DEX, mediated by the *t-PA* enhancer.

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