NF-κB-mediated regulation of urokinase gene expression by PMA and TNF-α in human A549 cells

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Abstract Binding sites for different transcription factors have been identified in the regulatory region of the human *uPA* gene. We investigated the role of NF-κB and AP-1 families of transcription factors in the induction of uPA mRNA by TNF-α and PMA in the A549 cell line constitutively expressing uPA mRNA and protein. Using the protein synthesis inhibitor cycloheximide and the antioxidant PDTC, that have an opposite effect on NF-κB and AP-1 activation, we showed that uPA mRNA induction by TNF-α and PMA in the A549 cell line is mainly due to NF-κB activation.

Key words: uPA; NF-κB; HIV; TNF-α

1. Introduction

Urokinase-type plasminogen activator (uPA) is involved in regulating biochemical phenomena leading to migration and invasiveness of normal and cancer cells. Although not essential for embryonic and fetal development [1], an important role of uPA seems established in promoting fibrinolysis, chemotaxis, angiogenesis and invasiveness in normal as well as pathologic processes like inflammation and cancer [2]. uPA expression can be induced or enhanced in culture and in vivo by physiological stimuli (cytokines, growth factors), phorbol esters (PMA) and during neoplastic transformation [3].

The upstream regulatory region of the human *uPA* gene has been studied in some detail and two distinct regions able to mediate responsiveness to phorbol esters have been identified: an upstream enhancer located between −2100/−1870 and a κB-like sequence element at −1592/−1582 [4–7]. The enhancer region contains several binding sites for known transcription factors: an octameric AP-1 binding site combined with a PEA3 site is present at the 5′ of the enhancer region while a consensus heptameric AP-1 site is located at the 3′ end. Both these sites are required for basal and induced enhancer activity [6,8]. The second phorbol esters responsive region, located at −1592, binds members of the NF-κB/Rel family of transcription factors [9], in particular the two heterodimers NFκB1/RelA and c-Rel/RelA [7,10,11].

The A549 is a human pulmonary epithelial cell line derived from an alveolar cell carcinoma that constitutively expresses uPA mRNA [12], in which both uPA mRNA and protein levels can be stimulated by TNF-α [13]. In order to determine the individual roles of NF-κB and AP-1 transcription factors in regulating *uPA* gene expression, we have analyzed uPA mRNA expression in the A549 cell line following treatment with TNF-α or a phorbol ester, the 12-*O*-tetradecanoyl-phorbol-13-acetate (PMA). Our data indicate that the Rel family factors play an important role in TNF-α as well as PMA induction of *uPA* gene transcription.

2. Materials and methods

2.1. Cell culture

The human pulmonary epithelial cell line A549 [12] was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Stimulations were carried out with recombinant human TNF-α (specific activity: 3.2×10^7 U/mg) at a final concentration of 100 U/ml; PMA at 100 ng/ml and cycloheximide at 10 μg/ml.

2.2. Plasmid probes and Northern blot assay

The following fragments were used as probes in Northern blotting analyses: the 1.5 kb *Pst*I fragment of human uPA cDNA [14]. Human cDNAs for NF-κB1 (p50), RelA (p65) and IκB-α have been previously described [10]. Murine 1.3 kb c-Fos cDNA was from the plasmid T7βc-Fos [15]; the human 350 bp c-Jun cDNA probe was from the plasmid pUN21c-Jun [16]. cDNA probes were labeled with [α-^{32}P]dCTP by the Random Primed DNA labeling Kit (Boehringer). Total RNA was extracted from cells by the guanidinium isothiocyanate method [17]. RNAs (20 μg) were loaded on 1% formaldehyde-containing agarose gels and transferred to a nitrocellulose membrane (BioRad). The intensity of RNA bands in the autoradiographs was quantitated by a video densitometer (Molecular Dynamics).

2.3. Nuclear extract and electrophoretic mobility shift assay

Nuclear and cytoplasmic extracts from cultured cells were prepared according to published methods [7,18]. DNA binding ability of 10 μg of nuclear extracts was analized by the electrophoretic mobility shift assay (EMSA) [7]. The oligonucleotides used for EMSA were labeled with [γ-^{32}P]ATP and were:

κB : 5′−GCTGCCTGCTGGGGAAAGTAC−3′,

corresponding to nucleotides −1592/−1571 of the human uPA gene;

AP−1 : 5′−GAGCAACATGAATCATGACGG−3′,

corresponding to nucleotides −1893/1872 of the human uPA gene.

3. Results

3.1. Induction of uPA mRNA by PMA and TNF-α

To determine the effect of TNF-α and PMA on the expression of the endogenous *uPA* gene in A549 cells, total RNA from control, TNF-α- or PMA-treated cells was analyzed by Northern blotting and hybridized with the uPA cDNA probe. The same filters were sequentially hybridized to a GAPDH probe, for loading control. Unstimulated cells expressed detectable levels of the 2.5 kb uPA mRNA (Fig. 1A, lane C). Treatment of the cells with TNF-α (100 U/ml) resulted in increase of uPA mRNA which peaked at 3 h (5-fold compared

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to untreated cells), and declined thereafter. Treatment of the cells with PMA resulted in a strong increase of uPA mRNA (10-fold at 3 h) (Fig. 1C). Quantitation of the data by densitometric analysis of the filters is shown in Fig. 1B,D.

3.2. *Effects of PMA and TNF-α on expression of the Rel/NFκB and Jun/Fos family members*

We next analysed the effect of TNF-α and PMA on the expression of genes of the Rel family and c-Jun and c-Fos families that may be involved in basal and inducible expression of the *uPA* gene [7,8]. As shown in Fig. 2, the 2.6 kb mRNA of RelA, was induced by TNF-α (Fig. 2A) and to a lesser extent by PMA (Fig. 2B). The low levels of the two c-Rel mRNA (3.5 kb and > 10 kb) were essentially unaffected by PMA or TNF-α (data not shown).

On the other hand both NFκB1 and IκB-α mRNAs, known to be regulated through NF-κB sites [19,20], were strongly induced by treatment with TNF-α and PMA; the 3.8 kb mRNA for NFκB1 reached a maximum 15-fold increase with PMA and 6.5-fold with TNF-α (Fig. 2A,B). The mRNA levels of IκB-α (Fig. 2A) upon TNF-α stimulation dramatically increased at 1 h, reached a maximum at 3 h, and decreased at 5 h. PMA was less effective than TNF-α to induce IκB-α mRNA (Fig. 2B).

In addition to the κB, the AP-1 site located in the uPA enhancer region is also a candidate in mediating up-regulation of uPA mRNA by TNF-α and PMA [8]. We therefore investigated the effects of TNF-α and PMA on the expression of the AP-1 subunits c-Jun and c-Fos. The filters were sequentially hybridized to cDNA probes for c-Jun and c-Fos, which in HepG2 cells bind as an heterodimeric complex to the heptameric AP-1 binding site at the 3′ of the uPA enhancer region [8]. Results showed that c-Jun mRNA was undetectable in untreated cells (Fig. 2A); TNF-α and PMA treatment had little effect on its level (Fig. 2A,B). c-Fos mRNA was also undetectable in untreated cells (Fig. 2A), and PMA and TNF-α had no effect on its expression (Fig. 2A,B).

From the data of Figs. 1 and 2 we conclude that uPA induction by TNF-α and PMA better correlates with activation of NF-κB family members.

3.3. *uPA mRNA induction by TNF-α and PMA does not require de novo protein synthesis*

To investigate whether induction of uPA mRNA by TNF-α or PMA was dependent on de novo protein synthesis, we employed the protein synthesis inhibitor cycloheximide (CHX). Treatment of the cells with CHX (10 μg/ml) for 1, 3 and 5 h resulted in an increase of uPA mRNA (Fig. 1A); co-treatment of cells with CHX and TNF-α or CHX and PMA showed a further 10- and 30-fold increase, respectively, of the uPA mRNA levels (Fig. 1A,C).

CHX alone induced or, together with TNF-α or PMA, superinduced RelA, NF-κB1 and IκB-α mRNAs (Fig. 2A,B); CHX has been reported to directly activate NF-κB [21]. CHX treatment also induced c-Jun mRNA, the levels of which increased in a time-dependent manner; TNF-α in combination with CHX determined an early and transitory stimulation of c-jun mRNA levels (Fig. 2A). PMA/CHX treatments revealed a 2.2 kb c-Fos mRNA at 5 and 8 h (Fig. 2B) but no c-Fos mRNA band was observed with CHX or PMA alone.

From these data we concluded that uPA mRNA induction

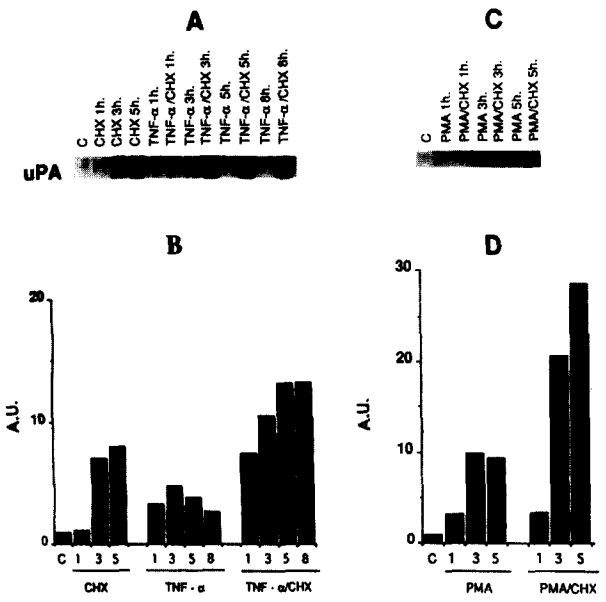

Fig. 1. uPA mRNA induction in A549 cells by TNF-α and PMA. Northern blot analysis of A549 cell mRNAs; 20 μg of mRNA for each sample was loaded on a 1% formaldheyde-agarose gel. A and C: Northern blots. B and D: Quantitation of the data in (A) and (C) after densitometric scanning of the autoradiographs: mRNA intensities were calculated relative to the intensities of the GAPDH internal mRNA control (A.U., arbitrary units). C = control cells (untreated). CHX = cycloheximide.

by TNF-α and PMA does not require de novo protein synthesis. Hence again the correlation between increase of uPA mRNA and RelA/c–Rel mRNA, may only be relevant upon long-term stimulations.

3.4. *Effects of TNF-α and PMA on DNA binding activity of NF-κB and AP-1*

NF-κB activation, resulting in nuclear translocation, does not need protein synthesis. To test NF-κB DNA binding activity, nuclear protein extracts from untreated and treated cells were analized by EMSA, using as a probe an oligonucleotide corresponding to the degenerate κB site present at −1592 of the human *uPA* gene. This sequence has been previously shown to bind several members of the Rel gene family [7]. The A549 nuclear extract had a very low constitutive binding activity (Fig. 3, lane 1). Following stimulation with PMA (lane 3) and TNF-α (lane 5) DNA binding was induced; TNF-α was a better inducer of NF-κB DNA binding activity. Interestingly, CHX synergized with PMA (lane 4) and with TNF-α (lane 6): this effect may possibly be due either to the prevention of IκB-α synthesis, a protein with a very short half-life [20], or to a direct activation of NF-κB by CHX [21]. The specificity of the binding activity was shown by the competition with an unlabeled κB oligonucleotide (lanes 7 and 8); an AP-1 binding oligonucleotide (lanes 9 and 10) did not compete. Identical data were obtained using a κB site derived from the HIV enhancer (data not shown) [22].

Next, we examined the effects of PMA and TNF-α on AP-1 binding activity. An oligonucleotide corresponding to the consensus AP-1 binding site at the 3′ of the uPA enhancer was used as a probe. The results (Fig. 3) showed that the A549 cell line had a consistent specific basal AP-1 activity (lane 1),

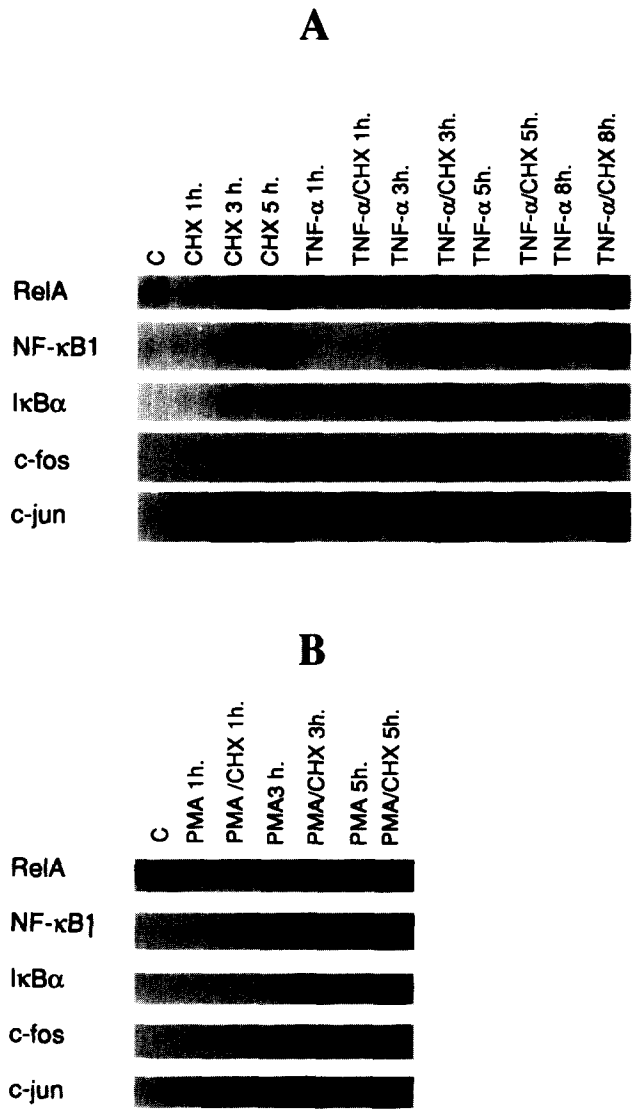

Fig. 2. The mRNAs of NF-κB and AP-1 subunits are differently regulated by TNF-α and PMA. The same filters of Fig. 1A,C were sequentially hybridized with cDNA probes for RelA, NFκB1, IκB-α, c-fos and c-jun. C = control cells (untreated).

which was specifically competed for by the unlabeled AP-1 (lanes 7 and 8) but not by the κB oligonucleotide (lanes 9 and 10). The AP-1 binding activity was increased after treatment with PMA (lane 3) but not with TNF-α (lane 5). Co-treatment of PMA with CHX abolished PMA induction (lane 4).

In conclusion, NF-κB was activated by PMA and TNF-α also in the presence of CHX; activation of NF-κB therefore is coincident with the lack of cycloeximide sensitivity of the uPA mRNA induction by PMA and TNF-α. On the other hand AP-1 responded poorly to PMA and not at all to TNF-α, and CHX abolished the modest PMA induction.

3.5. *Effects of PDTC treatment on DNA binding activities and uPA mRNA levels*

In order to investigate whether NF-κB DNA binding activity was involved in up-regulating uPA mRNA level, we employed the antioxidant PDTC (pyrrolidinedithiocarbamate) that has been reported to inhibit NF-κB activation while inducing AP-1 activity [23]. Nuclear extracts were prepared from A549 cells treated for 1 h with PDTC (100 and 150 μM) prior to the addition of PMA or TNF-α. As shown in Fig. 4, treatment with PDTC alone (lanes 2 and 3) did not induce NF-κB binding activity. On the other hand the PMA-induced κB binding (lane 4) was abolished by PDTC treatment (lanes 5 and 6); the same results were obtained with TNF-α/PDTC co-treatments (compare lane 7 to lanes 8 and 9). Again the different extent of NF-κB activation by PMA and TNF-α (compare lane 4 to lane 7) is noticeable. When the same extracts were incubated with an AP-1 oligonucleotide, the opposite result was obtained. AP-1 binding activity was induced by PDTC alone (Fig. 4, compare lane 1 with lanes 2 and 3) and co-treatment of PMA with PDTC (lanes 5 and 6) resulted in even greater induction of AP-1 binding activity than with PMA alone (lane 4); PDTC did not increase, however, the activity of cells treated with TNF-α (lanes 7 and 8).

We then analysed uPA mRNA levels in PMA- or TNF-α-treated cells for 3 h, in the presence and absence of PDTC. As shown in Fig. 5A PDTC alone weakly induced uPA mRNA levels (3-fold, lane 2) possibly through AP-1 activation; PMA/PDTC co-treatment, however, resulted in a decrease of uPA mRNA (lane 4) compared to treatments with PMA alone (lane 3). Similar results were obtained with TNF-α/PDTC co-treatments (lanes 5 and 6). Quantitation of the data (Fig. 5B) showed that the presence of PDTC decreased the level of uPA mRNA in PMA- and TNF-α-treated cells by 60 and 50%, respectively. We then reprobed the same filter with the *IκB-α* probe, a gene known to be under NF-κB transcriptional control [20]. The results showed that also in the case of IκB-α PMA and TNF-α inductions could be partially blocked by PDTC (Fig. 5A, lanes 3–6).

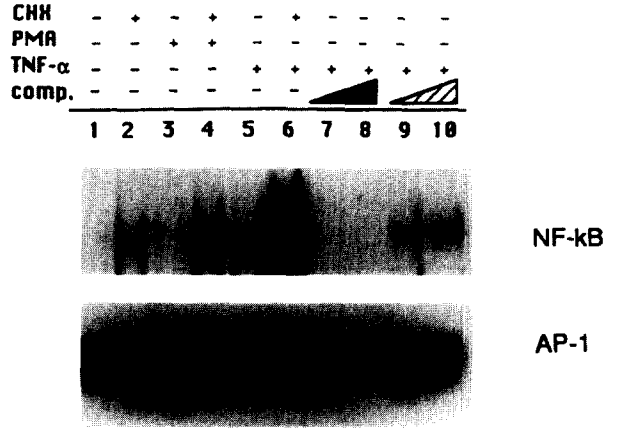

Fig. 3. PMA and TNF-α specifically induce NF-κB binding to a κB site of the human *uPA* gene. An oligonucleotide corresponding to the κB site at −1590 was used as probe in experiment of electrophoretic mobility shift assay (EMSA) with nuclear extracts derived from A549 cells. Untreated cells (lane 1). CHX treatment for 3 h (lane 2); PMA treatment was for 3 h (lane 3). Lane 4; co-treatment of PMA/CHX for 3 h. TNF-α treatments (lane 5, 3 h). Co-treatment of TNF-α/CHX for 3 h (lane 6). DNA binding specificity of TNF-α-treated nuclear extract was assesed with 20- and 60-fold excess of unlabeled oligonucleotide (lanes 7 and 8) and with 20- and 60-fold excess of the unrelated AP-1 oligonucleotide (lanes 9 and 10). The same nuclear extracts used were used in an EMSA experiment with a oligonucleotide spanning the canonical AP-1 of the human *uPA* gene as probe.

4. Discussion

The data reported in this paper show that in A549 cells induction of uPA mRNA by PMA and TNF-α requires NF-κB, a factor already present in an inactive form in the cytoplasm of these cells. Although induction of uPA mRNA correlated with that of RelA and NF-κB1 mRNAs, the lack of sensitivity of uPA mRNA induction to the block of protein synthesis indicates that inductions of RelA and NFκB1 mRNAs were not influential. This may possibly be due to the strong induction of IκB-α mRNA observed, a result already described by others and in line with NF-κB regulation of the *IκB-α* gene [20]. Indeed, despite the induction of IκB-α, treatment of A549 cells with PMA and TNF-α resulted in activation of the NF-κB DNA binding activity, suggesting that the increase in factors and inhibitors had a mutually exclusive effect. On the other hand, PMA and TNF-α had no effect on the levels of expression of the two major subunits of the AP-1 transcription factor, c-Jun and c-Fos. This result parallels the failure to demonstrate a major induction of DNA binding activity to the AP-1 sequence (Fig. 3) in particular by TNF-α. Since the antioxidant PDTC induces AP-1 binding, while inhibiting NF-κB activity [23], we have used this compound to discriminate between the two sets of transcription factors (Figs. 4 and 5). Both in the case of PMA and TNF-α, uPA mRNA induction was inhibited by the antioxidant PDTC and a similar effect was observed for the NF-κB-induced *IκB-α* gene [20].

The AP-1 family of transcription factors, which has been shown to be important for PMA and EGF induction of uPA mRNA in other types of cells [5,6,8] does not appear to be involved in uPA mRNA induction in the A549 cell line. In fact, while c-Jun mRNA was present in treated cells, no c-Fos mRNA could be detected. AP-1 binding activity, possibly due to other members of the Jun, ATF and Fos families, was present in untreated cells and was only weakly induced by PMA and TNF-α. Finally, while pretreatment with the antioxidant PDTC slightly stimulated AP-1 binding activity, it had an inhibitory effect on uPA mRNA induction by PMA and TNF-α. Overall, our data lead to the conclusion that the NF-κB transcription factor is involved in the activation of *uPA* gene transcription by PMA and TNF-α in human A549 cells. This result is in agreement with the block of *uPA* gene expression by a RelA antisense oligonucleotide in a human ovarian carcinoma cell line [24].

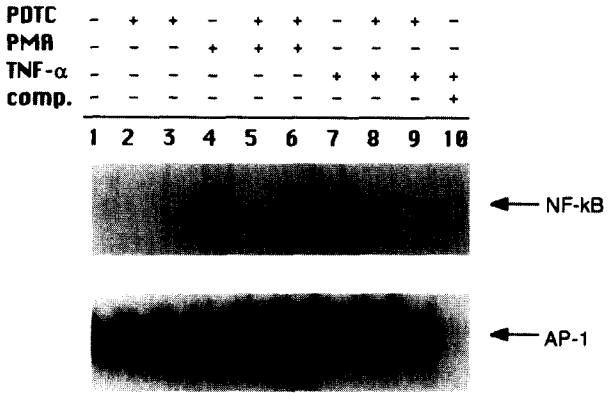

Fig. 4. PDTC blocks activation of NF-κB DNA binding activity by PMA and TNF-α. Nuclear extracts of A549 cells treated with TNF-α or PMA in conjunction with the antioxidant pyrrolidinedietilcarbamate (PDTC), were used for EMSA with the κB probe (Fig. 4). PDTC treatment alone (lanes 2 and 3, 100 and 150 μM). PMA and TNF-α-treated cells (lanes 4 and 7, respectively), PMA and TNF-α co-treatment with 100 and 150 μM PDTC (lanes 5, 6, 8 and 9). Lane 10; competition with a 60-fold excess of specific oligonucleotide. The same extracts were tested for AP-1 binding activity.

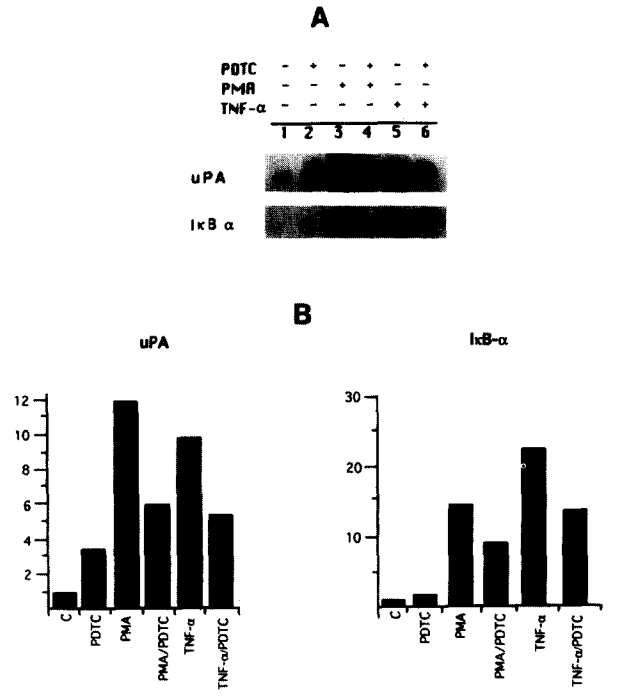

Fig. 5. PDTC inhibits PMA and TNF-α induction of uPA and IκB-α mRNAs. A: Northern blots. B: Quantitation by comparison to GAPDH mRNA levels and densitometric scanning. Concentration used: PMA, 100 ng/ml nM; TNF-α, 100 U/ml; PDTC, 150 μM.

In the 5′ flanking region of the human *uPA* gene, κB sites are present in at least two positions: immediately downstream of the enhancer (at −1865) and at −1590 [4,7,25]. The site at −1590 has an unusual κB sequence, but has been shown to mediate PMA induction of *uPA* gene transcription and to bind both the RelA–NF-κB1 and the RelA/c-Rel heterodimers [7]. The site at −1865 is highly homologous to the Igκ and HIV classical sites, but its activity on *uPA* gene transcription has not been rigorously tested. In fact, in human HeLa, HepG2 and HT1080 cells, the deletion of this site did not prevent PMA induction [6,26]. However, in these cells the AP-1 sites appear particularly important in inducing *uPA* gene transcription [8,26].

Taken together these data suggest that the same transcription factor can play different roles in different cell lines providing a mechanism to control gene expression in a cell-specific manner.

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